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ECOLOGY AND EVOLUTION OF ANTIMICROBIAL RESISTANCE IN *ENTEROCOCCUS*: A MULTILAYERED MOLECULAR APPROACH WITH EMPHASIS IN THE PLASMID DIVERSITY

Tese do 3.º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas na Especialidade de Microbiologia

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(Wallace Wattles, 1860-1911)

Abstract

The genus *Enterococcus* constitutes one of the best examples of bacterial adaptation to different environments due to the ability to survive in harsh conditions and their intrinsic resistance to different antibiotics, biocides and metals. Enterococci are well recognized commensals and opportunistic pathogens of humans and animals since the early 1900's. However, nosocomial enterococcal infections have increasingly been reported during the last 30y with these microorganisms becoming one of the most common pathogens causing severe nosocomial infections worldwide. This situation has been facilitated by the acquisition of resistance to all antibiotics available in the therapeutic arsenal including vancomycin, the last resort for the treatment of multiresistant Gram-positive organisms, and all new antibiotics recently licensed in the pharmaceutical market as tigecycline. The emergence of vancomycin-resistant enterococci is of particular concern because of the limited therapeutic options to treat multiresistant enterococci, and the possibility to transfer this trait to more pathogenic bacteria as *Staphylococcus aureus*.

The emergence and recent increase of enterococcal strains with new adaptive traits (inferred by their antibiotic resistance, colonization, or virulence phenotypes and genotypes) illustrate the role of lateral genetic transfer (LGT) in the evolution of these microorganisms. *The global goal of this work was to establish the diversity and the influence of different mobile genetic elements (MGE) belonging to the enterococcal mobilome in the transfer of adaptive traits between different hosts and in the selection of particular enterococcal lineages.* With this purpose, the role of clonal expansion and lateral genetic transfer in the dissemination of vancomycin resistance among *Enterococcus faecium* and *Enterococcus faecalis*, recovered from hospitalized persons in different countries of the five continents (1986-2009) and from different hosts or community environments (healthy humans, foodborne animals, distinct aquatic environments) (1995-2008) was analyzed.

The analysis of the population structure of *E. faecium* and *E. faecalis* isolates from different origins revealed an ***overrepresentation of particular clonal complexes*** (CC) in swine (*E. faecium* CC5), poultry (*E. faecium* CC9) and humans (*E. faecium* CC17, CC22 and CC94 and *E. faecalis* CC2), all of them comprising a diversity of sequence types (ST). The results highlighted the presence of different reservoirs for antibiotic-resistance. Also, the interhost transmission (swine-Human) of clones belonging to host-adapted clonal complexes of *E. faecium* (CC5, CC17) and *E. faecalis* (CC2), confirms the relevance of reverse and alternative routes for dissemination of opportunistic bacteria.

The analysis of the enterococcal ***plasmidome*** of *E. faecium* and *E. faecalis* isolates from different origins showed a ***species specific plasmid pool*** with differences in the plasmid content among strains from different hosts and habitats. The presence of broad host (e.g. Inc18) or particular narrow host plasmids (megaplasmids and small plasmids) encoding traits associated with specialized environments could have contributed to such differences.

A detailed study of the genetic elements encoding resistance to glycopeptides showed that *vanA*-Tn*1546*-carrying plasmids are *an outstanding driver of the* ***pandemic spread of vancomycin resistance among enterococci****.* ***Transferable plasmids*** carrying replication, conjugation and/or maintenance systems related to plasmid families of narrow host range as RepA_N (pRUM in *E. faecium* and pheromone-responsive in *E. faecalis*) or broad host range as Inc18 would have facilitated the successful dissemination of Tn*1546* among enterococci. Besides the host specificity of plasmids of *E. faecalis* and *E. faecium*, the diversity and frequency of LGT/recombination events occurring within each species provided interesting clues to understand why *vanA* plasmids remain confined to this genera. Of particular interest were the results related to *E. faecalis*. The narrow host range of pheromone plasmids and the frequent rearrangements observed within plasmids of this species might explain the maintenance of pAD1-Tn*1546* within this bacterial host. The ***unsuccessful transference*** *of these plasmids* ***to Staphylococcus aureus*** seems to be associated with the presence/absence of the broad host conjugative module of Inc18 plasmids.

Finally, the cumulative acquisition of adaptive traits by these species-specific plasmids would have conferred an increased fitness relevant for the selection and successful expansion of certain lineages in the predominant enterococcal species.

The recovery of enterococcal isolates resistant to tigecycline, among enterococci non-exposed to this antibiotic, highlights the possible selection by other antibiotics in non-clinical settings as in farms for animal production.

In summary, this work illustrated the selection of epidemic clones and the diversity and influence of enterococcal mobile genetic elements in the current expansion of specific clonal lineages and high prevalence of vancomycin-resistant enterococci worldwide and in Portugal in particular. The diversity of plasmids (*plasmidome*) encompassing different replication, transfer and maintenance systems highlights the wide possibilities of adaptation of these microorganisms.

Keywords: enterococci, LGT, plasmid, vancomycin resistance, Tn*1546*

Resumo

O género *Enterococcus* constitui um dos melhores exemplos de adaptação bacteriana a diferentes ambientes devido à sua capacidade de sobrevivência em ambientes hostis e à resistência intrínseca a diferentes antibióticos, biocidas e metais. Ainda que reconhecidas como bactérias comensais e oportunistas patogénicos de humanos e animais desde os anos 1900, as infecções hospitalares causadas por *Enterococcus* têm vindo a aumentar progressivamente durante os últimos 30 anos. Actualmente, são um dos agentes de infecções nosocomiais graves mais comuns a nível hospitalar e mundial. Tal tem sido facilitado pela aquisição de resistência a múltiplos antibióticos disponíveis no arsenal terapêutico incluindo a vancomicina, um antibiótico considerado de última escolha para o tratamento de Gram-positivos multiresistentes, e todos os novos antibióticos recentemente aprovados para uso em ambiente hospitalar como é o caso da tigeciclina. O aumento de isolados de *Enterococcus faecalis* e *Enterococcus faecium* resistentes à vancomicina (ERV) tem suscitado particular interesse devido à escassez de opções terapêuticas de tratamento e à possibilidade de transferência desta resistência para outras bactérias mais patogénicas como *Staphylococcus aureus*.

O aumento dramático e recente de *Enterococcus* com novas características adaptativas (inferidas pela sua resistência aos antibióticos, colonização, ou fenótipos e genótipos de virulência) ilustra o papel da transferência genética horizontal (TGH) na evolução deste género bacteriano. *O objectivo geral deste trabalho foi estabelecer a diversidade e a influência de diferentes elementos genéticos móveis (EGM) na transferência de características adaptativas entre diferentes hospedeiros e na selecção de linhagens clonais específicas*. Com este propósito, foram analisadas a importância da expansão clonal e da TGH na disseminação da resistência à vancomicina em isolados de *E. faecium* e *E. faecalis* obtidos de hospitais em diversos países dos cinco continentes (1986-2009) e da comunidade (humanos saudáveis, animais de produção alimentar, ambientes aquáticos) (1995-2008).

A análise da estrutura populacional de isolados de *E. faecium* e *E. faecalis* de diferentes origens revelou a ***predominância de determinados complexos clonais*** (CC) em suínos (CC5 *E. faecium*), aves (CC9 *E. faecium*) e humanos (*E. faecium* CC17, CC22 e CC94 e *E. faecalis* CC2). Adicionalmente, a transmissão entre hospedeiros (suínos-Homem) de clones pertencentes a determinados CCs de *E. faecium* (CC5, CC17) e *E. faecalis* (CC2) confirma a relevância de modos alternativos na disseminação de bactérias oportunistas.

A análise dos plasmídeos presentes em isolados de *E. faecium* e *E. faecalis* de diferentes origens revelou um reservatório de ***plasmídeos específicos de espécie*** com diferenças observadas entre diferentes hospedeiros e habitats. A presença de plasmídeos de largo espectro de hospedeiro (Inc18) ou de plasmídeos de espectro reduzido (megaplasmídeos e plasmídeos muito pequenos) que conferem vantagens adaptativas (ex.: bacteriocinas, virulência) pode ter contribuído para essas diferenças.

Um estudo detalhado dos elementos genéticos de resistência aos glicopéptidos demonstrou a grande relevância dos plasmídeos que albergam o transposão Tn*1546* (*vanA*) na ***disseminação pandémica da resistência à vancomicina em Enterococcus***. ***Plasmídeos conjugativos*** com sistemas de replicação, conjugação e/ou manutenção relacionados com famílias de plasmídeos de espectro reduzido, como a RepA_N (pRUM em *E. faecium* e plasmídeos de resposta a feromonas em *E. faecalis*), ou de espectro alargado, como os Inc18, deverá ter facilitado a rápida disseminação de Tn*1546* em *Enterococcus*. Para além da especificidade do conteúdo plasmídico de *E. faecalis* e *E. faecium*, a diversidade e a frequência de eventos de TGH que ocorre em ambas as espécies, indiciou sobre o facto de a resistência aos glicopéptidos permanecer confinada a este género. De particular relevância foram os resultados relacionados com os isolados de *E. faecalis*. Os plasmídeos de resposta a feromonas (PRF) de espectro reduzido de hospedeiro assim como os frequentes eventos genéticos observados entre plasmídeos desta espécie pode explicar a manutenção de PRF-Tn*1546* nesta espécie bacteriana. A ***transferência*** *destes plasmídeos* ***para Staphylococcus aureus*** parece estar associada à presença do módulo de conjugação de plasmídeos de largo espectro do tipo Inc18.

Finalmente, a aquisição e acumulação de características adaptativas por estes plasmídeos específicos de espécies pode ter conferido um *fitness* acrescido, relevante na selecção e expansão dramática de certas linhagens das espécies de *Enterococcus* mais importantes.

A detecção da resistência à tigeciclina, em isolados de *Enterococcus* não expostos a este antibiótico, indica a sua possível selecção por outros antibióticos usados em ambientes não hospitalares, como os locais de produção animal.

Em resumo, este trabalho permitiu demonstrar a selecção de clones epidémicos, a diversidade de EGM e a sua influência na actual expansão de determinadas linhagens clonais assim como na elevada prevalência de ERV em diferentes países e, em particular, em Portugal. A diversidade plasmídica demonstra ainda as amplas possibilidades de adaptação destes microorganismos.

Palavras-chave: *Enterococcus*, plasmídeos, resistência à vancomicina, transferência genética horizontal, Tn*1546*

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMEs	Aminoglycoside-modifying enzymes
BHI	Brain Heart Infusion
CC	Clonal complex
CCC	Covalently Closed Circular
CLSI	Clinical and Laboratory Standard Institute
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphates
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GEC	Genetic exchange community
GI	Genomic Island
ICE	Integrative Conjugative Element
IS	Insertion Sequence
LGT	Lateral Genetic Transfer
MBC	Minimum bactericidal concentration
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
MGE	Mobile Genetic Element
MLST	Multi-Locus Sequence Typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface component-recognizing adhesive matrix molecules
NCBI	National Center for Biotechnology Information
OC	Open Circular
PAI	Pathogenicity island
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
RAPD	Rapid Amplification of Polymorphic DNA
RCR	Rolling-circle replicating
RFLP	Restriction fragment length polymorphism

LIST OF ABBREVIATIONS *(continued)*

Rel	Relaxase
Rep	Replication-initiator protein
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
ST	Sequence type
TA	Toxin-Antitoxin
Tn / CTn	Transposon / Conjugative Transposon
VRE	Vancomycin-resistant enterococci
VREfs	Vancomycin-resistant *Enterococcus faecalis*
VREfm	Vancomycin-resistant *Enterococcus faecium*
VRSA	Vancomycin-resistant *Staphylococcus aureus*

Chapter 1

Introduction

It is the theory which decided what can be observed.

Albert Einstein

1.1. The genus *Enterococcus* spp.

1.1.1. Taxonomy and Historical Perspective

Enterococcus is a genus of Gram positive cocci that fall within the phylum *Firmicutes,* class *Bacilli*, order *Lactobacillales*, family *Enterococcaceae*.

The term “entèrocoque” was firstly used by Thiercelin in 1899 to describe the intestinal origin of bacteria in pairs and short chains recovered from human faeces (117). In the same year, MacCallum and Hastings reported an organism obtained from a case of endocarditis which they called *Micrococcus zymogenes*. The term “micrococcus” was by that time used to refer to a very hard bacterium isolated from different environments (252). The name *Streptococcus faecalis* was firstly used in 1906 also from a patient with endocarditis, and in the subsequent years other *Streptococcus* species (*S. faecium, S. durans, S. avium)* were described as differing in the fermentation patterns of *S. faecalis*. In 1919, Sherman proposed a classification scheme which separated streptococci into nonenterococcal streptococci (pyogenic, viridans, lactic) and “the enterococcal group”, based on the growth at different temperatures and pH conditions (379). This classification’s scheme also correlated with the serological Lancefield scheme and in the mid-1930s, enterococci were classified as *Group D Streptococcus*. It was only in 1984 that all *Group D Streptococcus,* with exception of *S. bovis*, was considered genetically different from the *Streptococcus*, and *Enterococcus* was globally considered a separate genus. Studies based on DNA-DNA hybridization, 16S rRNA gene sequencing (Figure 1), whole-cell protein analysis, and conventional phenotypic tests allowed the establishment of many enterococci species (293, 366, 409).

Over forty *Enterococcus* species have been described to date (www.bacterio.cict.fr/e/enterococcus.html), of which the most common species associated with the human host are *Enterococcus faecium* and *Enterococcus faecalis. E. faecium* and *E. faecalis* are genetically not closely related as demonstrated by 16S rRNA and *atpA* housekeeping gene (α-subunit of ATP synthase) sequences (301).

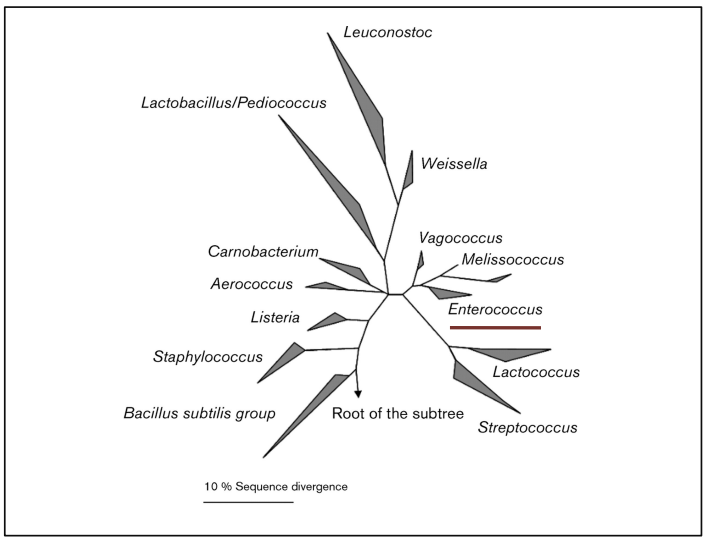


Figure 1. Phylogenetic dendrogram of Gram-positive genera by 16S rRNA sequencing (adapted from 213).

1.1.2. Physiology of Enterococci

Enterococci are non-spore-forming, facultative anaerobes able to survive in a wide range of environmental conditions, both at extreme temperatures (10-45ºC) and pH (4.8-9.6) conditions; they grow in concentrations of sodium chloride as high as 6.5% and hydrolyze esculin in the presence of 40% bile salts (117, 409).

Enterococci have metabolic features allowing them to grow within diverse environments such as gastrointestinal compartments of different mammal's intestine, reptils and birds and a diversity of environmental sources (e.g. food, water, inanimate surfaces). They are extraordinary tolerant to oxidative stress, azide, detergents, heavy metals, and ethanol as well as prolonged desiccation. In addition, they are able to catabolize a variety of energy sources that include complex carbohydrates, glycerol, lactate, malate, citrate, the diamino acids arginine and agmatine, and many α-keto acids. *E. faecalis* contain abundant surface membrane proteins able to degrade mucin which can be advantageous by providing additional sugar for growth and by facilitating bacterial adhesion (27, 182). Enterococci produce bacteriocin peptides and some are used as starter or protection cultures including probiotics in food and feed supplements (129, 302). All these metabolic and physiologic features enhance enterococcal competitiveness in diverse environments. However, there are physiologic differences between enterococcal species which might contribute to the adaptation to different hosts and environments (182, 229).

Different factors can mediate the establishment, the colonization and further clinical infection by *E. faecalis* and *E. faecium.* They include microbial surface component-recognizing adhesive matrix molecules (MSCRAMM) and cell-wall-anchored LPxTG

surface proteins, and the ability to form biofilms; biofilm production seems to be greater among *E. faecalis* species due to the high number of surface-exposed proteins (145, 173, 363, 377). Numerous proteins with MSCRAMM-like structure and several genes or gene clusters involved in biofilm formation, are increasingly described, some of which are especially abundant in clinical isolates from dominant *E. faecium* and *E. faecalis* clonal complexes. Virulence determinants of *E. faecalis* include a bile salt hydrolase, the cytolysin toxin, *gelE* within the *fsr* cluster (coding for gelatinase), capsular polysaccharides, glycolipids, the collagen-binding Ace (adhesin of collagen of *E. faecalis*), *esp* (coding for an enterococcal surface protein), the *ebp* cluster (coding for a sortase associated with surface pili formation), the *epa* gene cluster (encoding the polysaccharide Epa), *atn* (coding for an autolysin), *bopD* (sugar-binding transcriptional regulator), *bee* (coding for a cell-anchored protein), and the LPxTG surface protein aggregation substance. *E. faecium* associated with human infections seem to be particularly enriched in putative virulence genes encoding a glycoside hydrolase (hyl_{Efm}), Esp, PilA- and PilB-type pili, and several cell wall-anchored LPxTG surface proteins including Acm (adhesin of collagen from *E. faecium*) and Scm (second collagen adhesin of *E. faecium*). Enterococci also possess environment-sensing systems consisting of a large repertoire of two-component signal transduction systems, some of which are regulated by quorum-sensing mechanisms (e.g. the *fsrABCD* system related to biofilm formation), extra-cytoplasmic function sigma factors (ECF σ), and transcriptional regulators that modulate gene expression in response to environmental changes and control diverse bacterial physiological processes (71).

Understanding the factors involved in the commensal relationship between bacteria and the host is crucial to understand the different colonization ability and host immune responses between different enterococcal species as is in the development of new and valuable therapeutic strategies to prevent human infections caused by enterococci.

1.1.3. Resistance to compounds with antibacterial activity (antibiotics, biocides, heavy metals)

Enterococci show resistance to antibiotics, heavy metals, antiseptics, disinfectants, ethidium bromide and ultraviolet radiation (35, 67, 233, 285, 303). Such resistances may be associated with multidrug efflux pumps located in the chromosome (often coding for resistance to different compounds) or different genetic traits located on mobile genetic elements (MGEs) as plasmids or conjugative transposons (often coding for resistance to a single compound or family of a given compound). Efflux pumps, permeability changes and transferable mosaic genetic platforms can encode resistance to biocides/heavy

metals/UVR/EtBr and antibiotics and therefore, the selective pressure exerted by the first ones may favour the expression and co-dissemination of resistance to different antibiotics (277, 278, 356).

Enterococci show *intrinsic* and *acquired* resistance against antibiotics of different families. While intrinsic resistance is an inherent property of a given species and thus is chromosomally encoded, acquired resistance results from a mutation or acquisition of foreign DNA (293). The compounds used to treat or prevent the appearance of bacteria can be classified according to their effects on the bacterial growth in *bacteriostatic*, those that prevent the growth by keeping bacteria at the stationary phase, and *bactericidal*, those that kills bacteria. However, some broad classes of antibacterial agents considered bactericidal can exhibit bacteriostatic activity *in vitro* and vice-versa (320). In addition, bacteria can develop *tolerance* to a given antibiotic when they are inhibited but not killed by concentrations manyfold higher than the MIC (minimum inhibitory concentration). By definition, tolerance involves a reduction in the rate of antibiotic-induced killing of a whole bacterial population which evade only the killing action of an antibiotic. Virtually, all studies on this phenomenon have been restricted to tolerance to β-lactam antibiotics or other cell wall inhibitors. In addition to *genotypic tolerance*, which is a property restricted to some bacterial mutants that specifically evade only the killing effect of β-lactam antibiotics without increase in the MIC, every bacterial cell can evade the killing effect of β-lactam antibiotics under certain physiological conditions, the *phenotypic tolerance*, as is the most universal example of nongrowing (dormant) or slowly growing bacteria. *In vitro* phenotypic tolerance has been defined as a minimum bactericidal concentration (MBC) that is 32 times the MIC (293, 320, 432).

1.1.3.1. ***Intrinsic resistance*** in enterococci includes resistance to β-lactams, aminoglycosides, and trimethoprim-sulfamethoxazole. Additional resistances to lincosamides and vancomycin are characteristics of specific enterococcal species (231, 293, 294).

Enterococci show intrinsic resistance to most **β-lactam** antibiotics (MIC of 1 to 8 μg/mL) due to the low affinity of specific cell wall proteins to bind penicillin. Such proteins required for the synthesis of the enterococcal cell wall are the target for beta-lactam compounds and are known as penicillin-binding proteins (PBPs). More specifically, intrinsic resistance of *E. faecium* and *E. hirae* has been attributed to the presence of PBP5, which is highly expressed and has marked lower affinity for β-lactams than the other PBPs (MIC of 16 to 64 μg/mL). The level of resistance varies among β-lactams, with penicillins showing the highest *in vitro* activity (ampicillin being the most active) followed

by carbapenems and cephalosporins which are not used in the clinical practice for the treatment of enterococcal infections. In addition, enterococci are typically tolerant to all β-lactams, and *in vitro* MBCs of penicillins and cephalosporins are usually >100 μg/mL (231, 293).

Enterococci exhibit intrinsic low level resistance to all **aminoglycosides** (MIC of 8-64 μg/mL) conferred by the low uptake or permeability of these agents. In *E. faecium,* intrinsic resistance to aminoglycosides is conferred by the chromosomal genes *efmM*, encoding the *E. faecium* methyltransferase, and the *aac(6')-Ii* that encodes a aminoglycoside 6'-N-aminoglycoside acetyltransferase (139, 293). However, the aminoglycoside poor active transport, which is due to poor membrane energization, is enhanced when enterococci are exposed to β-lactams or any antibiotic that act against the cell wall. When antibiotics which act by different mechanisms, such as cell membrane disruption (β-lactams) and inhibition of ribosomal protein synthesis (aminoglycosides), the disruption of the bacterial cell wall permits the invasion and enhanced uptake of the aminoglycoside resulting in a synergistic and bactericidal effect (231, 283). Serious enterococcal infections like endocarditis and meningitis require bactericidal therapy and this effect must be achieved by the synergistic combination of a cell wall active agent (penicillin or vancomycin) with an aminoglycoside (7, 293).

Enterococci appear susceptible *in vitro* to **trimethoprim-sulfamethoxazole** (TMP/SMX) when tested in appropriate conditions (media devoided of thymidine which reverses the action of TMP/SMX), but this combination should not be considered bactericidal *in vivo* as demonstrated by clinical failures during treatment with this antibiotic. This discrepancy may be due to the fact that enterococci can use exogenous folates in biological fluids and escape the inhibition of folate synthesis produced by the antibiotic. However, the reversal of this antifolate effect seems to be more important for *E. faecium* than for *E. faecalis* (231, 293). Resistance to trimethoprim species-specific of *E. faecalis* is due to the enzyme dihydrofolate reductase (DHFR) codified by *drfE* (MIC 16 to 512 μg/mL) (74).

E. faecalis, E. avium, E. gallinarum, and *E. casseliflavus* exhibit low level resistance (MIC of 12.5-100 μg/mL) to **lincosamides** (clindamycin and lincomycin) and **streptogramins A-type** due to the putative efflux of these antibiotics. All *E. faecalis* are intrinsically resistant to streptogramin A compounds due to the species specific gene *lsa* ("lincosamide and streptogramin A resistance") which confers resistance to both clindamycin and Quinupristin-Dalfopristin (MIC of 4-32 μg/mL) (293, 384).

E. gallinarum, *E. casseliflavus* and *E. flavescens* are intrinsically resistant to glycopeptides due to the chromosomal genes *vanC1*, *vanC2 and vanC3*, respectively, that produce a pentapeptidoglycan precursor terminating in D-Ala-D-Ser instead of D-Ala-

D-Ala. This low-level resistance to glycopeptides may be inducible or constitutive (MIC of vancomycin 2-32 µg/mL and MIC of teicoplanin 0.5-1 µg/mL) (79, 293).

1.1.3.2. *Acquired resistance* has been successively observed in enterococci over the past three decades and includes different classes of antibiotics such as phenicols, tetracyclines, macrolides, lincosamides, streptogramins, aminoglycosides, β-lactams, glycopeptides, and fluoroquinolones (200, 231, 293) (Figure 2).

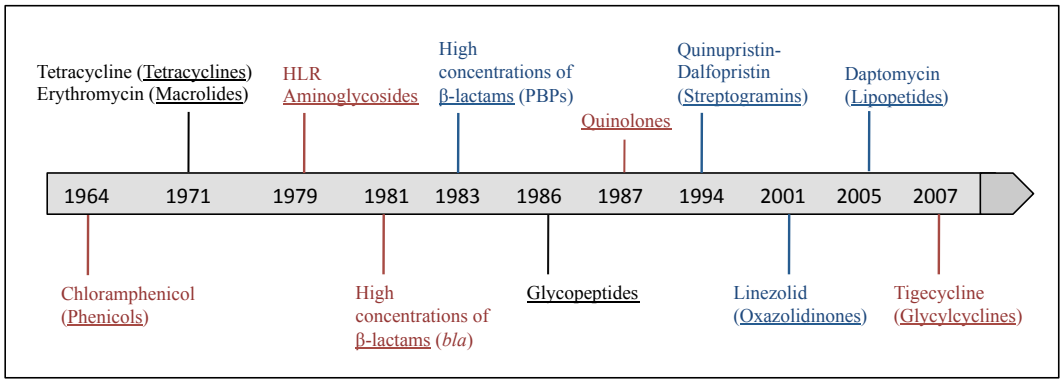


Figure 2. Chronologic representation of first descriptions of acquired resistance to different antibiotic families in enterococci. Enterococcal species where resistance first was reported appear in red for *E. faecalis*, in blue for *E. faecium*, and in black for both *E. faecalis* and *E. faecium.*
HLR, high-level resistance; PBPs, penicillin-binding proteins.

The first report of transferable resistance in enterococci in 1964 showed the transfer of **chloramphenicol** resistance between *E. faecalis* strains, which is generally mediated by a chloramphenicol acetyltransferase enzyme (CAT; MIC of >16 µg/mL) (324, 334). An active efflux pump has also been described in four *E. faecalis* and one *E. faecium* strains (251).

Acquired resistance to tetracyclines (MIC of >8 µg/mL) and macrolides (MIC of ≥8 µg/mL) was detected in clinical *E. faecalis* strains in early 1970's (11).

Two major mechanisms of **tetracycline** resistance are described in enterococci: i) active efflux of the antibiotic (*tetK, tetL*); and ii) ribosomal protection (*tetM, tetO, tetT, tetS*). The mechanism of action linked to *tetU* from *E. faecium* remains unknown (58, 279).

Acquired resistance to **macrolides, lincosamides and streptogramines** (MLS phenotype) in enterococci can be achieved by: i) modification of the target action by a rRNA methylase (*ermA, ermB, ermC, ermF, ermT*); ii) inactivating enzymes such as lyases (*vgbA*) and transferases (*lnuB, vatB, vatD, vatE*); or iii) antibiotic active efflux through ATP binding transporters (*msrA, msrC*, *msrD, lsaA, vgaB*) or major facilitators (*mefA*) (350) (http://faculty.washington.edu/marilynr/).

Enterococci can acquire high-level resistance (HLR) to **aminoglycosides** by three mechanisms: i) modification of the ribosomal target; ii) alteration of antibiotic transport (expulsion by efflux pumps or decreased cell membrane permeability); and iii) antibiotic modification by aminoglycoside-modifying enzymes (AMEs) (200, 293). The first two are due to chromosomal mutations whereas the last, generally mediated by MGEs, confers the highest levels of resistance: MIC of ≥500 µg/mL for gentamicin, MIC of ≥1000 µg/mL for streptomycin, and MIC of ≥2000 µg/mL for kanamycin. Acquired HLR to aminoglycosides makes bacteria resistant to the synergism of an aminoglycoside with a cell active agent as β-lactams and the bactericidal treatment of serious infections compromised. Of note, some AMEs acting in particular aminoglycosides do not confer HLR in the host (MICs generally of 64 to 256 µg/mL) but can prevent synergism and even yield antagonism between aminoglycosides and cell wall active agents (231).

AMEs are the most frequent mechanism of resistance to aminoglycosides. They can be acetyltransferases (AACs), phosphotransferases (APHs), or nucleotidyltransferases (ANTs) depending if the antibiotic inactivation occurs through *N*-acetylation, *O*-phosphorylation or *O*-nucleotidylation, respectively. They vary in their aminoglycoside's spectrum of action which often overlap (Table 1).

Table 1. Aminoglycoside-modifying enzymes described in enterococci (adapted from 231).

Enzyme group	Enzyme	Phenotype[d]	*Enterococcus* range of species
Acetyltransferases	AAC(6')-Ie[a]	KAN, TOB, AMI, NEO, FOR	*E. faecalis, E. faecium, E. casseliflavus, E. gallinarum*
	AAC(6')-Ii[b]	KAN, TOB, AMI, NEO	*E. faecium*
	AAC(6')-Im	KAN, TOB, AMI, DIB, NET	*E. faecium*
Phosphotransferases	APH(3')-III	KAN, AMI, ISE, NEO, BUT, RIB, LIV	*E. faecalis, E. faecium, E. casseliflavus, E. gallinarum*
	APH(2'')-Ia[a,c]	GEN, NET, SIS, KAN, TOB, AMI, ISE, NEO, BUT, RIB, LIV	*E. faecalis, E. faecium, E. casseliflavus, E. gallinarum*
	APH(2'')-Ib[c]	GEN, NET, KAN, TOB, DIB, AMI, ISE	*E. faecium, E. durans*
	APH(2'')-Ic[c]	GEN, NET, KAN, TOB	*E. faecalis, E. faecium, E. gallinarum*
	APH(2'')-Id[c]	GEN, NET, KAN, TOB	*E. faecalis, E. faecium, E. casseliflavus*
Nucleotidyltransferases	ANT(3')-Ia	STR	*E. faecalis*
	ANT(6)-Ia	STR	*E. faecalis*
	ANT(9)-Ia	SPE	*E. faecalis, E. durans*
	ANT(9)-Ib	SPE	*E. faecalis*
	ANT(4')-Ia	KAN, TOB, AMI, NEO, DIB	*E. faecalis, E. gallinarum*

[a]Part of the bifunctional enzyme AAC(6')-APH(2'') which confers HLR to all available aminoglycosides except streptomycin. It is widespread in enterococci from both animal and human origins and accounts for more than 90% of clinical enterococci that have HLR to gentamicin (usually MIC of ≥2000 µg/mL).
[b]Intrinsic resistance in *E. faecium*.
[c]All these APH enzymes confer HLR to gentamicin, often with intermediated MIC values of <500 µg/mL but with the synergy between ampicillin and gentamicin supressed.
[d]AMI, amikacin; BUT, butirosin; DIB, dibekacin; FOR, fortimicin; GEN, gentamicin; ISE, isepamycin; KAN, kanamycin; LIV, lividomycin; NEO, neomycin; NET, netilmicin; RIB, ribostamycin; SIS, sisomicin; SPE, spectinomycin; STR, streptomycin; TOB, tobramycin.

Acquired resistance to **β-lactams** can be due to the production of a β-lactamase (*E. faecalis*) or modifications of PBPs that include the mutation, hyperproduction or transferability of *pbp* genes associated to composite genetic platforms (*E. faecalis, E. faecium).* The production of β-lactamase confers resistance to penicillin, aminopenicillins (ampicillin) and ureido-penicillins (piperacillin) and low level of resistance to imipenem. The enzyme is inhibited by clavulanic acid, sulbactam and tazobactam. The enterococcal β-lactamase was firstly identified in 1981 and although has been detected in different countries of different continents, its prevalence remains low (292).

HLR to β-lactams (MIC of ≥32-≥256 μg/mL) in most clinical isolates of *E. faecium* has been associated with mutations or overproduction of *pbp5* with increased low affinity for penicillins, which has proved to be transferable in some instances (294, 340). This HLR of *E. faecium* may be an extreme example of intrinsic resistance associated with low affinity PBPs or may represent acquired resistance. Alterations in different PBPs can also confer resistance to β-lactams in *E. faecalis*. They include mutations in the *pbp4* gene, apparently increased production of PBP 5 and decreased penicillin binding of PBPs 1 and 6 (52, 315).

Acquired resistance to **glycopeptides** (vancomycin and teicoplanin) was detected in clinical isolates of *E. faecium* and *E. faecalis* collected in Europe in 1986 and the USA in 1988, respectively (232, 357, 437). The mechanism of resistance involves modification of the target site via a switch in the cell wall composition from pentapeptide ending in D-Ala:D-Ala to other aminoacid termination, resulting in peptidoglycan precursors with low affinity for these antibiotics.

To date, seven types of acquired glycopeptide resistance (VanA/B/D/E/G/L/M) have been described based not only in the genetic level (gene similarity and synteny) (Figure 3) but also in the phenotype they confer (33, 326, 464, 475). The *van* gene cluster *vanF* has been described in a biopesticide, *Paenibacillus popilliae*, but has not yet been found in enterococci (322). The different clusters or types of vancomycin resistance are designated according to the name of the ligase gene, which encodes either a D-Ala:D-Lac or a D-Ala:D-Ser ligase for the synthesis of peptidoglycan precursors with low affinity for glycopeptides (Table 2). The mechanism of resistance is complex but in all cases, the genetic element consists on a cluster of 5-7 genes encoding similar enzymatic functions. The *vanA*, *vanB*, *vanD* and *vanM* gene clusters contain genes for a two-component regulatory system (*vanR* and *vanS*), three resistance genes (*vanH*, encoding dehydrogenase; *vanA*, *vanB*, *vanD* or *vanM*, encoding ligase; *vanX*, encoding DD-dipeptidase); an accessory gene (*vanY*); and the *vanZ* gene, which is present in the *vanA* gene cluster, whereas the *vanW* gene is found only in the *vanB* operon (Figure 3). The *vanC, vanE* and *vanL* operons are organized similarly: *vanC, vanE* or *vanL, vanXY* (D,D-

dipeptidase-D,D-carboxypeptidase), *vanT* (serine racemase; two *vanTm* and *vanTr* in the case of *vanL*), *vanR* (response regulator), and *vanS* (sensor histidine kinase). The *vanG* operon begins with a three-component regulatory system, *vanU* (transcriptional regulator), *vanR, vanS, vanY* (D,D-carboxypeptidase), *vanW* (unknown function), and then *vanG, vanXY*, and *vanT* (78, 79).

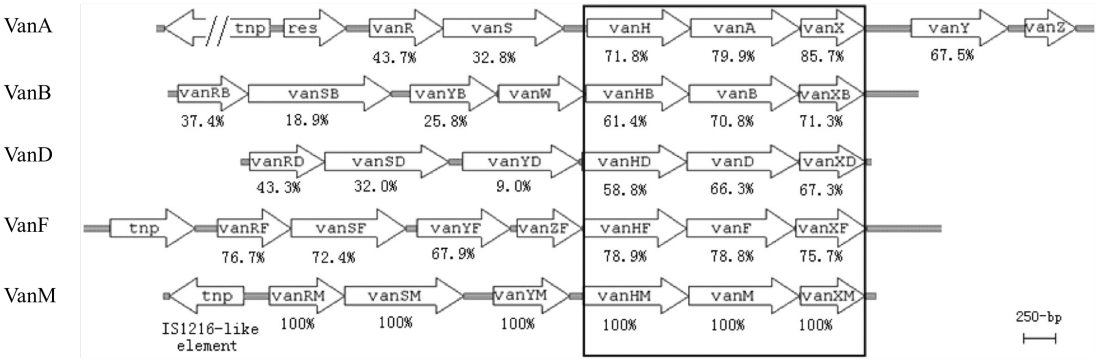


Figure 3. Alignment of vancomycin resistance gene clusters of *vanA, vanB, vanD, vanF* and *vanM* (adapted from 475). The percent amino acid identities with *vanRM*, *vanSM*, *vanYM*, *vanHM*, *vanM*, and *vanXM* products are shown below the respective genes.

Acquired glycopeptide resistance is mainly due to VanA and VanB phenotypes which are the most frequently reported in clinical isolates. The *vanA* gene cluster mediates high-level inducible resistance to both vancomycin and teicoplanin, while *vanB* causes variable levels of resistance to vancomycin but none to teicoplanin, being resistance only inducible by vancomycin (464). The genotypes VanA and VanB are generally associated with mobile genetic elements (transposons and/or plasmids), which are described in sections 1.3.2.2.-1.3.2.4. and 1.3.3. of this chapter.

Table 2. Characteristics of acquired *van* gene clusters in enterococci.

Type	Resistance (expression)	Modified target	MIC (µg/mL) VAN / TEC	Location (mobile element)
VanA	High level (inducible)	D-Ala-D-Lac	64-1000 / 16-512	Plasmid/Chromosome (Tn*1546*)
VanB	Variable level (inducible)	D-Ala-D-Lac	4-1000 / 0.5-1	Plasmid/Chromosome (Tn*1547*, Tn*1549*, Tn*5382*)
VanD	Moderate level (constitutive)	D-Ala-D-Lac	64-128 / 4-64	Chromosome
VanE	Low level (inducible/constitutive)	D-Ala-D-Ser	8-32 / 0.5	Chromosome
VanG	Low level (inducible)	D-Ala-D-Ser	16 / 0.5	Chromosome
VanL	Low level (inducible)	D-Ala-D-Ser	8 / susceptible	Chromosome
VanM	High level (inducible)	D-Ala-D-Lac	>256 / 96	Plasmid

Abbreviations: MIC, minimal inhibitory concentration; VAN, vancomycin; TEC, teicoplanin.

Acquired high-level resistance to **quinolones** is associated with amino acid changes in the "quinolone-resistance determining region" (QRDR) of the genes encoding topoisomerase IV (*parC, parE*) and DNA gyrase (*gyrA, gyrB*), which are essential for replication. The ParC mutation Ser-80→Ile alone or combined with the GyrA mutation Ser-83→Tyr confers high-level ciprofloxacin resistance (MIC of 64 to >128 µg/mL) (200, 438). Resistance by active efflux of quinolones or increased expression of endogenous efflux pumps has also been suggested for *E. faecalis* (194, 233).

Linezolid (a synthetic oxazolidinone) shows good activity against most clinical isolates of enterococci and it is restricted to the treatment of multidrug resistant isolates. However, resistance to this antimicrobial agent due to mutations of the 23S rRNA modifying the target interaction site has been reported (7, 119).

Daptomycin (a cyclic lipopeptide) is active against most multidrug resistant enterococci including vancomycin-resistant enterococci, being the only antibiotic with *in vitro* bactericidal activity against VRE (vancomycin-resistant enterococci). Rare occurrences of isolates resistant to this antibiotic have been reported (MIC of >4 µg/mL), but the mechanisms underlying the reduced susceptibility to daptomycin were not investigated (7, 48).

Tigecycline (a glycylcycline) is used to treat a broad spectrum of multidrug resistant isolates. Only an *E. faecalis* resistant to tigecycline (MIC of 2 µg/mL) has been recently described although the exact mechanism of resistance was not elucidated (458).

1.1.4. Clinical enterococcal infections

Enterococci have been recognized as a cause of endocarditis and urinary tract infections since the early 1900's, and have rapidly emerged as one of the most common nosocomial pathogens since the 1980s. They may also be responsible for other severe healthcare-associated infections that includes intra-abdominal, pelvic and soft tissue infections, and bacteremia. Rare infections caused by enterococci include meningitis, hematogenous osteomyelitis, septic arthritis, and pneumonia (260, 293, 294, 403). As opportunistic pathogens, they are often isolated from elderly and debilitated patients who have received multiple courses of antibiotics and/or have been hospitalized for long periods or time (162, 287, 293, 294).

Last reports provided by the National Nosocomial Infections Surveillance System (NNISS) and the National Healthcare Safety Network (NHSN) in the United States, from the European Antimicrobial Resistance Surveillance Network (EARS-Net), and from the SENTRY program show that rates of serious enterococcal infections remained relatively stable through the last years (22, 174, 403) (www.rivm.nl/earss). However, a progressive

inversion of the ratio of *E. faecalis* and *E. faecium* causing disease (from 10:1 before 1990, to 3:1 in the late 1990s, and currently 1.5:1) has been reported (174, 431). This coincided with an increasing trend towards multiantibiotic-resistant *E. faecium*, for which ampicillin and vancomycin resistance are much more prevalent than in *E. faecalis* recovered from clinical infections (57, 102, 152, 265, 291, 431, 471). Also a higher mortality rate has been associated with *E. faecium* than with *E. faecalis* independent of the antibiotic profile (244, 305). Human infections caused by *E. durans, E. avium, E. gallinarum, E. casseliflavus, E. hirae, E. muntdii*, and *E. raffinosus* have been rarely documented (69, 165, 202, 293, 360, 406). Multidrug-resistant enterococci are a major cause of mortality and morbidity in hospitalized patients worldwide posing increased therapeutic and economic challenges to healthcare institutions.

1.1.5. Treatment

Ampicillin and penicillin remain the antibiotics of choice for susceptible isolates but tetracyclines, chloramphenicol and quinolones are alternatives against these strains. Fosfomycin and nitrofurantoin are restricted to treat urinary tract infections.

A bactericidal effect is required for severe enterococcal infections which is achieved by the combination of a cell active agent (β-lactam, vancomycin) and an aminoglycoside as stated above (section 1.1.3.1; 293). Nonetheless, the treatment of ampicillin- and vancomycin-resistant enterococcal infections is nowadays compromised with the increasing acquisition and accumulation of different mechanisms of resistance to these agents.

New treatment options have been introduced in the therapeutic arsenal in the last years. Several combination therapies using different antibiotics have been recently used in the treatment of enterococcal infections, though the frequent lack of clinical experience, the increased rates of resistance to some compounds, and their variable activity depending on the strain, demand their use for multidrug resistant enterococci. The determination of the bactericidal effect by time-kill curves might be needed for selection of the most appropriate therapeutic regimen when options are limited (7).

1.2. Ecology and population structure of *Enterococcus*

1.2.1. Ecology

Enterococci are a normal inhabitant of the intestinal tract in most mammals and birds, and they can be also recovered from reptiles, insects, soil, plants, food and water (3).

The healthy human intestinal microbiota are dominated (>90%) by species from the phyla *Firmicutes* and *Bacteroidetes* (Figure 4; 112, 264). The concentration of enterococci in faeces is about 6-8 $\log_{10}$ cells/g of faeces, which is significantly lower than that for the most abundant orders of bacteria in faeces such as the *Clostridiales (Firmicutes)*, *Bifidobacteriales* (*Actinobacteria*) and *Bacteroidales (Bacteroidetes)* (9-10 $\log_{10}$ cells/g feces). However, *E. faecium* and *E. faecalis* were found to be one of the predominant members of the small intestine (jejunum and ileum) (167).

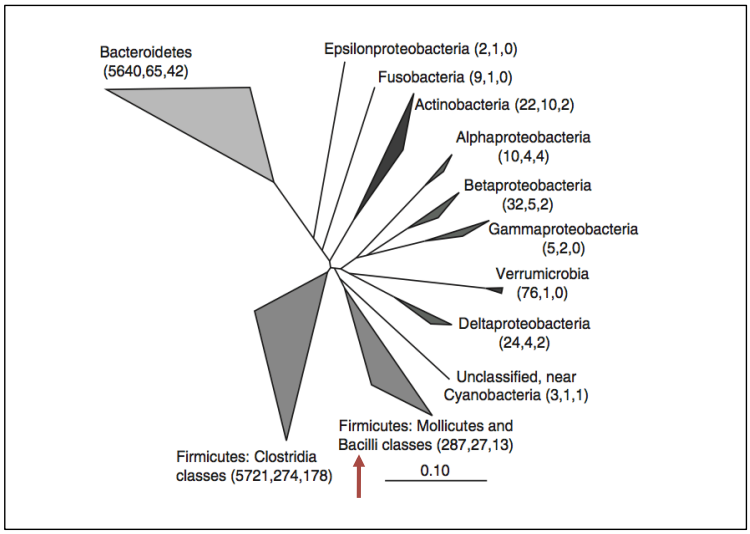


Figure 4. Phylogenetic tree of human intestinal 16S rRNA sequences (adapted from 264). The arrow indicates the clade that includes *Enterococcus*.

The genus *Enterococcus* comprises several species some of which showing adaptation to different host or environmental conditions. While *E. faecium* and *E. faecalis* are widely distributed, yellow-pigmented species *E. mundtii*, *E. casseliflavus* or *E. sulfureus* are associated with plants and *E. columbae* and *E. asinii* are apparently associated with pigeons and donkeys, respectively. Variations in the distribution of widespread enterococcal species varies depending on the hosts (*E. faecalis* predominates in cats, dogs, calves and pigs, while *E. faecium* are more abundant among poultry), and the age (*E. cecorum* is particularly associated with older poultry), or the intestinal compartments (*E. faecium* predominates in the caecum and *E. faecalis* in the recto-sigmoid colon of human intestine) within a given host (3, 167, 213). In addition to *E. faecium* and *E. faecalis*, *E. hirae, E. durans* and *E. gallinarum* are the most common

species recovered from farm animals and pets; and *E. durans* from dairy products (fermented food products as cheeses and milk), fish, olives, vegetables, raw minced beef and pork (129, 213, 313). A variable occurrence of different enterococci species has been described among wild animals (250, 267, 328, 329). *Enterococcus raffinosus* has only been detected in pathological materials and its habitat is unknown (406).

E. faecium and *E. faecalis* are abundant in the small intestine, whereas *E. avium* and *E. durans* only occasionally colonize the human intestine (30, 273, 374, 406). Enterococci may also colonize the oropharynx, mouth, vagina, perineal region and soft tissue wounds of asymptomatic humans, where *E. faecalis* is generally more abundant than *E. faecium* (56, 293).

1.2.2. Population structure

The geographic and ecologic expansion of different clones or clonal complexes of enterococci from different origins have been documented in different studies. The diversity and evolution of the main enterococci species found in humans is greatly influenced by homologous recombination related with the acquisition of different genetic traits. Main definitions related with the study of population structure are included in Box 1 (for reviews see 122, 157, 391, 392, 436).

1.2.2.1. *Enterococcus faecium*

Different studies have demonstrated that *E. faecium* species comprises different ecovars o genogroups showing common genotypic and phenotypic features (177, 442, 466, 469). Multilocus sequence typing (MLST) of a high number of isolates from different origins, hosts and geographical areas has shown that *E. faecium* has a highly recombinant population structure that comprises a large number of sequence types (STs) at linkage equilibrium with overrepresentation of specific clonal complexes (CCs).

The first population structure analysis of *E. faecium* performed with isolates from different origins and using the techniques amplified fragment length polymorphism (AFLP) and ribotyping, grouped isolates in different genogroups A (non-hospitalized persons and pigs), B (poultry), C (hospitalized patients - designated by that time as “lineage C1” - and pets), and D (veal calves) (26, 31, 38, 41, 76, 196, 197, 442, 466).

Box 1. Population structure glossary

Population structure: it ranges from clonal to panmictic

- *Clonal* - not influenced by horizontal genetic exchange and recombination, best modelled by a tree-like phylogeny
- *Panmictic* - non-clonal, all recombination is possible, best modelled by a phylogeny network

Tree-like phylogeny: a branching phylogeny where each member has a single ancestor.
Phylogeny network: a phylogeny where the nodes are connected by multiple links and members can have multiple ancestors.

Linkage disequilibrum: clonal populations are a consequence of asexual reproduction, typically at linkage disequilibrum, e.g., with non-random association among alleles at different loci.
Linkage equilibrum: non-clonal populations are generated by frequent recombination and characterized by linkage equilibrum, e.g., random association among alleles at different loci.

Epidemic clone: A rapid and transitory clonal expansion can occur in a population where recombination is frequent within all members generating descendants of a particularly successful variant.

Meroclone: A dominant clone may diversify by accumulation of mutations or horizontal transfer becoming a meroclone.

Ecovar: The term “ecovar” is used in the literature to name, based on phenotypic or genotypic characteristics, particular groups within a bacterial species associated with specific hosts and environments.

Afterwards, analysis by MLST identified different predominant clonal lineages recovered from swine (CC5), poultry (CC9), veal calf (CC1), hospitalized humans (CC17, preliminary designated as CC1), and healthy humans (CC22, CC94) (94, 107, 177, 468) thus confirming preliminary results by AFLP (466). Currently, isolates from major human-adapted clones are increasingly recovered from farm and companion animals, and strains from clonal complexes commonly found among animals are also collected from humans (21, 88, 89, 469).

Most of the *E. faecium* isolates causing human infections and outbreaks in hospitals of different continents belong to CC17 (140, 468, 469). CC17 is a meroclone comprising a high number of related STs which were thought to evolved from a ST17 founder (226, 468). Recombination, besides mutation, seems to have played a primary role in the genetic diversification of *E. faecium*, along with the acquisition and accumulation of adaptive mechanisms through horizontal transfer. CC17 isolates are enriched in traits conferring resistance to antibiotics (high levels of ampicillin, streptomycin, kanamycin, erythromycin and quinolones) or enhancing invasiveness as Esp (a protein involved in colonization and formation of biofilms which is coded by genes located in a pathogenicity

island) and Hyl (a glycoside-hydrolase enabling the metabolism of complex carbohydrates). The cumulative gain of different traits might have contributed to the adaptation and further global expansion of CC17 (75, 76, 211, 225-227, 336, 456, 465). Selection, diversification and global expansion of seven ampicillin-resistant CC17 genotypes (ST16, ST17, ST18, ST78, ST117, ST192, and ST203) seem to have occurred since at least 1982 (23, 29, 37, 46, 49, 76, 87, 100, 114, 140, 177, 179, 205, 206, 211, 215, 216, 237, 240, 319, 429, 438, 455, 468, 470, 479, 482). CC17 has facilitated the global spread of VRE in hospitals of different continents as most of VRE isolates from hospitalized humans belong to this lineage (226). Outbreaks of CC17 *E. faecium* susceptible to glycopeptides but resistant to ampicillin have been reported (76, 197, 237, 429).

The genetic relationship among *E. faecium* STs has traditionally been analyzed by using the eBURST algorithm which groups STs differing in not more than two of the seven loci and thought to be descendant from a common ancestor (121). Nonetheless, phylogenetic inferences by using eBURST may result unreliable for species with high recombination rates in combination with a relatively low mutation rate as shown for *E. faecium* (434, 468). An optimized implementation of eBURST is goeBURST which has corroborated the clustering of most CC17-STs (132). ClonalFrame, which is based on nucleotide sequences of the 7 *E. faecium* housekeeping gene sequences rather than alleles for a Bayesian-based phylogenetic analysis (103), indicated that the CC17-STs does not share a recent common ancestry but should be split into different CCs as founder of distinct clonal complexes. This further suggests that hospital-derived *E. faecium* may not have evolved from a single founder but rather have evolved independently from different ancestral clones (469).

1.2.2.2. *Enterococcus faecalis*

A number of molecular methods including PFGE, AFLP, ribotyping, capsule typing, RFLP analysis, rep-PCR and RAPD, have been applied to the epidemiological analysis of *E. faecalis* (150, 180, 261, 371, 418, 447).

Different MLST schemes have been developed for analysing the *E. faecalis* population structure (298, 300, 354). An alternative trilocus sequence typing scheme based on antigenic or cell surface proteins revealed to have equivalent discriminatory power to that of PFGE and MLST (59). All these analysis have shown that *E. faecalis* has an epidemic population structure at linkage equilibrium due to high levels of recombination and that apparently lacks relevant host specificity. The contemporary *E. faecalis* population structure comprises a diversity of STs with overrepresentation of clonal

complexes CC2, CC9, CC21, CC40 and CC87 and the prominent singleton ST16 (122, 203, 226, 275, 354, 470).

ST6/CC2 seems to be particularly enriched in mobile genetic elements and genes encoding surface structure-proteins and is one of the most widespread clonal complexes, while specific CCs (CC40, CC87) might be endemic in particular geographical regions (203, 236, 272, 274, 275, 332, 355, 376, 395). ST21/CC21 and ST16 have been also identified among hospital-derived isolates. However, these prevalent clones have been frequently found in the community as well, including farm animals and food products. Also other less prevalent singletons as ST116 have been identified both in animal and human strains indicating that *E. faecalis* clones are more often shared between different reservoirs (5, 42, 221, 248, 275, 376, 400). The finding of virulence genes such as cytolysin, the enterococcal surface protein Esp and aggregation substance among animals, food and healthy babies further confirms the lack of relevant host specificity of *E. faecalis* clones (373, 376, 394). In contrast to that described for *E. faecium*, an association among vancomycin resistance and a particular clonal complex has not been identified among *E. faecalis*. However, CRISPR-cas loci which are regularly interspaced short palindromic repeat (CRISPR) loci with associated genes (*cas* genes) providing bacteria with a sequence-specific, acquired defense against plasmids and phages, are not uniformly distributed among different MLST lineages. In contrast to other lineages (e.g., ST21 and ST40), they were found to be absent in ST6/CC2, ST9/CC9 and CC8 indicating that loss of CRISPR-cas in these widespread lineages might have contributed to the acquisition of genetic traits facilitating their hospital adaptation (318).

The relationships among *E. faecalis* STs have been exclusively analyzed by using the eBURST algorithm which seems to be reliable and sensible for the typing of clonal populations (121, 434). Nevertheless, recent studies indicate the need to revisitate the *E. faecalis* population structure, since novel STs are increasingly being described, both among human and non-human sources (42, 332, 394, 400).

1.3. Mobile genetic elements in *Enterococcus*

1.3.1. Lateral genetic transfer

Lateral genetic transfer (LGT) seems to be crucial in the evolution of *Firmicutes*, which present a broad host spectrum of phyla and prokaryotes for exchanging DNA (18). Lactobacilli bacterial genera appear particularly prone to participate in transfer genetic events (Figure 5). Horizontal transfer seems to be more influenced by the physical proximity among organisms sharing similar genomic properties such as genome size,

genome G/C composition, and carbon utilization, than by phylogenetic proximity. The dynamic interplay between groups of bacterial species, genetic elements and environments constructs communities of genetic exchange (GEC, *genetic exchange community*) (188, 385). We can consider each enterococcal species as constituted by different populations of clones with specific properties enabling adaptation to particular conditions and therefore diversely influenced by LGT with microorganisms of different GECs.

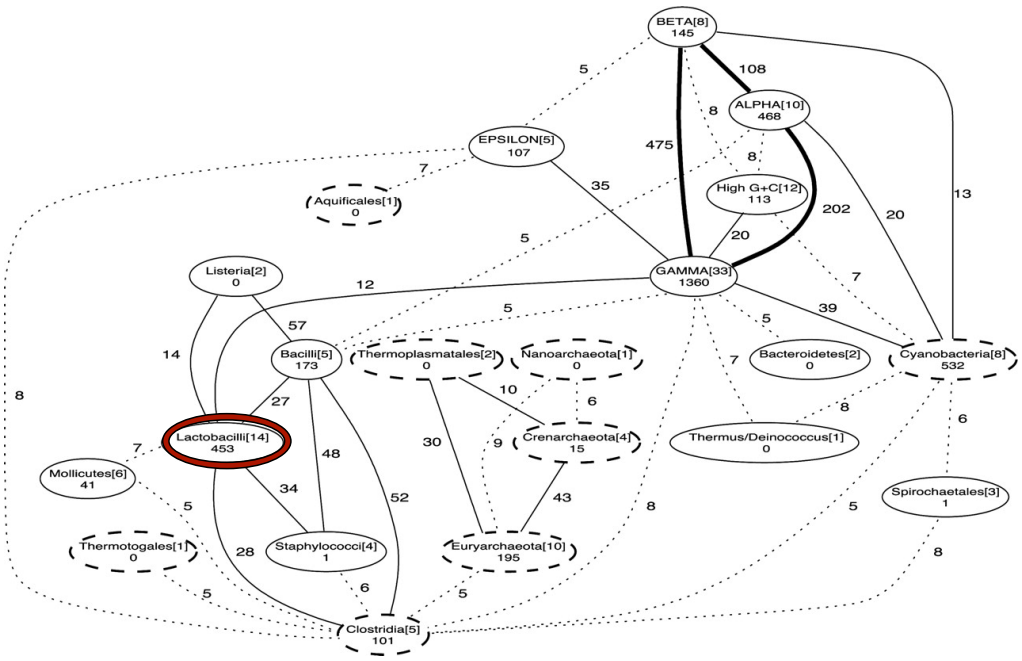


Figure 5. Highways of obligate gene transfer within and among phyla and divisions of prokaryotes (adapted from 18). Each oval represents a prokaryotic group, with the name and number of taxa in that group indicated on the first line. Numbers below taxon names report inferred transfers within that taxon, whereas numbers on the linking edges report inferred transfers between taxa.

LGT seems to be important in the adaptation of *E. faecium* and *E. faecalis* to specific environments as different traits involved in enhancing colonization, virulence or persistence are located on MGEs (Figure 6). They include genes coding for adhesins, resistance to antibiotic, biocides or metals, social traits (bacteriocins, restriction-modification systems) or metabolic pathways (92, 136, 316, 430). A diversity of MGEs has been identified among enterococci which includes transposons or transposable elements, genetic islands, plasmids, bacteriophages and self splicing elements. We will revise the main characteristics of transposons and plasmids which were further studied in this thesis.

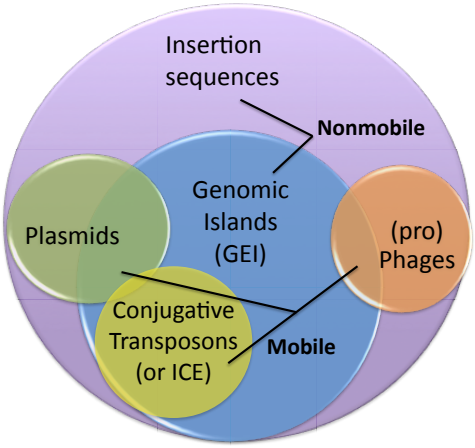


Figure 6. Organization of the mobile genome pool (adapted from 195). The accessory/flexible genetic elements are represented in coloured circles. ICE, Integrative Conjugative Elements.

1.3.2. Transposable elements

Transposable elements (TE) are widely distributed among enterococci and comprise insertion sequences, composite transposons, Tn*3*-derivatives transposons, and conjugative transposons. Composite and Tn*3* transposons are widespread among gram-positive and gram-negative bacteria while CTns are mostly found among gram-positive microorganisms (64, 451).

1.3.2.1. Insertion sequences

Insertion sequences (IS) are the simplest TE consisting of short DNA sequences (0.7-2,5-kb in length) codifying for a transposase necessary for their mobilization and insertion (Figure 7). ISs can exist as autonomous TEs or as part of composite transposons (257, 381).

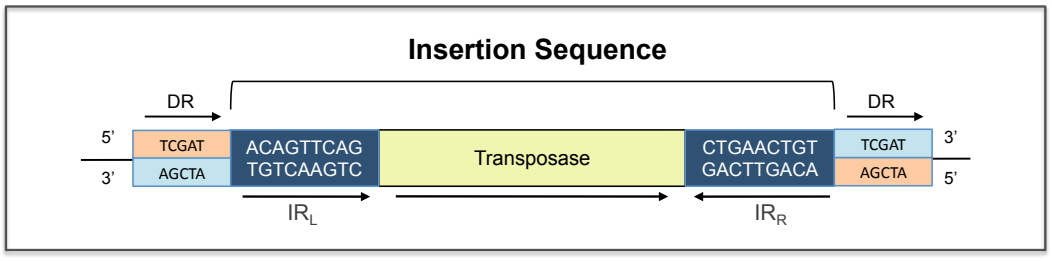


Figure 7. Organization of a typical insertion sequence (IS). Most ISs have terminal inverted repeats (IR_L, left Inverted Repeat; IR_R, right Inverted Repeat) at their ends (10 – 40-bp) flanking the gene encoding the transposition function. On insertion, most ISs generate short directly repeated sequences (DRs of 2 – 14-bp length) of the target DNA flanking the IS. The presence of these DRs is linked to the transposition, whether replicative or non-replicative/conservative, although they are very similar at the mechanistic level (53, 256, 280).

Based on the nature of the transposase catalytic site, two types of ISs have been identified among enterocci: i) containing a conserved DDE motif; and ii) related to serine site-specific recombinases similarly to the rolling circle replicases (124, 256). ISs corresponding to 10 families[1] widespread among *Firmicutes* have been identified in enterococci from different ecological niches (Table 3). Specific types are largely represented in both *E. faecalis* and *E. faecium* such as IS*16* and IS*256* (IS*256* family), IS*1216* (IS*6* family), IS*1476* and IS*1251* (IS*L3* family), and IS*Ef1*/IS*6770* (IS*30* family) (47, 181, 253, 309, 339, 344). IS*Ecp1*, often reported to be involved in the mobilization of CTX-M genes among *Enterobacteriaceae*, was recently described in *E. casseliflavus*, further demonstrating the relevance of LGT among very phylogenetically distant species (55).

To date, only specific ISs, such as IS*256* and IS*1216* have been associated with the formation of novel IS-based composite transposons (28, 342). IS*256*-like sequences enable Tn*1549* to transpose into conjugative plasmids while IS*1216* favors either transposition of nonconjugative plasmids carrying Tn*1542* into a conjugative plasmid or integration of Tn*5385* into the chromosome (168, 333, 337). The *aac6-aph2* gene mediating high-level gentamicin resistance has been associated with different Tn*4001*–like elements flanked by IS*256*, but the role of these flanking ISs remains to be characterized (51).

On the contrary, a variety of genetic elements is frequently interrupted by ISs. Homologous recombination across ISs can generate modular genetic platforms (e.g. Tn*5382*, Tn*5385*, and Tn*5384*) (28, 337), or modify MGE such as Tn*1546* or plasmids (309, 389, 411, 467), thus greatly contributing to their plasticity besides spread.

The wide distribution of particular ISs among the genomes of some enterococcal species has been exploited for typing purposes. The frequent presence of IS*Ef1*/IS*6770* among *E. faecium* and *E. faecalis* hospital strains has made to use this trait as an epidemiological marker of strains causing nosocomial infections (416). IS*16* seems to be widespread among CC17 *E. faecium* strains (228, 457). Changes in the whole genome organization of miniature insertion sequences (EFAR repeats) was proved to be useful to establish clonal relationships among *E. faecalis* isolates (444). Finally, the involvement in the formation of functional promoters for biofilm formation has been linked to IS*1191* in virulent *E. faecalis* strains (68).

[1]An IS family is defined as a group of ISs with related transposases, strong conservation of the catalytic site, conservation of organization and similar IRs. More than 3,000 individual ISs corresponding to 25 family groups in over 300 eubacterial and archaeal species are recorded at the website ISfinder (http://www-is.biotoul.fr; 124, 382). The transposition of ISs, which act as repetitive homologous sequences, is implicated in the disruption or activation of genes, as well as deletions, inversions or reorganizations of the genome. ISs are therefore an important source of genetic plasticity in prokaryotes with great impact in the assembly of gene clusters with adaptive functions such as antibiotic resistance, virulence or new catabolic pathways (99, 257, 381).

Table 3. Insertion sequences found among enterococcal mobile genetic elements.

Family group	Nature of the catalytic site	Insertion Sequence	Enterococcal genetic element
IS*6*	DDE	IS*1216*	Tn*1546*, Tn*5405,* Tn*5385* pIP816, pRE25, pRUM, pTEF1, pTEF3, pVEF1-2-3-4, pLG1, pS177, p5753cA, p5753cB, pUW1965, pUW786, pTW9, pSL1, pBEE99, pEF418, pEF-01, Efs-PAI, pA17Sv1, pJH1, pEJ97-1, pJM01, pHKK701
		IS*257*	Tn*5384,* Tn*5385,* Tn*924*
IS*256*	DDE	IS*256*	Tn*4001*, Tn*5384*, Tn*5382*, Tn*1547*, p5753cB pTEF1, pTEF2, pTEF3, pVEF1-2-3, pWZ1668
		IS*16*	Tn*1546*, Tn*1547,* pRUM
		IS*1191*/IS*905*[b]	Efs-PAI
		IS*1542*	Tn*1546*
IS*3*	DDE	IS*3*/IS*911*	pWZ1668, pBEE99, pLG1, pVEF4
		IS*150*	Tn*5382*, pUW786
		IS*Enfa3*	Tn*5382*
		IS*153*	
		IS*1485*	Tn*1546*, pRE25, p5753cB
		IS*981*	pEF1, pRE25[d], p5753cB
IS*982*	DDE	IS*19* (IS*Efm1*)[c]	Tn*1546*, *vanD* cluster
IS*30*	DDE	IS*6770 (*IS*Ef1*)[c]	Tn*1546*, Tn*1549*, pMRC01, pLG1, pBEE99
		IS*1252*	pHKK701, pHKK702, pIP816
		IS*1678*	Tn*1546*, pS177
		IS*1062*	pRE25, pPD1
IS*5*	DDE	IS*Efa5*	Tn*1546*
IS*110*	Site-specific recombinase	IS*110*/*116/902*	pEF01-1, pLG1
		IS*Enfa110*	Tn*5382*
IS*605*	Complex organization[a]	IS*Efa4*	*vanD* cluster, pRI1
IS*200*		IS*Enfa200*	Tn*5382*
IS*L3*	Unknown[a]	IS*L3*	
		IS*204*/IS*1001*/ IS*1096*/IS*1165*	Tn*1546*, pLG1
		IS*1251*	Tn*1546*, pS177
		IS*1476*	Tn*1546*
IS*1380*	Unknown[a]	IS*Ecp1*	
IS*NYC*	Unknown[a]	IS*1182*	Tn*5405*, pRUM, pUW786, pS177, p5753cB

Abbreviations: PAI, Pathogenicity island.
[a]The active sites of IS*200*/IS*605*, IS*L3*, IS*1380*, and IS*NYC* families have not been defined.
[b]IS*905* is a isoform of IS*1191*.
[c]IS*19* and IS*Efm1* and IS*6770* and IS*Ef1* are synonyms.
[d]The truncated version of IS*981* is present on pRE25.

1.3.2.2. Composite transposons

Composite transposons consist on a variable number of genes, mainly encoding antibiotic resistance or catabolic functions, which are flanked by terminal IS elements (in the same or opposite direction) (Figure 8; 280, 304).

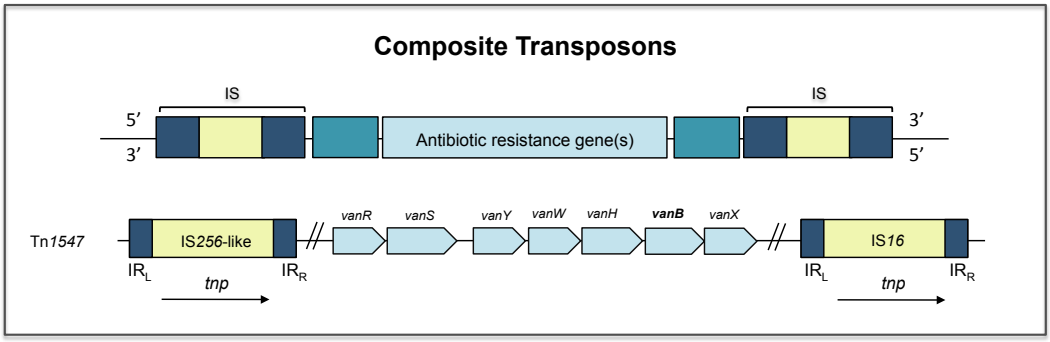


Figure 8. General organization of a composite transposon. Tn*1547*, which harbors the van*YWHB*$_1$*X* gene cluster encoding vancomycin resistance (333), is represented. The left end of Tn*1547* is identical to the 1324-bp IS*256* while the right end consists of IS*16* (both of IS*256* family). Abbreviations: *tnp*, transposase; IR_L, left Inverted Repeat; IR_R, right Inverted Repeat. Arrows represent IS orientation.

A wide diversity of enterococcal composite transposons (4.7 - 65.0-kb) associated with antibiotic resistance has been described (Table 4). Some representative examples are Tn*1547* (IS*256* family, *vanB*) conferring vancomycin resistance, Tn*5405* (IS*1182, aadE-sat4-aphA-3*) encoding aminoglycoside-streptothricin resistance, and highly related Tn*5281/5384/5385* encoding high-level gentamicin resistance (171, 459). Most of them are disseminated among strains of different species and origins due to their location on transferable broad host plasmids (411, 460).

Table 4. Characteristics of composite transposons identified in *Enterococcus*.

Transposon	Size (kb)	Resistance genes and flanking ISs (Tn backbone)	Host range	Refs.
Tn*5281*	4.7	IS*256-aac6'-aph2''*-IS*256* (inverted copies of ISs)	*Enterococcus, Staphylococcus aureus* (Tn*4001*), *Streptococcus agalactiae* (Tn*3706*), *Mycoplasma*	(176, 234, 383)
Tn*924*	27	IS*257-aac6'-aph2''*-IS*257*	*Enterococcus faecalis*	(412)
Tn*1547*	64	IS*256*-like-*vanB1*-IS*16* (ISs in direct orientation)	*Enterococcus*	(333)
Tn*5384*	26	IS*256*-Tn*4001*-ΔTn*917-mer*-IS*256* (*aac6'-aph2'', ermB, merX;* directly repeated copies of ISs)	*Enterococcus faecalis*	(28, 339)
Tn*5385*	65	IS*1216*-Tn*4001*-ΔTn*917-mer*-IS*1216* (*aac6'-aph2'', ermB, mer, aadE, tetM, blaZ;* directly repeated copies of ISs)	*Enterococcus faecalis*	(337)
Tn*5405*	12	IS*1182-addE-sat4-aphA-3*-IS*1182* (inverted copies of ISs)	*Enterococcus, Staphylococcus, Streptococcus, Campylobacter*	(101, 460)
Tn*5482*	26-30	IS*1216V-vanA*-IS*1216V*	*Enterococcus*	(163)
Tn*5506*	39	IS*1216V2-vanA*-IS*1252*-IS*1216V1* (IS*1216V2* in inverted direction of IS*1216V1*)	*Enterococcus faecium*	(168)

The presence of related transposable elements is favourable for the occurrence of homologous recombination across ISs located in transposons, plasmids, and chromosomes contributing to the transfer and spread of particular enterococcal transposons or influencing DNA rearrangements. Such genetic events might result in the acquisition of different traits (e.g. antibiotic resistance, virulence or bacteriocins) for adaptation to different environments (217, 266, 323).

1.3.2.3. Tn*3* derivatives

Tn*3*-like transposons typically encode a transposase (TnpA, enzyme involved in excision and integration) and a resolvase (TnpR, a site-specific recombinase for co-integrate resolution within the *res* site), and one variable DNA fragment (e.g. antibiotic resistance genes) in one genetic unit flanked by two inverted repeats (IR) (Figure 9; 153, 170, 212, 451). The mechanism of transposition is replicative meaning that after transposition both donor and recipient possess a copy of the genetic unit (238, 276).

The Tn*3* family of transposons is divided in two main subfamilies depending on whether their transposase and resolvase genes are transcribed oppositely (Tn*3* subfamily) or in the same direction (Tn*21* subfamily) (153). In enterococci, Tn*1546* and Tn*917*/Tn*3871* represent Tn*3* and Tn*21* subfamilies, respectively. The widespread of Tn*3*-transposons in enterococci is facilitated by location on conjugative plasmids (64).

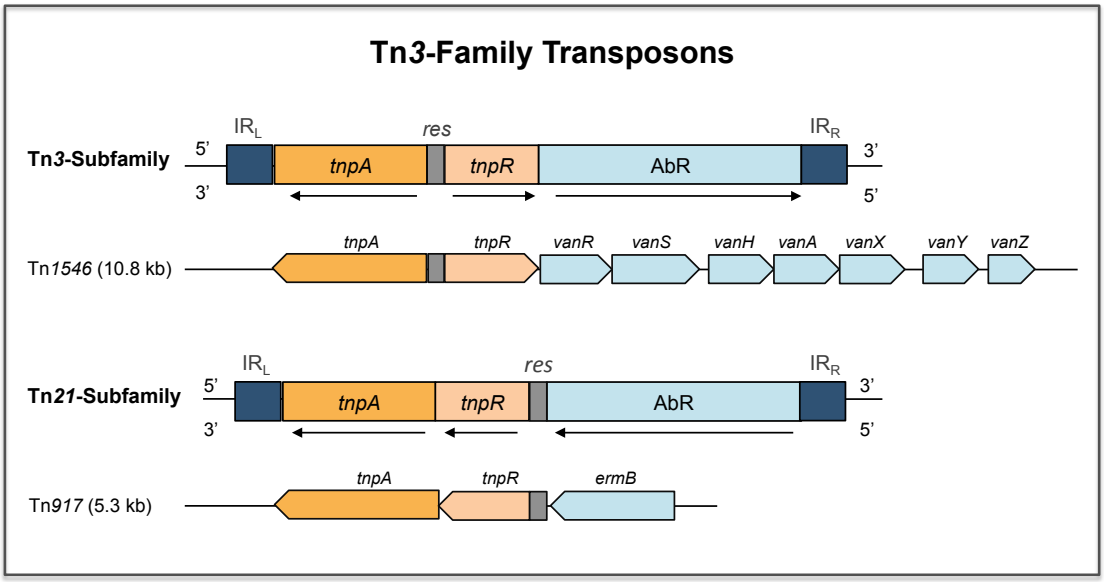


Figure 9. General organization of Tn*3*-family transposons. Both prototype Tn*3* and Tn*21* subfamilies are represented. The *res* site lies between the *tnpA* and *tnpR* genes in Tn*3*-subfamily and upstream of the *tnpR* and *tnpA* genes in Tn*21*-subfamily elements. Open reading frames (ORFs) correspond to arrow-shaped boxes and their names are given above the ORFs. Abbreviations: AbR, antibiotic resistance; *tnpA*, transposase; *tnpR*, resolvase; IR_L, left Inverted Repeat; IR_R, right Inverted Repeat.

Tn*1546* (10.8-kb) is a cluster of 7 *van* genes (*vanRSHAXYZ*) encoding high levels of resistance to vancomycin and teicoplanin (10, 464). The genes *vanHAX* are highly conserved and essential for the expression of glycopeptide resistance. Different Tn*1546* variants have emerged as a result of mutations, deletions, rearrangements and insertions within and between regions not essential for resistance (*orf1, orf2, van*Y, and *vanZ*) (47, 190, 309, 317, 321, 467, 472). Tn*1546* is the predominant genetic element coding for resistance to glycopeptides among enterococci from different hosts (62, 325, 453). The *vanA* gene has also been identified in other species recovered from gastrointestinal flora and from soil DNA mirroring a wide environmental dispersion (155, 156, 241, 262, 322, 330).

Tn*917* (5.3-kb) contains the *ermB* gene which codifies for macrolides-lincosamide-streptogramin B (MLS_B) resistance and is highly similar to Tn*551* from *S. aureus* (378, 419). Tn*917* is often located on hemolysin-bacteriocin plasmids of *E. faecalis* such as pAD1 and its transposition, as well as the expression of erythromycin resistance, is inducible by erythromycin (420). Tn*3871* (5.1-kb) is closely related to Tn*917* (15). These elements seem to be widely distributed among humans, food and foodborne animals (230, 351).

1.3.2.4. Conjugative Transposons /Integrative Conjugative Elements

Conjugative transposons (CTns) are self-transmissible elements that move from the chromosome of a donor bacteria to the chromosome of a recipient bacteria by a process that requires intercellular contact. Once in the recipient, they integrate in a site identical to that in which reside in the donor and they are capable of independent replication (for reviews see 44, 335, 348, 474). Site specific integration made to call them CONSTIN (*conjugative, self-transmissible, integrating element*) or ICEs *(integrative and conjugative element).* After years of discussions about the nomenclature of these elements, it was agreed that any new transposable elements would be named by using a modification of the previous Tn system. The registry for Tn numbers is currently hosted by University College London (www.ucl.ac.uk/eastman/tn/) (346) and it made possible to assign names in a logical way. It is now accepted that the terms CTn and ICE are interchangeable (346, 474). Alternatively any transposable element which is also conjugative can be given the prefix CTn. CONSTIN is a term now confined to CTns from Gram negative bacteria.

ICEs/CTns have a mosaic structure comprising modules involved in *integration/excision*, *conjugation*, and *regulation* (43, 44, 289, 346) (Figure 10). Despite the synteny observed for these elements, they differ in the integrase/excisionase sequences (serine and tyrosine recombinase families) and the specificity for the site of

insertion (elements that integrate in a single or in multiple sites) which can determine their promiscuity (348, 349). They have an extraordinary ability to undergo genetic exchange processes within a diversity of genomes or MGE which often result in mosaic platforms carrying fragments of transposons and plasmids. In addition, the independent expression of the modules involved in different basic functions, made the conjugation system able to mobilize a wide variety of non-conjugative MGE as plasmids, integrative and mobilizable elements (IMES) and *cis*-mobilizable elements (CIMES) into which they often insert (44, 335, 337, 451, 474).

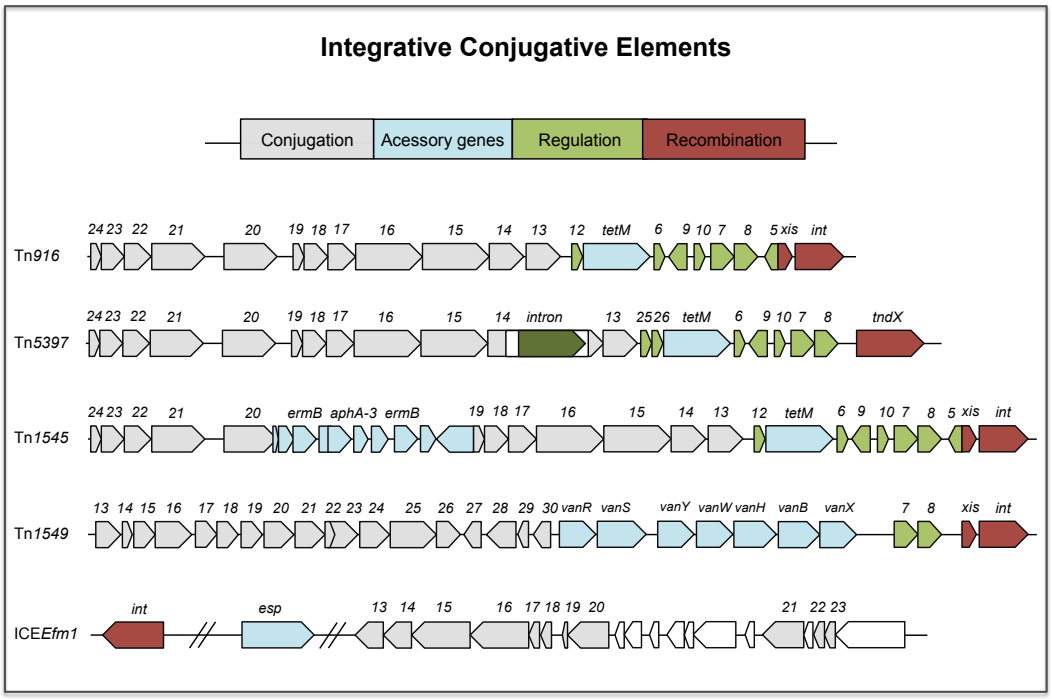


Figure 10. Structure of integrative conjugative elements (ICEs). Relevant examples of ICEs among enterococci are represented. Open reading frames (ORFs) correspond to arrow-shaped boxes and their names are given above the ORFs. *int*, integrase; *xis*, excisionase.

CTns greatly contribute to the dissemination of antimicrobial resistance among a diversity of environmental, commensal and pathogenic bacteria including enterococci (6, 307). Most enterococcal CTns carry tetracycline resistance genes (*tetM* or *tetS*) although they can also harbor genes coding for kanamycin and erythromycin (CTn*1545*), or glycopeptide resistance (CTn*1549* and its close homolog CTn*5382*) (50, 144, 335, 451). In addition, there are modular transposable structures containing CTn*916*-like transposons as CTn*5385* (CTn*5381*-Tn*5384*-Tn*552*), or CTn*5382* (*pbp5*-CTn*1549)* coding for resistance to multiple antibiotics (50, 337). Enterococcal CTns lacking antibiotic resistance genes have also been described (43, 44, 338). Although specific function has

not been assigned for them, interplay between different CTn*916* elements might also contribute to the genome evolution through catalysing large genomic deletions (338).

Elements belonging to the CTn*916*/Tn*1545* family (*tetM*) are the most frequently isolated among enterococci (335, 348, 349) (Table 5). They have a very low integration specificity, which may explain the wide dissemination and broad host range of Tn*916*-like elements (335, 348, 474). CTn*6000* (*tetS*), CTn*5801* (*tetM*) and CTn*5397* (*tetM*) have been recovered from isolates of different origins although its occurrence might still be underestimated (39, 307, 308).

Analysis of available sequenced elements and genomes revealed the wide spread of particular CTn among many gram positive microorganisms, and a high degree of module exchange among them, thus highlighting relevant genetic interplay among GECs (6, 349, 474). CTns have been recovered from gram-positive bacteria colonizing the gastrointestinal tract and oral cavity of different humans and food animals with and without tetracycline exposure, indicating a probable high transferability of these elements in natural conditions (220).

Genomic islands (GIs) are large non-transmissible chromosomal DNA regions (10 - 500-kb) with aberrant nucleotide composition that can be mobilized by the conjugation modules of plasmids, CTns or phages (34, 105, 311). They code for specific integrase and very often an excisionase, and are flanked by direct perfect repeats. They often exhibit a variable content that includes sequences from different MGE (34, 138, 367). GIs may derive from ICEs that for different reasons are defective of self-transfer (44, 448).[2] A few GIs have been described in *E. faecalis* and *E. faecium* which are associated with virulence genes (*pathogenicity islands*, PAIs) or metabolic traits. While the PAI of *E. faecalis* is widely distributed among isolates from human and animal origin (372, 376), the GIs of *E. faecium* remain confined to lineages mainly recovered from hospitalized patients (4, 111, 134, 160, 172, 225).

About one third of the *E. faecalis* PAI includes sequences homologous to that of the enterococcal pheromone-responsive plasmids pAD1 and pAM373, indicating the involvement of plasmid-meditated integration events in the origin of this PAI (367, 375).

[2]Genomic islands are often found integrated at conserved loci, which might facilitate the exchange of genes between bacteria by self-transmissible elements (as ICEs or phages) using similar integration sites (tRNA genes). They are also frequently flanked by 16 - 20-bp direct repeats, result of the site-specific integration of the GIs into the target site, which can act as recognition sequences for their excision. Their variable content can include integrases or factors related to plasmid conjugation or phages involved in GI transfer; as well as ISs or transposons involved in the mobilization or deletion of the genetic material (34, 138, 367).

The presence of highly similar PAI$_{E.\ faecalis}$ variants from human and animal isolates confirms the successful transferability of this element previously demonstrated *in vitro* (275, 312, 376). A PAI$_{E.\ faecium}$ (64 to 104-kb) that encodes an Esp protein similar to that of *E. faecalis* has been identified in *E. faecium* (225, 441). This element is now called ICE*Efm1* as it has recently been shown that it is self-transmissible and contains an integrase and a portion of Tn*916* (428).

GIs related to metabolism include a 8.5-kb element recovered from isolates of the CC17 lineage which is linked to a novel metabolic pathway possibly involved in carbohydrate transport and metabolism (172, 236).

Finally, a putative 17.8-kb GI harbouring the *hyl*$_{Efm}$ gene has been associated with increased colonization in a mouse peritonitis model (8, 223, 341). It is a highly conserved genetic structure located within megaplasmids ranging from 150 to 350-kb with a potential role in carbohydrate metabolism that has been exclusively associated with *E. faecium* clinical/outbreak strains in several countries (76, 140, 336).

Table 5. Conjugative transposons (ICEs) identified in enterococci[a]

Transposon	Family	Size (kb)	Integrase type	Integration sites	Characterized function (gene)[b]	Host range[c]	Larger element	Reference
Tn*916*	Tn*916*	18	Tyr	A + T rich	TET-MIN (*tetM*)	Gram$^+$, Gram$^-$, anaerobes (35 bacterial genera)	Tn*3872*	(64, 335)
Tn*918*	Tn*916*	16	Tyr	A + T rich	TET-MIN (*tetM*)	*Enterococcus faecalis*, staphylococci	NI	(63, 64)
Tn*919*	Tn*916*	15.4	Tyr	A + T rich	TET-MIN (*tetM*)	*E. faecalis*, *S. sanguis*, *Lactococcus lactis*	NI	(125)
Tn*920*	Tn*916*	23	Tyr	A + T rich	TET-MIN (*tetM*)	*Enterococcus faecalis*	NI	(295)
Tn*925*	Tn*916*	18	Tyr	A + T rich	TET-MIN (*tetM*)	*Enterococcus faecalis*, *Leuconostoc*	NI	(64)
Tn*3702*	Tn*916*	18	Tyr	A + T rich	TET-MIN (*tetM*)	*Enterococcus faecalis*	NI	(178)
Tn*5381*	Tn*916*	18	Tyr	A + T rich	TET-MIN (*tetM*)	*Enterococcus faecalis*	Tn*5385*	(337, 343)
Tn*5383*	Tn*916*	18	Tyr	A + T rich	TET-MIN (*tetM*)	*Enterococcus faecalis*	NI	(343)
Tn*5031-5033*	Tn*916*	NK	Tyr	A + T rich	TET-MIN (*tetM*)	*Enterococcus faecium*	NI	(128)
Tn*1545*	Tn*916*	25.4	Tyr	A + T rich	TET-MIN (*tetM*), ERY *(ermB)*, KAN [*aph(3')*]	*Streptococcus, Enterococcus, Staphylococcus, Bacillus, Bacteroides, Clostridium, Lactococcus, Listeria,* Gram$^-$ species	NI	(64, 451)
Tn*5382*	Tn*916*	34	Tyr	A + T rich	VAN (*vanB2*), AMP (*pbp5*)	*Enterococcus*, *Streptococcus*, anaerobes	NI	(14, 50)
Tn*1549*	Tn*916*	34	Tyr	A + T rich	VAN (*vanB2*)	*Enterococcus*, anaerobes	Tn*5382*	(144, 222)
Tn*5386*	Tn*916*	29	Tyr	A + T rich	Lantibiotics (*spa*); collagen adhesion protein	*Enterococcus faecium*	NI	(338)
Tn*5397*	Tn*916*	21	Ser	Single site	TET-MIN (*tetM*)	*Enterococcus (E. faecium, E. hirae), Streptococcus, Clostridium difficile, Bacillus subtilis*	NI	(6)
Tn*5801*, CW*459*, (Tn*6086*, Tn*6014)*	Tn*916*	25	Not related To Tn*916*	GMP synthase	TET-MIN (*tetM*)	*Enterococcus, Staphylococcus, Streptococcus mitis, Clostridium perfringens*	NI	(95, 98)
Tn*6000*	Tn*916*	33.2	Related to *S. aureus* PAI SaPIbov	3' gene end (ribosomal protein L31)	TET *(tetS)*	*E. faecium*, *E. faecalis*, *E. casseliflavus*	NI	(39, 307, 308, 347)
Tn*6009*	Tn*916*	18	Tye	NI	TET-MIN (*tetM*), mercury *(mer)*	*Enterococcus, Streptococcus,* Gram$^-$ species	NI	(393)

Table 5. *Continued*

Transposon	Family	Size (kb)	Integrase type	Integration sites	Characterized function (gene)[b]	Host range[c]	Larger element	Reference
Tn*950*	NI	47	NI	NI	ERY *(ermB)*	*Enterococcus faecium*	NI	(404)
EfaC1	NI	25.3	Tyr	3'end of one gene encoding tRNA	None identified	*Enterococcus faecalis*	NI	(43, 323)
EfaC2	NI	32.7	Tyr	3'end of one gene encoding GMP synthase	None identified	*Enterococcus faecalis*	NI	(43, 323)
EfaD2	NI	ND	Tyr	Unknown	None identified	*Enterococcus faecalis*	NI	(43, 323)
OG1RF-CTn homologue	NI	49	Putative phage-related integrase	Unknown	Putative adhesin proteins	*Enterococcus faecalis*	NI	(32)

[a]AMP, ampicillin; ERY, erythromycin; KAN, kanamycin; MIN, minocycline; TET, tetracycline, VAN, vancomycin; Tyr, tyrosine recombinase; Ser, serine recombinase; ND, not determined; NI, not identified.

[b]Functions encoded not involved in integration/excision.

[c]Host range includes both host into which the elements have been introduced and hosts in which they occur naturally.

1.3.3. Plasmids

Bacterial plasmids are extra-chromosomal genetic elements that are able to replicate and to be maintained independently of the host chromosome. They have a modular structure that includes a *constant* region containing genes encoding essential functions (replication, conjugation, and establishment), and a *variable* region containing a variable set of genes or gene clusters encoding adaptive functions (e.g. antibiotic resistance, toxic heavy metal resistance, virulence, bacteriocin production, metabolic or symbiotic functions, radiation resistance) (Figure 11) (20, 115, 407, 413).

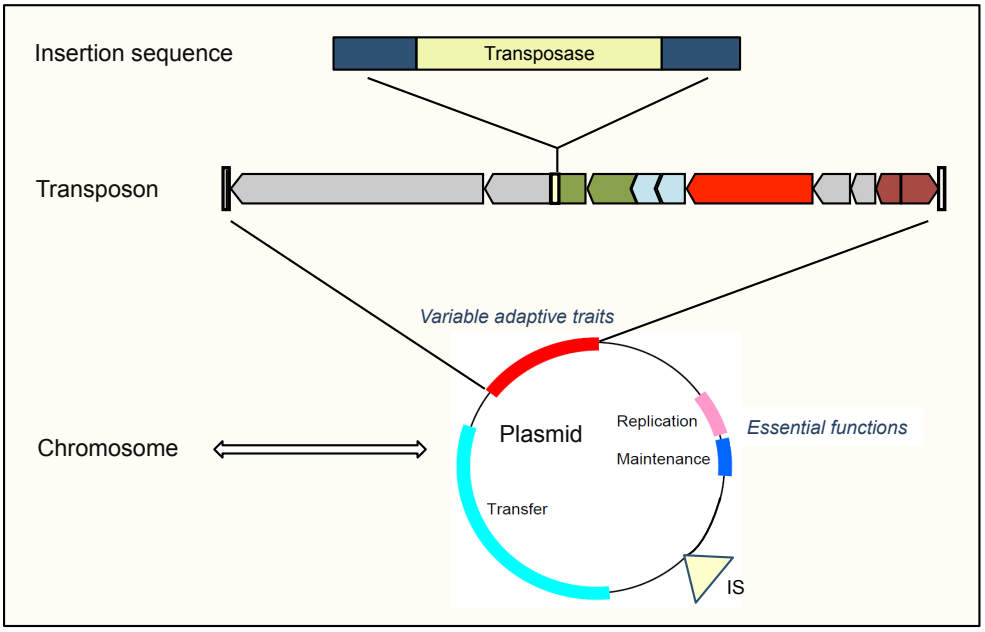


Figure 11. Hierarchal composition of MGEs. Transposons may be inserted into a dispersive element such as a conjugative plasmid, which in turn can integrate into, or exchange DNA with the chromosome.

Plasmids greatly vary in size, from less than 1-kb to 2.09-Mb (http://www.genomics.ceh.ac.uk/plasmiddb/) (284), and it has been speculated that this size range can depend on ecological pressures, as complex environments would select for larger plasmids if they encode traits conferring the bacterial host with a competitive advantage (386).

The classification of plasmids has been performed based on different criteria such as the number of copies, host range, replication and mobility (131, 142, 191, 310, 407). The *replication* of plasmids occurs via one of two general strategies: rolling-circle (RC) or theta and strand displacement (96). Replication of RC plasmids initiates by a site-specific nick and typically produces single-stranded DNA replication intermediates (207), whereas replication of theta-type plasmids produces double-stranded DNA replication intermediates (115). There are different plasmid-encoded control mechanisms (antisense

RNA, proteins, or DNA sequences) during replication in order to maintain the average copy plasmid number in a growing bacterial population. Other typical stabilizing systems of plasmids include multimer resolution, active partitioning and postsegregational killing systems (12, 358, 414, 440).

According to *mobility*, plasmids can be classified in *conjugative, mobilizable* and *non-mobilizable*. The mobility machinery includes a set of mobility (MOB) genes also named relaxosomal components that allows conjugative DNA processing, and a mating pair formation (MPF) complex which is a type 4 secretion system (T4SS). The relaxosomal components comprise an origin of transfer (*oriT*) and a relaxase which is a key enzyme catalyzing the initial and final stages in conjugation. They are common in both conjugative and mobilizable plasmids ***Conjugative***_plasmids encode the functions necessary to promote their own transfer, MOB and MPF, and therefore tend to be large (>30 kb) with low copy number. ***Mobilizable*** plasmids use an MPF of another genetic element present in the cell. They are smaller with high copy number. ***Non-mobilizable*** plasmids are not conjugative neither mobilizable as they spread naturally by transformation or transduction. They have not been described among enterococci (247, 390). Because of the universal distribution of relaxases in all known plasmids, this trait has been used for classification purposes (131, 143).

Enterococcal plasmids were first described in 1972 and evidence for conjugative transfer among humans and among animals was reported in the early 80´s (64, 80).

To date, different enterococcal plasmid types have been described which differ in the mechanism of replication, conjugation abilities, and host range. According to the mechanism of replication, enterococcal plasmid families replicate by a rolling-circle (pRI1-like family) or a theta mechanism (RepA_N, Inc18-type, pHTβ-like) (141, 191, 352, 389, 449, 451). Four families of relaxases (MobC, MobP, MobQ, MobV) have been associated with different plasmid types and CTns. The large $MobP_7$ clade is the largest relaxase family and the unique to extend over several bacterial phyla as *Proteobacteria, Firmicutes* and *Fusobacteria,* and is also the most commonly identified among enteroccal plasmids (143). Plasmid free strains seem to be rare among enterococci (451).

1.3.3.1. Rolling-Circle Replicating plasmids

RCR plasmids constitute a large group of small (<10kb), high-copy number plasmids, able to replicate in a broad range of gram-positive and gram-negative bacteria (97, 209). The mechanism of rolling-circle replication and its regulation is one of the best characterized in gram-positive bacteria (96, 116, 208, 209, 451). Briefly, all RCR plasmids encode initiator proteins that presumptively make a single-strand nick in the plasmid

leading or double-strand origin (*dso* containing different sequences for binding and nicking by the Rep proteins). After covalent extension of the 3'hydroxyl end by a DNA polymerase and a round of replication, synthesis through the origin regenerates a new copy of the *dso* and the release of ssDNA intermediates which are essential for the conversion of the displaced leading strand to the double-stranded form exclusively by host proteins. The level of synthesis of Rep proteins and specific transcriptional repressors are limiting factors in the replication and copy number of these plasmids. The RC mechanism of replication may also impose the small size of this plasmid family.

To date, seven different families of RCR plasmids have been described based on on the sequence of replication initiator proteins (Rep) and the *dso* of replication (208, 451). Many of them are phenotypically cryptic in nature, while many others carry antibiotic resistance genes or additional genes associated with mobilization and transfer, or recombinases involved in plasmid multimer resolution (207, 209). Plasmids from pE194/pLS1 (or pMV158) and pC194/pUB110 families are known to replicate in *E. faecium* or *E. faecalis*, but a functional RCR replicon native to *Enterococcus faecium* has been reported only very recently. It corresponds to a new group of RCR plasmids (pRI1-like) represented by pRI1 and derivatives, either cryptic (pRI1, pJS42, pEFNP1) or conferring resistance to tetracycline (pKQ10::*tetU*) (Table 6). These plasmids are widely spread since they have been detected in isolates from farm animals (poultry, turkey and pigs) and hospitalized patients of different countries (141, 191, 345). These plasmids can be mobilized by conjugative theta plasmids resistant to glycopeptide and streptogramin (141).

Table 6. Diversity, epidemiological background and main genetic characteristics of plasmids identified among *Enterococcus*.

Plasmid family	Plasmid	Size (kb)	Original host (source)	Host range[b]	Genomic markers	Plasmid content						GenBank accession number (Reference)
						Rep1[d]	Rep2[d]	Rel[i]	TA	Bac	VRL	
ROLLING-CIRCLE REPLICATING PLASMIDS												
pT181	pJS42	4,147	*Enterococcus faecium* JH95	*Efm*	cryptic	Rep_{14}		$MobV_4$				NC_010291
(pRI1-like)	pRI1	6,038	*Enterococcus faecium* 9631160-1 (poultry, UK)	*Efm*	cryptic	Rep_{14}		$MobV_4$				EU327398 (141)
	pEFNP1	ND	*Enterococcus faecium* N15	*Efm*	cryptic	Rep_{14}						AB038522
	pKQ10	ND	*Enterococcus faecium*	*Efm*	*tet(U)*	Rep_{14}						EFU01917 (345)
pC194 / pUB110	pIP823	3,712	*Listeria monocytogenes* BM4293	*Efs*	*dfrD*	Rep[e]		MobV				U40997 (54)
	pWC1	2,846	*Lactococcus lactis* 2204	*Efs*	cryptic	Rep[e]						L75827 (327)
pE194 / pLS1	pMV158	5,541	*Streptococcus agalactiae* (clinical isolate, USA)	*Efs*	*tet(L)*	Rep[e]		$MobV_1$				NC_010096 (249)
	pJB01	2,235	*Enterococcus faecium* JC1 (pathogenic)	*Efm*	cryptic	Rep[e]						AY425961 (210)
	pLFE1	4,031	*Lactobacillus plantarum* M345 (raw-milk cheese, France)	*Efs*	*erm(B)*	Rep[e]		$MobV_1$				FJ374272 (123)
THETA REPLICATING PLASMIDS												
Small	pAMα1	9,759	*Enterococcus faecalis* DS5 (USA)	*Efs*	*tet(L)*	Rep_6		$MobV_1$				NC_005013 (109)
mobilizable	pS86	5,149	*Enterococcus faecalis* S-86 (clinical isolate, Spain)	*Efs*	cryptic	Rep_6		$MobV_1$				AJ223161 (270)
plasmids	pEF47	5,5	*Enterococcus faecalis* 47 (cow rumen, Slovakia)	*Efs*	cryptic	Rep_6		$MobV_1$				AY842500 (397)
	p703/5	ND	*Enterococcus faecalis* KBL703 (milk, Korea)	*Efs*	Unknown	Rep_6						AF109375
	pCIZ2	7,383	*Enterococcus faecium* L50 (sausage, Spain)	*Efm*	cryptic	Rep_u		$MobP_7$		*ent*Q		DQ832184 (81)
	pB82	6,173	*Enterococcus faecium* VR82 (clinical isolate, USA)	*Efm*	cryptic	Rep_{11}		$MobP_7$		*bac*43		AB178871 (417)
	pEF1071	9,328	*Enterococcus faecalis* BFE 1071 (minipig feces, Germany)	*Efs*	cryptic	Rep_{11}		$MobP_7$		*ent*1071A/B		AF164559 (13)
	pEFR	3,176	*Enterococcus faecium*	*Efm*	cryptic	Rep_{11}						AF511037
	Plasmid	>3,206[a]	*Enterococcus faecalis* FAIR-E 309 (cheese, Argentina)	*Efs*	cryptic	Rep_{11}				*ent*1071A/B		AY063485 (133)
	p200B	12,463	*Enterococcus faecium*	*Efm*	cryptic	Rep_{18}		$MobP_7$		*bac*32		AB158402

Table 6. *Cont.*

Plasmid family	Plasmid	Size (kb)	Original host (source)	Host range[b]	Genomic markers (pheromone response[c])	Plasmid content						Reference; GenBank accession number
						Rep1[d]	Rep2[d]	Rel[i]	TA	Bac	VRL	
THETA REPLICATING PLASMIDS (cont.)												
	pEF418	>15,9[a]	*Enterococcus faecalis* 418	*Efs, Efm*	*aadE*	Rep_{18}						AF408195
	pGL	8,347	*Enterococcus durans* 41D (cheese product, USA)	*Edu*	cryptic?	Rep_u		$MobP_7$		*durA, durB*		HQ696461
Inc18	pIP501	30,603	*Streptococcus agalactiae* B96 (sputum, France)	*Efs, Efm*	*cat, erm(B)*	Rep_1		$MobQ_3$	ω–ε–ζ			AJ505823, X17655 (415)
	pRE25	50,237	*Enterococcus faecalis* RE25 (dry sausage, Europe)	*Efs, Efm*	*cat, erm(B), sat4, aph(3')-III, aadK*	Rep_1	Rep_2	$MobQ_3$	ω–ε–ζ			X92945 (369)
	pIP816	34,616	*Enterococcus faecium* BM4147 (clinical isolate, France)	*Efm*	*vanA, str*	Rep_1	Rep_2					AM932524 (389)
	pAMβ1	27,815	*Enterococcus faecalis* DS5	*Efm*	*erm(R)*	Rep_1						GU128949
	pVEF1	39,626	*Enterococcus faecium* 399/F99/H8 (farmer isolate, Norway)	*Efm*	*vanA*	Rep_2	Rep[g]		ω–ε–ζ			AM296544 (388)
	pVEF2	39,714	*Enterococcus faecium* 399/F99/A9 (poultry isolate, Norway)	*Efm*	*vanA*	Rep_2	Rep[g]		ω–ε–ζ			AM410096 (388)
	pVEF3	63,135	*Enterococcus faecium* 399/S99/A7 (poultry isolate, Norway)	*Efm*	*vanA*	Rep_2	Rep[g]		ω–ε–ζ			AM931300 (387)
	pVEF4	>44[a]	*Enterococcus faecium* 399/F98/A4 (poultry isolate, Norway)	*Efm*	*vanA*		Rep[g]		ω–ε–ζ			FN424376 (389)
	pEF1	21,344	*Enterococcus faecium* 6T1a (olive fermentation, Spain)	*Efs*	*uvrA*	Rep_2		$MobP_7$		*ent*I, *ent*J, L50, AS-48		NC_010880 (353)
	pEF-01	35,975	*Enterococcus faecalis* EF-01 (bovine, China)		*cfr*	Rep[f]	Rep[g,h]		ε–ζ			CP002208
	pTEF1	66,32	*Enterococcus faecalis* V583 (clinical isolate, USA)	*Efs*	*Tn4001, erm(B), qacZ* (cAD1)	Rep_1	Rep_9	$MobC_2$	*par*			AE016833 (323)
	pPPM1000	UK	*Enterococcus faecium*	Unknown	Tn*mer1*, *str*							(90)
RepA_N	*Pheromone-responsive plasmids*											
	pAD1	59,3	*Enterococcus faecalis* DS16 (clinical isolate, USA)	*Efs*	*uvrB* (cAD1)	Rep_9		$MobC_2$	*par*		*cyl*	(64, 130, 420)
	pJH2	59	*Enterococcus faecalis* JH1 (clinical isolate, UK)	*Efs*	(cAD1)						*cyl*	(64, 186)

Table 6. *Cont.*

Plasmid family	Plasmid	Size (kb)	Original host (source)	Host range[b]	Genomic markers (pheromone response[c])	Plasmid content						Reference; GenBank accession number
						Rep1[d]	Rep2[d]	Rel[i]	TA	Bac	VRL	
RepA_N	*Pheromone-responsive plasmids (cont.)*											
	pIP964	65	*Enterococcus faecalis*	*Efs*	(cAD1)						*cyl*	(64, 224)
	pAMγ1	60	*Enterococcus faecalis* DS5	*Efs*	*uvrB* (cAD1)						*cyl*	(64, 109)
	pBEM10	70	*Enterococcus faecalis* HH2 (clinical isolate, USA)	*Efs*	*bla,* Tn*4001* (cAD1)							(295)
	pCF10	67,673	*Enterococcus faecalis* SF-7 (clinical isolate, USA)	*Efs*	*tet(M)*-Tn*925, uvrA* (cCF10)	Rep_9		$MobP_7$	*par*			AY855841 (64, 108)
	pTEF2	57,66	*Enterococcus faecalis* V583 (clinical isolate, USA)	*Efs*	(cCF10)	Rep_9		$MobP_7$	*par*			AE016831 (323)
	pAMS1	130	*Enterococcus faecalis* (root canal isolate, Sweden)	*Efs*	(cCF10)				*par*	MC4-1		(127, 370)
	pMB1	90	*E. faecalis* S-48 (human wound exudate, Spain)	*Efs*	(cCF10)					*bac*, BC-48		(64, 268)
	pHKK703	55	*Enterococcus faecium* R7 (clinical isolate, USA)	*Efs, Efm*	(cCF10)							(168)
	pBRG1	50	*Enterococcus faecium* LS10 (clinical isolate, Italy)	*Efs, Efm*	*vanA* (cCF10-like)							(255)
	pPD1	59	*Enterococcus faecalis* 39-5 (oral cavity, USA)	*Efs*	(cPD1)	Rep_9				*bac*21		(137, 476)
	pYI14	61	*Enterococcus faecalis* YI714 (clinical isolate, Japan)	*Efs*	(cPD1)					*bac*41		(423)
	pMB2	56	*Enterococcus faecalis* S-48 (human wound exudate, Spain)	*Efs*	(cPD1)					*bac*21, AS-48		(64, 269)
	pEJ97-1	11,334	*Enterococcus faecalis* EJ97 (wastewater, Spain)	*Efs*	(cPD1?)	Rep_8				*ent*EJ97		AJ490170 (361)
	pAM373	36,804	*Enterococcus faecalis* RC73 (clinical isolate, USA)	*Efs*	(cAM373)	Rep_8					*cyl*	AE002565 (91)
	pAM368	107	*Enterococcus faecalis* 368 (clinical isolate, USA)	*Efs*	*vanA* (cAM373)			$MobC_2$				(380)
	pBEE99	80,6	*Enterococcus faecalis* E99 (clinical isolate, USA)	*Efs*	*bee, uvr*	Rep_9			*par*	*bac*41-like		GU046453 (67)
	pMG2200	106,527	*Enterococcus faecalis* NKH15 (hospital outbreak, Japan)	*Efs*	*vanB2, uvr*	Rep_9		$MobP_7$		*bac*44		(478)
	pAM323	66	*Enterococcus faecalis* HH2 (clinical isolate, USA)	*Efs*	(cAM323)			$MobP_u$[j]				(295)
	pAM324	53	*Enterococcus faecalis* HH2 (clinical isolate, USA)	*Efs*	(cAM324)				*par*			(295)

Table 6. *Cont.*

Plasmid family	Plasmid	Size (kb)	Original host (source)	Host range[b]	Genomic markers (pheromone response[c])	Plasmid content						Reference; GenBank accession number
						Rep1[d]	Rep2[d]	Rel[i]	TA	Bac	VRL	
RepA_N	*Pheromone-responsive plasmids (cont.)*											
	pHKK10	55	*Enterococcus faecium* 228 (clinical isolate, Spain)	*Efs, Efm*	*vanA* (cHKK100)						*cyl*	(161)
	pSL1/2	128,1	*Enterococcus faecalis* KV1/2 (clinical isolate, Korea)	*Efs*	*vanA, ermB,aac(6')-aph(2"), ant(6)-Ia, aph(3')-IIIa* (cSL1)							(243)
	pOB1	71	*Enterococcus faecalis* 5952 (clinical isolate, USA)	*Efs*	(cOB1)						*cyl*	(297, 314)
	pYI1	58	*Enterococcus faecalis* (clinical isolate)	*Efs*	(cOB1)						*cyl*	(183)
	pYI2	56	*Enterococcus faecalis* (clinical isolate)	*Efs*	(cYI2)						*cyl*	(183)
	pYI17	57,5	*Enterococcus faecalis* YI717 (clinical isolate)	*Efs*	(cYI17)							(421)
	pAMγ2	~60	*Enterococcus faecalis* DS5	*Efs*	(cAMγ2)							(64)
	pAMγ3	~60	*Enterococcus faecalis* DS5	*Efs*	(cAMγ3)							(64)
pRUM-like	pRUM	24,873	*Enterococcus faecium* U37 (clinical isolate, USA)	*Efm*	*cat,* ΔTn*5405, uvr*	Rep_{17}		$MobP_7$	Axe-Txe			NC_005000 (151)
	pS177	39	*Enterococcus faecium* S177	*Efm*	*vanA*	Rep_{17}			Axe-Txe			(159)
	P5753cB	56,689	*Enterococcus faecium* 5753c (clinical isolate, USA)	*Efm*	*aac(6')-aph(2"), tetM,* Δ*Tn5405, erm(B)*	Rep_2	Rep_{17}			Lactococcin 972		GQ900487
Megaplasmids	pLG1	281,02	*Enterococcus faecium* 64/3xUW2774 (clinical isolate, Germany)	*Efm*	*vanA, erm(B), tcrYAZB, ctpA*	Rep[f]		$MobP_7$		*hyl*		HM565168-565236 (223)
pHTβ-like	pHTβ	52,89	*Enterococcus faecium* (clinical isolate, Japan)	*Efm, Evm*	*vanA*	Rep_u		$MobP_u$				AM296544 (422, 425)
	pMG1	65,029	*Enterococcus faecium* FH (clinical isolate, Japan)	*Efm*	*aac(6')-aph(2"), aacA-aphD* (Tn*4001*)	Rep_u		$MobP_u$				AB206333 (405)
	pTEF3	17,963	*Enterococcus faecalis* V583 (clinical isolate, USA)	*Efs*	cryptic?	Rep_u						NC_00467 (323)

Table 6 Footnote.
Abbreviations: ND, not determined; UK, unknown; Rep, replication; Rel, relaxase; Mob, mobilization; TA, toxin-antitoxin system; Bac, bacteriocin; VRL, virulence.
[a]Only partial sequence of the plasmid is available.
[b]*Enterococcus* species where these plasmids have been identified to date or have been shown to be able to replicate in. Efm, *Enterococcus faecium*; Efs, *Enterococcus faecalis*; Evm, *Enterococcus avium*; Edu, *Enterococcus durans*;
[c]Only applicable to pheromone-responsive plasmids.
[d]Rep families are named RepX where X indicates different rep-families according to Jensen *et al*. (191). RepU means unique.
[e]Rep proteins displaying <50% homology with other RCR plasmids and not included in Jensen's scheme.
[f]Replication protein RepB homologuous to that of several plasmids from *Lactococcus* spp. ("pEF-01_024") and not included in Jensen's scheme.
[g]Rep proteins not included in Jensen's scheme.
[h]Replication protein (pEF-01_001") 100% homologuous to that from pVEF1, pVEF2 and pVEF4 plasmids.
[i]Plasmid relaxases are grouped in different MOB families (capital letters) and subfamilies (numbers in subscript) according to Garcillan-Barcia *et al*. (143). Pu means that the phylogenetic position of the protein is out of the clades of the MobP tree.
[j]Also designated as MOB(MG) according to Zheng *et al*. (478).

1.3.3.2. Theta-replicating plasmids

Replication by the theta-type mechanism was firstly described in Gram-positive bacteria for the streptococcal/enterococcal Inc18 group (40). It involves the opening of the DNA strands at an A+T rich region of the origin, synthesis of a primer RNA (pRNA) and initiation of leading DNA strand synthesis by covalent extension of pRNA. This DNA replication mode is termed “theta” due to the resemblance of the replication intermediates to the Greek letter θ. With some exceptions, these plasmids require a plasmid-encoded Rep initiator protein, which shares identity between plasmids replicating via the theta mechanism (96, 115).

Enterococcal theta-replicating plasmids comprise small mobilizable (ca. <15 kb) plasmids which are mostly cryptic and do not encode conjugal functions, and large conjugative (>30 kb) plasmids, which may encode a wide diversity of adaptive functions as resistance to antibiotics, biocides, metals, metabolic functions and virulence traits.

a) Small theta-replicating plasmids

Small theta-replicating plasmids (<15 kb) are being increasingly reported among *Enterococcus* (Table 7). Although many of them are cryptic, they are frequently associated with the production of bacteriocins[3], which exhibit activity against a variety of gram-positive genera including other enterococci strains. Bacteriocin-producing enterococci have been isolated from diverse environments such as foods, animals, and humans, although we lack data about their prevalence among hospital strains (71, 82, 302).

The replication proteins of these plasmids are highly similar to theta-type gram-positive plasmids belonging to the pUCL287 family from lactic acid bacteria (e.g. pIP501,

pAMβ1) (19). However, they may contain mobilization systems commonly associated with RCR plasmids (MOBV) or theta replicating plasmids (MOBP) (143). The mobilization proteins from pAMα1-like (pS86, pEF47) plasmids, which replicate via RC mechanisms in *Bacillus* and via a theta mode in *Enterococcus*, belong to MobV whereas that from pCIZ2, pB82, pEF1071, p200B and pGL belong to a sub-cluster of $MobP_7$ in which a variety of small theta-replicating plasmids from *Firmicutes* are included (143, 270). These plasmids have been isolated from enterococcal isolates recovered from hospitalized patients, foods and foodborne animals (Table 6).

Table 7. Characteristics of bacteriocins previously associated with small theta-replicating plasmids in enterococci.

Bacteriocin	Type[a]	Source		Activity spectrum	Observations	Refe-rences
		Plasmid	Host			
Bac 32	Class IIa, pediocin-like	pTI1 (or p200B) 12.5-kb	*E. faecium*	*E. faecium, E. hirae, E. durans* (narrow spectrum)	Novel bacteriocin widely disseminated among *E. faecium* (both VRE and VSE)	(185)
Bac 43	Class IIa, pediocin-like	pDT1/pB82 6.2-kb	*E. faecium*	*E. faecium, E. faecalis, E. hirae, E. durans, Listeria monocytogenes*	Only identified in vancomycin-resistant clinical *E. faecium*	(417)
Enterocin Q	Class IIc, leaderless	pCIZ2 7.4-kb	*E. faecium L50*	*Listeria monocytogenes, Clostridium spp., Streptococcus spp.*	*E. faecium* L50 produces 3 different bacteriocins (EntP, EntL50 and EntQ)	(81)
Enterocin 1071A **Enterocin 1071B**	Class IIb, two peptide bacteriocin	pEF1071 9.3-kb	*E. faecalis*	*E. faecium, E. faecalis, E. durans, Listeria innocua, Lactobacillus salivarius, Streptococcus agalactiae, Peptostreptococcus aerogenes, Micrococcus sp., Propionibacterium*		(13)
Duracin GL	Class II	pGL 8.3-kb	*E. durans*	Antilisterial mode of action	From a cheese product	GenBank accession number HQ696461

The heat-stable peptide bacteriocins from lactic acid bacteria have so far been grouped into two major classes: class I, the lantibiotics, and class II, the heat-stable nonlantibiotics. The bacteriocins from enterococci belong almost exclusively to the heat-stable, nonlantibiotic class II bacteriocins, with the exception of cytolysin. Class II bacteriocins are usually sub-divided in pediocin-like bacteriocin (IIa), the two-peptide bacteriocins (IIb), the leaderless peptide bacteriocins (IIc), and the circular bacteriocins (IId).

[3]Most bacteriocins in gram-positive bacteria show a small length, heat stability (peptide bacteriocins), and their antimicrobial activities are directed against a broader spectrum of bacteria than is seen for bacteriocins of gram-negative bacteria (for reviews see 77, 302).

b) Inc18 family

It is a group of low copy number plasmids (25 - 50-kb) that exhibit a very broad host range (219, 369, 411). Outstanding representatives of this family include pIP501 from *Streptococcus agalactiae*, pRE25 and pAMβ1 from *E. faecalis,* and pIP186 from *E. faecium*.

The paradigm of this family is pMS19035 which comprises two identical parts, each onecontaining a module involved in replication, a SegA region involved in the multimer plasmid resolution, and a SegB region that have two transcriptional units related to partition (δ–ω) and the post-segregational killing system (ε–ζ) which contributes to plasmid stabilization and maintenance (93, 369). They are under a global control exerted by ω on essential functions such as copy-number and stable inheritance (93, 104, 154, 369, 483). The size and backbone of the Inc18 plasmids is highly variable due to both acquisition of DNA (insertion sequences, transposons, genetic islands) but also to DNA losses which seem to occur by spontaneous deletion after conjugal transfer to different hosts (415, 484).

Plasmids of the Inc18 family replicate by a unique antisense-RNA-mediated transcriptional attenuation mechanism which has so far not been identified in gram-negative bacteria (36, 93, 451). The relaxase is the prototype of the clade $MobQ_3$ within MOBQ family which only comprises plasmids of phylum *Firmicutes* (143) (Table 6). Plasmids lacking replication and transfer modules from the Inc18 family but possessing remnants of Inc18 rep genes are increasingly reported (352, 387-389). Also, the presence of genes coding for Rep and post-segregational killing systems (PSK) from Inc18 plasmids are commonly found amongst plasmids and bacterial chromosomes showing they are frequently involved in LGT processes (70, 286, 440).

Inc18 plasmids usually confer resistance to macrolides, lincosamides and streptogramins and often, to kanamycin, streptomycin, cloramphenicol and mercury due to the presence of different transposons or genetic islands (90, 230, 369). They have also been linked to the spread of Tn*1546*-*vanA* among enterococci from different origins (191, 352, 389, 480) (Table 6). Different studies indicate a role of the Inc18 plasmid family in the emergence of vancomycin-resistant *Staphylococcus aureus* by transfer of Inc18::Tn*1546* plasmids from *E. faecalis* (126, 480, 481).

c) RepA_N family

The family of RepA_N plasmids comprises a wide diversity of plasmid families of narrow host range which are broadly distributed among low G+C Gram-positive bacteria (449). The RepA proteins from the RepA_N family of plasmids fell into five large clusters

with strong correlation with the host genus (*Enterococcus, Streptococcus, Lactobacillus, Lactococcus and Staphylococcus*) suggesting that the replication system has evolved in different host backgrounds. In enterococci, members of this family include pheromone-responsive plasmids from *E. faecalis*, and derivatives of pRUM and pLG1 plasmids from *E. faecium* (223, 449). Two phylogenetically distant relaxase families, $MobC_2$ and $MobP_7$, have been identified among the enterococcal pheromone-responsive, pRUM and pLG1 plasmids (143).

Pheromone-responsive plasmids constitute a paradigm of the conjugation systems in Gram positive organisms. These plasmids encode a response to pheromones, linear heat-stable peptides of 7-8 amino acids that are produced by plasmid free recipient strains. Pheromones are chromosomally encoded while genetic determinants for signal detection and response are located on the sex pheromone plasmid. This conjugal mechanism is highly specific since each plasmid only respond to a specific pheromone (64, 218). Thus, pheromone-responsive plasmids have been subgrouped into different incompatibility groups, according to the sex pheromone to which they respond (Table 6).

The most common families are pAD1, pCF10, pPD1 and pAM373 plasmids which are mainly isolated from nosocomial human infections and encode antibiotic resistance (Tn*1546_vanA, Tn1549_vanB2*, Tn*4001_aac6'-aph2'',* Tn*916*-like*_tetM*), and/or putative virulence traits (bacteriocins and cytolysins). *E. hirae, S. aureus* and *Streptococcus gordonii* have already been described to produce cAM373-like peptides with activity to a pAM373-specific pheromone, promoting the interspecies spread of antibiotic resistance (63, 65, 445). Some *E. faecium* may also exhibit a response when present in an *E. faecalis* host (161, 168, 255). The transfer and cointegration of pheromone plasmids with others able to replicate in *S. aureus* has been described, which reflects the possibility of acquisition and spread of vancomycin resistance among clinical staphylococci (380, 454). The mobilization of pheromone-responsive plasmids has recently been reported to involve transfer of antibiotic resistance and MLST markers, and capsule genes, and thus to promote chromosomal diversification in *E. faecalis* (263).

Enterococcal pheromone-responsive plasmids with different rep and mobilization proteins have been identified. Two different rep homologs have been identified to date (pAD1/pCF10 and pAM373-like; Table 6) (191, 449). Jensen *et al*. have recently identified rep from pAD1/pCF10 plasmids among a set of *E. faecalis* isolates expressing tetracycline resistance and recovered from poultry, pigs and humans (191). Replicases are not necessarily correlated with the same relaxases in each plasmid since pAD1 and pCF10 carry mobilization proteins belonging to different families, the $MOBP_7$ (pcfG_pCF10) and $MOBC_2$ (TraX_pAD1/pAM373) (143). Derivatives of pAD1 can also contain an unique

antisense-regulated toxin-antitoxin system *par* that ensures their stable inheritance within *E. faecalis* cells. The *par* locus is encoded by the plasmid pAD1 but related loci have been identified on other *E. faecalis* plasmids (e.g. pCF10, pTEF2, pAM373, pAMS1, pMG2200) and chromosomes (*E. faecalis* V583), as well as on plasmids (VRSAp, pRC18) and chromosomes of *Staphylococcus* and *Lactobacillus* species. The occurrence of these *par*-like loci may be relevant in plasmid stability and metabolic regulation in different Gram-positive bacteria (450, 452).

pRUM-like derivatives (e.g. pRUM, pS177) are mosaic plasmids generated by the recombination processes between transposons, ISs and small plasmids. They contain a specific PSK system system named Axe-Txe that acts as a segregational stability module in a wide spectrum of bacterial hosts (151, 159) (Table 6). Plasmids containing RepA and Axe-Txe from pRUM are widespread among *E. faecium* isolates from different countries, mainly in CC17 of nosocomial origin (352). Vancomycin or tetracycline resistant isolates from humans and poultry frequently harbour pRUM-like plasmids, sometimes carrying Tn*1546* (151, 159, 191, 286, 401).

RepA_N megaplasmids. Megaplasmids are commonly found among *E. faecium* isolates from different origins (21, 166, 223, 352, 462, 477). The only megaplasmid fully sequenced is pLG1, a ~281-Kb plasmid isolated from a vancomycin resistant *E. faecium* nosocomial strain in Germany (223). It contains a rep gene coding for a protein with similarity with $RepA_{pAD1}$ and thus has been consider as a member of the RepA_N family (449). The relaxase identified in pLG1 belongs to $MOBP_7$ subfamily to which also belong other RepA_N plasmids (143).

d) pMG1-like family

This family comprise derivatives of pHT plasmids (pHTα, pHTβ and pHTγ) and pMG1 which have been associated with the spread of resistance to glycopeptides (Tn*1546_vanA*) and high levels of resistance to aminoglycosides (Tn*4001*-like elements) among *E. faecium* and *E. avium* in the United States and Japan (405, 424, 425) (Table 6).

Plasmids of this family show a high homology with the virulence pXO2 plasmid from *Bacillus anthracis*, suggesting they may have a common ancestor. Nevertheless, many ORFs of pHT and pMG1 plasmids do not show significant homology with any reported proteins, having been categorized as a new type of highly efficient conjugative plasmids. Its transfer system belongs to the MobP family based on the *oriT* and relaxase sequences (143, 422). To date, these plasmids types have been exclusively identified among *E. faecium* recovered from humans.

e) Megaplasmids

The presence of megaplasmids as a component of the bacterial mobilome is being increasingly reported, even though differentiation between large and megaplasmids is often controversial. Plasmids larger than 150-kb usually associated with metabolic functions are classically defined as megaplasmids (254).

Megaplasmids are common among *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* and confer on these microorganisms the ability to utilize complex carbohydrates from vegetables in the diet, host-derived glycoproteins and glycoconjugates that could enhance their adaptation and persistence in the colon (239, 365). The diversity of megaplasmids among enterococci remains largely unexplored. However, the existence of large plasmids in *E. faecium* strains with human and animal origins recovered from the mid- to late 1990s may be inferred from different recent studies, suggesting they encode essential functions to the adaptation in different hosts or environments (21, 166, 352, 462). These plasmids can codify for genes involved in sugar metabolism (mannitol, glycerol, sorbitol, and raffinose) with a great variability in their phenotypic expression (408, 477). *E. faecium* megaplasmids have been recently found to encode resistance to different antibiotic families (macrolides, glycopeptides, aminoglycosides) and heavy metals (copper-*tcrYAZB*) and enhanced epidemicity (glycoside hydrolase hyl_{Efm}) (8, 223).

The origin of these megaplasmids remains undetermined since the plasmid classification based on Rep proteins was designed upon the sequences of small- or medium-size plasmids from known families of Gram-positive bacteria and pLG1 is the only enterococcal megaplasmids fully sequenced (RepA_N; $MobP_7$) (191). MobP (as MobQ) is uniformly distributed in plasmids of all size ranges (390).

To date, the diversity of megaplasmids among *E. faecium, E. durans and E. hirae* remains largely unexplored, and megaplasmids have not been described in *E. faecalis* species.

Chapter 2

Objectives and outline of the study

The mere formulation of a problem is far more essential than its solution.

Albert Einstein

2.1. Statements of Objectives

The emergence and spread of antibiotic resistance has been facilitated by lateral genetic transfer (LGT) events involving different types of mobile genetic elements (MGE) and bacterial clonal lineages adapted to specific ecological conditions. The analysis of the MGE and LGT events occurring in species widely distributed among hosts and living in a variety of environments becomes essential not only for understanding the ecology and adaptation of bacterial pathogens but also for designing strategies to control the fittest members of a species able to acquire a wide diversity of genetic traits.

The emergence and spread of enterococcal strains with new adaptive traits (inferred by their antibiotic resistance, colonization, or virulence phenotypes and genotypes) suggests a relevant role of LGT events in the evolution of the species of this genus. In fact, the cumulative acquisition of determinants enhancing the fitness of certain clonal complexes seems to have contributed to the ramping success of *Enterococcus* as a leading nosocomial pathogen worldwide. This thesis is intended to define the importance and diversity of MGE in the capture, expression and dissemination of antibiotic resistance genes in enterococci and to detect well adapted enterococcal populations in hospital and community settings. The work will contribute to identify the MGE responsible for the epidemicity and the adaptation of these bacterial populations to different environments and to understand the recent genotypic and phenotypic changes observed in this genus.

Our ***hipothesis*** is that LGT contributes to the adaptation of enterococci to specialized ecological communities influencing the selection of particular clonal lineages in which the acquisition and persistance of antibiotic resistance is favoured. The ***goal*** of this work is to establish the ***diversity and the influence of different MGE belonging to the enterococcal mobilome in the transfer of adaptive traits between different hosts and in the selection of particular enterococcal lineages.*** With this purpose, we have analyzed the role of clonal expansion and lateral genetic transfer in the dissemination of vancomycin resistance among *Enterococcus faecium* and *Enterococcus faecalis*, recovered from hospitalized persons in different countries of the five continents (1986-2009) and from different hosts living in diverse community environments (healthy humans, foodborne animals, distinct aquatic environments) (1995-2008).

This study focuses on the following ***specific aims***:

1. To analyse the population structure of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* recovered from clinical and non-clinical settings in Portugal (1995-2008) and other countries of the five continents (1986-2009).

2. To identify the plasmidome of antibiotic resistant enterococci from different environments.

3. To characterize plasmids and transposons responsible for the global spread of vancomycin resistance among *Enterococcus faecium* and *Enterococcus faecalis* from human and animals in order to establish the dissemination routes of Tn*1546* and to understand the role of LGT in the successful spread of antibiotic resistance.

4. To analyze the results obtained in the objectives 1, 2, 3 under the light of current knowledge on the topic in order to i) evaluate the relative contribution of particular clonal lineages of *E. faecium* and *E. faecalis* species and specific mobile genetic elements in the pandemic (global geographical distribution) and selective expansion (almost restricted to enterococci) of glycopeptide resistance and ii) to infer the impact of LGT in the clonal selection of enterococcal species.

5. To evaluate the *in vitro* activity of new antibiotics against selected enterococcal lineages adapted to different hosts and ecological conditions.

2.2. Outline of the thesis

The findings that would answer the *specific aims* of the thesis yielded different publications (n=13; 8 publications in peer review journals, 2 manuscripts under editorial revision, 2 in preparation, 1 book chapter). Such results have been organized throughout the papers, according to the rationale proposed as follows:

I. ***Insights on the population structure of vancomycin-resistant Enterococcus faecium and Enterococcus faecalis from clinical and non-clinical settings.*** The population structure of a representative and comprehensive collection of *E. faecium* and *E. faecalis* was analysed in isolates from humans, food products, animals and the environment recovered from different geographical regions of Portugal and other countries of Europe and the USA (1995-2008).

- Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L. 2009. Dispersion of multidrug resistant *Enterococcus faecium* belonging to major clonal complexes in different Portuguese settings. *Applied and Environmental Microbiology*. 75(14):4904-4908.
- Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L. 2009. Clonal expansion within CC2 and dissemination of vancomycin-resistant plasmids in different *Enterococcus faecalis* clonal lineages of Portugal. *Journal of Antimicrobial Chemotherapy*. 63(6):1104-1111.
- Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Jensen LB, Zervos MJ, Donadedian S, Francia MV, Baquero F, Peixe L. 2011. Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17, CC5, and *Enterococcus faecalis* CC2 clonal clusters harboring Tn*1546* on indistinguishable plasmids. *Journal of Clinical Microbiology*. 49(3):925-931.

II. ***Analysis of the plasmidome of enterococci from different environments with special emphasis on that encoding antibiotic resistance or adaptive traits. Characterization of vanA-Tn1546 and vanB-Tn1549/Tn5382 transposons responsible for the global spread of vancomycin-resistance among Enterococcus from humans and animals.***

IIa. *Analysis of plasmids responsible for the virulence/epidemicity of specific clonal lineages highly adapted to the hospital environment.*

- Freitas AR, Tedim AP, Novais C, Ruiz-Garbajosa P, Werner G, Laverde-Gomez JA, Cantón R, Peixe L, Baquero F, Coque TM. 2010. Global spread of the colonization-

virulence *hyl*$_{Efm}$ gene in megaplasmids of CC17 *Enterococcus faecium* polyclonal sub-cluster. *Antimicrobial Agents and Chemotherapy.* 54(6):2660-2665.

- Laverde Gomez JA, van Schaik W, Freitas AR, Coque TM, Weaver KE, Francia MV, Witte W, Werner G. 2011. A multiresistance megaplasmid pLG1 bearing a *hyl*$_{Efm}$ genomic island in hospital *Enterococcus faecium* isolates. *International Journal of Medical Microbiology.* 301(2):165-175.

IIb. *Analysis of plasmids and transposons in antibiotic resistant enterococci from humans and animals.*

- Freitas AR, Tedim AP, Francia MV, Peixe L, Novais C, Jensen L, Willems R, Baquero F, Coque TM. The spread of vancomycin resistant *Enterococcus faecium* (1986-2009): a historic view from the plasmid size. (*in preparation*)
- Freitas AR, Ruiz-Garbajosa P, Tedim AP, Francia MV, Novais C, Zervos MJ, Donabedian S, Peixe L, Baquero F, Coque TM. Influence of pAD1 and Inc18 plasmids in the worldwide spread of vancomycin-resistant *Enterococcus faecalis* outbreak strains (1987-2005). (*in preparation*)
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III. ***Integration of all data and multilayered analysis of the relative contribution of epidemic clones and mobile genetic elements in the current expansion of specific clonal lineages through the analysis of temporal and spatial differences of that biological units.*** The impact of the lateral genetic transfer in the diversification, selection and spread of different enterococcal lineages, the opportunities and constraints of LGT

processes among enterococci of different origins and species belonging to the same communities of *genetic exchange* and the diversity and the influence of different MGE belonging to the enterococcal mobilome in the transfer of adaptive traits between different hosts was analysed.

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IV. ***Screening of the in vitro activity of the new molecule tigecycline against Enterococcus spp. from different ecological niches in Portugal.*** The *in vitro* activity of tigecycline, an antibiotic of last resort to treat severe human infections, was tested against enterococci collected from humans, food products, animals and the environment in Portugal (1996-2008).

- Freitas AR, Novais C, Correia R, Monteiro M, Coque TM, Peixe L. Non-susceptibility to tigecycline in enterococci from hospitalized patients, food products and community sources. *Accepted for publication in International Journal of Antimicrobial Agents.*

Chapter 3

Results (manuscripts)

Every experiment proves something. If it doesn't prove what you wanted it to prove, it proves something else. **Prof. Anon**

3.1. Insights on the population structure of *Enterococcus faecium* and *Enterococcus faecalis* from clinical and non-clinical settings

3.1.1. Dispersion of multidrug-resistant *Enterococcus faecium* isolates belonging to major clonal complexes in different Portuguese settings

3.1.2. Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of *Enterococcus faecalis* from Portugal

3.1.3. Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17, CC5, and *Enterococcus faecalis* CC2 clonal clusters harboring Tn*1546* on indistinguishable plasmids

Dispersion of multidrug-resistant *Enterococcus faecium* isolates belonging to major clonal complexes in different Portuguese settings

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Dispersion of Multidrug-Resistant *Enterococcus faecium* Isolates Belonging to Major Clonal Complexes in Different Portuguese Settings

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The population structure of 56 *Enterococcus faecium* isolates selected from a collection of enterococci from humans, animals, and the environment in Portugal (1997 to 2007) was analyzed by multilocus sequence typing. We identified 41 sequence types clustering into CC17, CC5, CC9, CC22 and CC94, all clonal lineages comprising isolates from different hosts. Our findings highlight the role of community-associated hosts as reservoirs of enterococci able to cause human infections.

Enterococci are organisms widely distributed in nature that are recognized as one of the leading causes of nosocomial infections (1, 20). Despite their ubiquity, the population structure of *Enterococcus faecium* comprises a diversity of sequence types, with overrepresentation of particular clonal complexes (CCs) associated with swine (CC5), poultry (CC9), veal calf (CC1), or humans (CC17, CC22, and CC94) (1, 5, 15, 17, 30, 37). Particular host-specific human clonal lineages are considered high-risk CCs (HiRCCs) since they are recovered mostly from hospitalized patients (17). CC17 is the only recognized *E. faecium* HiRCC, nowadays globally disseminated, which has been sporadically isolated from nonhospitalized humans (3, 8–10). Different studies have analyzed the population structure of local enterococci, but they are focused mainly on clinical strains with a specific phenotype, such as vancomycin resistance, generally isolated in a short temporal frame (2, 7, 16, 18, 27, 29, 36).

We analyzed 56 representative *E. faecium* isolates from a Portuguese collection comprising 1,700 enterococci from different geographical locations (1997 to 2007), some included in previous studies (22–26). They were recovered from hospitalized patients in five hospitals of different regions (n 20), swine excrement (n 6) and environmental piggery samples (n 16), retail poultry (n 6) of four national commercial brands, feces from healthy humans (n 5), hospital wastewater (n 2), and the estuary of the river Douro (n 1). Susceptibility testing with 15 antibiotics was performed following CLSI guidelines (6). Species identification and detection of genes encoding vancomycin resistance were performed by using multiplex PCR (11). Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) as described previously (15, 22, 28). Sequence types (STs) differing in one or two of the seven housekeeping genes were considered single-locus variants (SLVs) and double-locus variants, respectively. Clusters of related STs differing in 2 loci that were thought to be descendants from a common ancestor were grouped into CCs by using the eBURST software program (12, 13) (http://www.mlst.net). Genes coding for virulence factors such as enterococcal surface protein (encoded by *esp*), hyaluronidase (encoded by *hyl*), cytolysin (encoded by *cyl*), gelatinase (encoded by *gel*), and aggregation substance (encoded by *agg*) and the backbone structure of Tn*1546* harbored by vancomycin-resistant *E. faecium* (VRE) were investigated by using PCR (21, 34, 38).

The isolates studied (35 VRE isolates and 21 vancomycin-susceptible *E. faecium* [VSE] isolates) corresponded to 49 PFGE types and 41 different STs, including 24 newly identified STs, which clustered into CC5, CC9, CC17, CC22, and CC94 (Fig. 1 and Table 1).

CC17 was identified in 24 isolates (18 VRE isolates and 6 VSE isolates) from hospitals, healthy volunteers, swine, piggeries, and the environment in different regions from 1997 to 2007. In agreement with other studies, the CC17 meroclone consisted of a high diversity of STs (ST16, ST18, ST78, ST80, ST125, ST132, ST280, ST368, ST369, ST390, ST393, ST430, and ST431), particularly enriched by ST18 (7, 18, 29, 36, 37) (Table 1). All CC17 isolates were resistant to ampicillin and erythromycin, and most of them were associated with resistance to glycopeptides (75%) and ciprofloxacin (71%) and a high level of resistance to kanamycin (58%) and streptomycin (50%). *esp* was detected among isolates from most sources (38%), while *hyl* (17%) was mostly associated with the clinical setting. The detection of CC17 among nonclinical sources might indicate a hospital input of community strains with different genetic contents besides contamination from the hospi-

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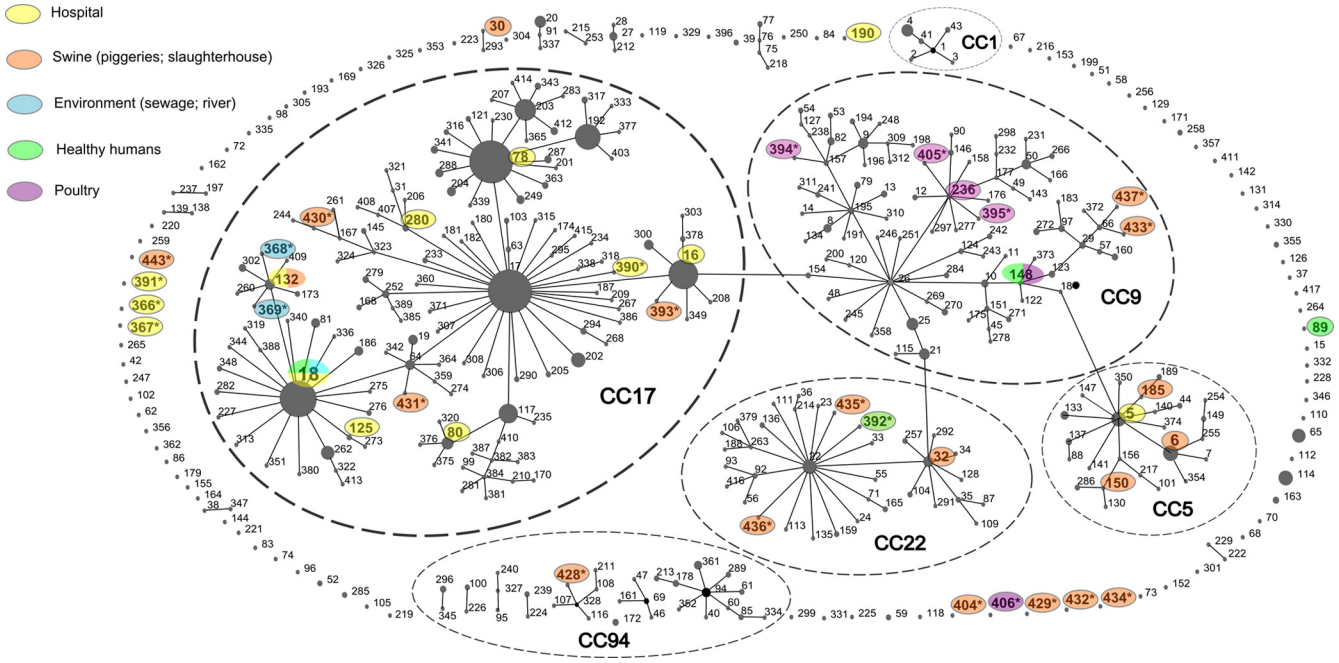


FIG. 1. Clustering of 41 *E. faecium* STs by use of eBURST. STs are indicated by colored circles, representing 56 isolates from the present study, with 439 MLST profiles representing 1,358 *E. faecium* isolates from the database (www.mlst.net). Each ST is represented as a node; the relative size of each node is indicative of its prevalence among the isolates, and lines connect SLVs. Colors indicate isolation sources, and CCs are represented by dashed circles (CC1, CC5, CC9, CC17, CC22, and CC94).

tal setting and could explain its high prevalence and global spread (3, 8–10).

Clonal dissemination and maintenance of particular resistant *E. faecium* strains among food animals have previously been documented, but MLST data are absent in almost all of these works (14, 19, 26, 32, 33). Although swine and poultry are considered potential reservoirs of pathogenic *E. faecium*, transmission to humans has been demonstrated in a few cases and is always linked to persons in close contact with farms (4, 19, 32, 33). CC5 comprised isolates of ST5 (*n* 1), ST6 (*n* 1), ST150 (*n* 1), and ST185 (*n* 2). Isolates classified as ST5 and ST185, which is an SLV of ST5, were recovered from two hospitals over 2 years (22) and from soil of an extensive piggery, respectively. They were clonally related to the ST6 isolate which represents the VRE strain "A" widespread among swine of four European countries since 1997 (26). CC5 isolates did not contain the tested putative virulence factors and exhibited various resistance phenotypes (Table 1).

CC9 included eight clonally unrelated isolates from community (ST148), swine (ST433 and ST437), and poultry (ST236, ST394, ST395, and ST405), generally resistant to ciprofloxacin (88%) and vancomycin (62%). Most of the CC9 poultry strains were persistently recovered from commercial brands of retail poultry products over the years and corresponded to different ampicillin-resistant VRE or VSE strains (23). All isolates lacked the virulence genes tested. CC9 strains associated with *purK6* (ST236 and their SLVs, ST395 and ST405) or *purK3* (ST148) have also been detected in chickens from Korea and Spain, respectively, which might mirror particular globally disseminated poultry lineages (http://efaecium.mlst.net/).

CC22 (ST32, ST392, ST435, and ST436) comprised isolates mostly resistant to tetracycline and ciprofloxacin and highly resistant to kanamycin from different piggeries and from human feces. This genogroup was recently associated with isolates from human origin (5, 17), with this study representing the first description of isolates among swine.

The remaining STs were recovered from humans and animals in nine different locations and corresponded to ST30, ST89, ST190, ST366, ST367, ST391, ST404, ST406, ST428 (CC94), ST429, ST432, ST434, and ST443, mostly identified as singletons. It is of note that ST366/ST367 and ST391 from hospitals are double-locus variants of STs belonging to CC17 and were resistant to ampicillin and contained *esp*. Also, ST443 from swine harbored *purK1*, which is associated with CC17, and contained the *esp* and *hyl* genes.

Tn*1546* among CC17 isolates was highly diverse, with a number of variants containing IS*Ef1*. Tn*1546* types A, D, and S, lacking insertion sequences and associated with animals, were detected in the non-CC17 VRE isolates CC5 and CC9 (21, 38; this study).

In summary, this is the first study describing the population structure of *E. faecium* from different origins and locations in Portugal, one of the European nations with the highest VRE rates in the nosocomial setting (see the EARSS Annual Report 2006 [http://www.rivm.nl/earss]) (35). Our results pose new insights into *E. faecium* host specificity, since all the identified clonal complexes comprised isolates from different host origins. The frequent recovery from the community of HiRCC17 and the emergence of CC5 in the hospital setting highlight the role of nonhospital hosts as possible reservoirs of pathogenic enterococci. This community reservoir of multidrug-resistant strains able to cause human infections might contribute to their

TABLE 1. Epidemiological features of *Enterococcus faecium* isolates from human, swine, poultry, and environmental samples in Portugal

CC[a]	ST	PFGE type[b]	Epidemiology/origin	Source (product)[c]	Region/yr of isolation	Antibiotic resistance profile[d,e]	Virulence trait(s)[e]	Tn*1546* type[f]	Reference
CC17	16	74	Clinical isolate	HP_A (catheter)	Center/2001	VAN, TEC, AMP, TET, ERY, STR	esp	PP-9	22
CC17	18	108	Clinical isolate	HP_A (blood)	Center/2000	VAN, TEC, AMP, TET, ERY, CIP, STR, KAN	esp	PP-4	22
CC17	18	E	Hospital sewage (different places)	HW_F (wastewater)	North/2001	VAN, TEC, AMP, ERY, CIP, GEN, STR, KAN, QD	hyl	PP-16	24
CC17	18	59	Community surveillance	HV (fecal swab)	North/2001	VAN, TEC, AMP, TET, ERY, CIP, GEN, KAN, CHL, QD	None	PP-5	25
CC17	18	**78**	Outbreak strain (n 17 isolates) disseminated in 2 hospitals (2001–2003)	HP_A (liver fluid)	Center/2002	VAN, TEC, AMP, ERY, CIP, KAN, NIT	None	PP-4	22
CC17	18	**70**	Outbreak strain (n 14 isolates) disseminated in 3 hospitals (2001–2003)	HP_B (unknown)	Center Eastern/2002	VAN, TEC, AMP, TET, ERY, CIP, QD	None	PP-2b	22
CC17	18	97	Clinical isolate	HP_A (unknown)	Center	VAN, TEC, AMP, ERY, CIP	None	PP-4	22
CC17	18	69	Community surveillance	HV (fecal swab)	North/2004	VAN, TEC, AMP, TET, ERY, STR, KAN	None	D	25
CC17	18	125	Clinical isolate	HP_D (catheter)	North/2007	VAN, TEC, AMP, ERY, CIP	None	PP-5	This study
CC17	18	128	Clinical isolate	HP_D (pus)	North/2007	VAN, TEC, AMP, ERY, CIP, NIT	None	PP-5	This study
CC17	78	127	Clinical isolate	HP_D (urine)	North/2007	AMP, ERY, CIP, GEN, KAN, NIT	None	-	This study
CC17	80	80	Clinical isolate	HP_A (urine)	Center/1997	VAN, TEC, AMP, ERY, CIP	esp	PP-4	22
CC17	125	126	Clinical isolate	HP_D (urine)	North/2007	VAN, TEC, AMP, ERY, CIP, NIT	esp, hyl	PP-5	This study
CC17	132	**88**	Outbreak strain disseminated in two hospitals (1999–2001)	HP_A (blood)	Center/1999	VAN, TEC, AMP, TET, ERY, CIP, GEN, STR, KAN, QD	None	PP-4	22
CC17	132	**119**	Clinical isolate	HP_C (urine)	North/2002	VAN, TEC, AMP, ERY, CIP, GEN, KAN, QD	esp	PP-13	22
CC17	132	**119.5**	Environmental isolate from an intensive piggery	PE_{II} (liquid manure)	South/2007	VAN, TEC, AMP, ERY, GEN, KAN	esp	**PP-31**	This study
CC17	280	**100** (2)	Outbreak strain disseminated in two hospitals (2002–2003)	HP_B (urine, n 2)	Center Eastern/ 2002–2003	(VAN), (TEC), AMP, (TET), ERY, CIP, GEN, (STR), KAN	(esp), hyl	PP-5	This study
CC17	368	H	Hospital sewage (different places)	HW_C (wastewater)	North/2001	VAN, TEC, AMP, ERY, CIP, GEN, STR, KAN	esp	PP-20	24
CC17	369	RP5	River Douro (different places)	R (water sample)	North/2003	VAN, TEC, AMP, ERY, CIP, STR, KAN, QD	esp	X	24
CC17	390	122	Clinical isolate	HP_B (unknown)	Center Eastern/2002	AMP, TET, ERY, STR, KAN, QD	None	-	This study
CC17	393/431	**SN208** (2)	From one intensive piggery	SW_{II} (solid manure) PE_{II} (dust)	South/2006–2007	AMP, TET, ERY, STR, NIT	None	-	This study
CC17	430	SN211	From an intensive piggery	PE_{II} (food)	South/2007	AMP, TET, ERY, STR, NIT	None	-	This study
CC5	6	**A**	Strain from a pig slaughterhouse spread in 4 European countries (1997–2000)	SW (feces)	Center/1997	VAN, TEC, TET, ERY, KAN	None	D	26
CC5	5	$\mathbf{A_3}$	Clinical strain disseminated in 2 hospitals during 2001–2002	HP_C (pus)	North/2002	VAN, TEC, ERY	None	D	22
CC5	185	$\mathbf{A_5}$ (2)	From the soil of an extensive piggery	PE_{VI} (soil)	South/2007	VAN, TEC, AMP, TET	None	D	This study
CC5	150	SN216	From an intensive piggery	SW_V (feces)	North/2007	AMP, TET, ERY, CIP, STR, KAN	None	-	This study
CC9	148	36	HLR-Gm strain from HV and poultry	HV (fecal swab)	2001	AMP, TET, ERY, CIP, GEN, STR, KAN	None	-	25
CC9	236	**1**	Strain (n 18 isolates) identified in 5 different national brands during 3 yr	RP (carcass)	North/1999	VAN, TEC, TET, ERY, CIP, NIT, QD	None	A	23
CC9	236	**4**	Strain (n 18 isolates) identified in 3 different national brands during 3 yr	RP (carcass)	North/1999	VAN, TEC, TET, ERY, CIP, KAN, NIT, QD	None	A	23
CC9	394	8	Retail poultry isolate	RP (carcass)	North/1999	VAN, TEC, TET, ERY, CIP, NIT	None	A	23
CC9	395	**2**	Strain (n 11 isolates) identified in 4 different national brands during 3 yr	RP (carcass)	North/2001	VAN, TEC, TET, ERY, CIP, NIT, QD	None	A	23
CC9	405	12	Retail poultry isolate	RP (carcass)	North/1999	VAN, TEC, ERY, CIP, STR, NIT, QD	None	S	23
CC9	433	SN214	From one facility of an intensive piggery	PE_V (air)	North/2007	AMP, TET	None	-	This study
CC9	437	SN219	From an intensive piggery	PE_{IV} (food)	North/2007	AMP, TET, ERY, CIP, STR, KAN	None	-	This study
CC22	32	SN210	From antibiotic preparation given in an intensive piggery	PE_{III} (antiseptic)	South/2006	TET, KAN	None	-	This study

CC22	32	SN213	From an extensive piggery	PE_{VI} (water for consumption)	South/2007	TET, CIP, NIT	None	-	This study
CC22	392	41	Community surveillance	HV (fecal swab)	Center/2001	TET, ERY, CIP, GEN, STR, KAN, QD	None	-	25
CC22	435	SN217	From swine excrements of intensive piggery	SW_V (feces)	North/2007	TET, ERY, CIP, GEN, STR, KAN	None	-	This study
CC22	436	SN220	Animal feed isolate	PE_V (stock food)	North/2007	TET, CIP, KAN	*agg*, *cyl*, *esp*	-	This study
CC94	428	SN207	From the surroundings of intensive piggery	PE_I (waste lagoon)	South/2006	TET, ERY, GEN, KAN	None	-	This study
ST	30	SN221 (2)	Swine and animal feed isolates from an intensive piggery	SW_{III} (feces)/ PE_{III} (food)	South/2006	TET, ERY, CIP, GEN, KAN	None	-	This study
CS	89	58	Community surveillance	HV (fecal swab)	North/2001	VAN, TEC, ERY, STR, QD	None	D	25
CS	190	98	Clinical isolate	HP_A (urine)	Center/1998	VAN, TEC, AMP, TET, ERY, CIP, STR, KAN	None	A	22
CS	366	99	Clinical strain recovered during 1999–2000 in hospital A	HP_A (urine)	Center/1999	VAN, TEC, AMP, ERY, CIP, GEN, STR, KAN	*esp*	PP-5	22
CS	367	84	Clinical isolate	HP_A (catheter)	Center/2000	VAN, TEC, AMP, ERY, CIP, GEN, STR, KAN	*esp*	X	22
CS	391	124	Clinical isolate	HP_E (pus)	Center Eastern/2007	VAN, TEC, AMP, ERY, QD	*esp*	PP-5	This study
CS	404	D	From a pig slaughterhouse	SW (feces)	Center/1997	VAN, TEC, TET, ERY, CIP	None	A	This study
CS	406	**3**	Strain identified in 3 different national brands during 1999–2001	RP (carcass)	North/2001	VAN, TEC, TET, ERY, CIP, GEN, KAN, STR	None	A	23
CS	429	SN209	From swine excrements of intensive piggery	PE_{II} (solid manure)	South/2006	TET, ERY, STR, KAN	*gel*	-	This study
CS	432	SN212	From swine excrements of extensive piggery	PE_{VI} (liquid manure)	South/2007	TET, ERY, QD	None	-	This study
CS	434	SN215	From one facility of an intensive piggery	PE_{IV} (air)	North/2007	TET, ERY, STR	None	-	This study
CS	443	SN221	From a swine in the final facility before the abbatoir	PE_V (rectal swab)	North/2007	VAN, TEC, TET, ERY, NIT	*esp*, *hyl*	A	This study

[a] CCs are shown according to eBURST clustering. CS, singletons.

[b] Persistent and/or disseminated PFGE types are in bold. Strains identified with different PFGE subtypes were included in some cases, and their number is designated in parentheses.

[c] HP, hospitalized patients; HV, healthy volunteers; RP, retail poultry; SW, swine; PE, piggery environment; HW, hospital sewage; R, river. The different hospitals are designated by capital letters (A to E) and piggeries by roman numerals (I to VI). Capital letters represent the cities where hospitals are located: A, Coimbra; B, Viseu; C and F, Porto; D, Matosinhos; E, Covilhã.

[d] VAN, vancomycin; TEC, teicoplanin; AMP, ampicillin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; HLR, high level of resistance; GEN, gentamicin; STR, streptomycin; KAN, kanamycin; NIT, nitrofurantoin; CHL, chloramphenicol; QD, quinupristin-dalfopristin.

[e] Variable presence of a given antibiotic and virulence trait among isolates belonging to the same PFGE type appear in parentheses.

[f] Tn*1546* designation is based on the results obtained by a PCR overlapping assay as described previously (21, 38); PP-31 appears in bold because this is a new Tn*1546* type accordingly to the scheme described by Woodford et al. (38).

spread in hospitals and counteract any containment measure at the hospital level.

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AUTHOR'S CORRECTION

Dispersion of Multidrug-Resistant *Enterococcus faecium* Isolates Belonging to Major Clonal Complexes in Different Portuguese Settings

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Volume 75, no. 14, p. 4904–4908. Page 4904, Abstract, line 3: "clustering into CC17, CC5, CC9, CC22, and CC94" should read "clustering into CC17, CC5, CC9, and CC22."

Page 4904, column 2, paragraph 2, line 4: "which clustered into CC5, CC9, CC17, CC22, and CC94" should read "which clustered into CC5, CC9, CC17, and CC22."

Page 4905: Figure 1 should appear as shown below. (ST428 should not be considered as belonging to CC94.)

Page 4905, column 2, paragraph 2, lines 3 and 4: "ST428 (CC94)" should read "ST428."

Page 4907, Table 1, column 1, line 8: "CC94" should read "ST."

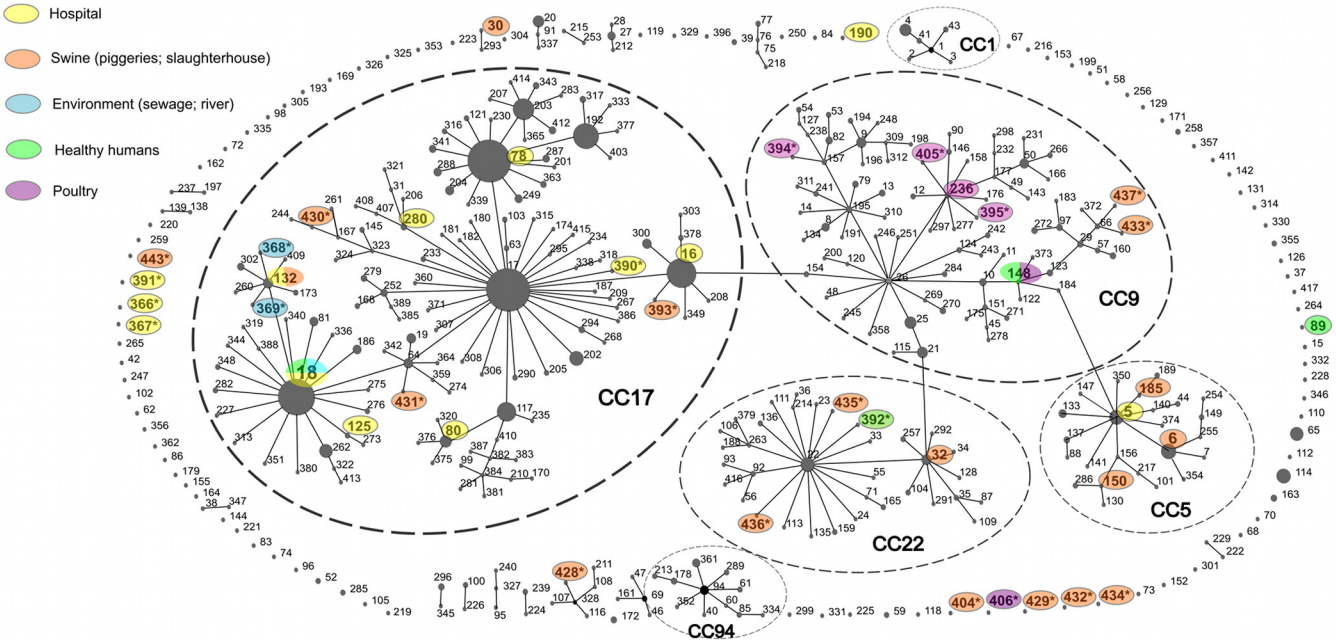


FIG. 1.

Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of *Enterococcus faecalis* from Portugal

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Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of *Enterococcus faecalis* from Portugal

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***Objectives*: The aim of this study was to assess the diversity of *Enterococcus faecalis* populations recovered in different regions of Portugal during the last decade (1996–2007) and to analyse their genetic elements associated with vancomycin resistance.**

***Methods*: Forty *E. faecalis* isolates (22 vancomycin-susceptible and 18 vancomycin-resistant) representing disseminated and/or multiresistant strains from different sources (humans, animals and the environment) were characterized by PFGE and multilocus sequence typing. Genes encoding putative virulence markers and the backbone of Tn*1546* were investigated by PCR. Plasmid analysis included determination of size, content (S1 hybridization) and comparison of restriction fragment length polymorphism patterns.**

***Results*: The 40 *E. faecalis* isolates (22 PFGE types) mostly clustered within the worldwide-spread clonal complexes (CCs) CC2 (13 ST6 mostly corresponding to an epidemic strain, where ST stands for sequence type), CC21 (3 ST21, 1 ST22 and 1 ST224) and ST16 ($n=7$), but also comprised ST159, ST35, ST19, ST26, ST30, ST41, ST55, ST59, ST117, ST160 and ST200. CC2 and CC21 were isolated from both hospital and community settings. Similar Tn*1546*-like elements encoding VanA were found on related plasmids within strains belonging to different clonal lineages and recovered in distinct hospitals over several years.**

***Conclusions*: The predominance of *E. faecalis* CC2 is mainly due to the dissemination of a particular clone persistently recovered for 11 years. The presence in the community of specific strains belonging to major clonal lineages highlights the role of community-associated hosts as possible reservoirs of putative human pathogenic enterococci. Both clonal expansion and dissemination of epidemic conjugative VanA plasmids seem to join forces in the establishment of pathogenic *E. faecalis* strains.**

Keywords: enterococci, MLST, CC2, CC21, ST16, VRE, mobile elements

Introduction

Enterococci are one of the most frequent causes of endocarditis, bacteraemia and urinary tract infections and have become one of the leading causes of nosocomial infections in recent years.[1] The species most frequently identified in human infections is *Enterococcus faecalis*, followed by *Enterococcus faecium*; however, vancomycin resistance (VanA-Tn*1546* and VanB-Tn*1549*) is more frequently associated with *E. faecium* than *E. faecalis*. Recent data show a progressive inversion of the ratio of *E. faecalis/E. faecium* causing infections, which seems to be associated with the increase in glycopeptide resistance of

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E. faecium.[2] Despite vancomycin resistance genetic elements being more diffused among *E. faecium*, the successful transfer of Tn*1546* from enterococci to more pathogenic bacteria such as *Staphylococcus aureus* has been associated with *E. faecalis*.[3]

Molecular epidemiological methods have contributed to the gain in significant knowledge on the population structure of the enterococcal species most commonly recovered from humans. The contemporary *E. faecalis* population structure comprises a diversity of clones with overrepresentation of clonal complexes (CCs) CC2, CC9, CC10, CC21, CC40 and CC87 and the prominent singleton ST16 (where ST stands for sequence type).[4,5] CC2, CC9 and CC87 are considered high-risk CCs, as they mostly include isolates causing infections in hospitalized patients.[4,6] The available data indicate that *E. faecalis* has an epidemic population structure that apparently lacks relevant host specificity, and recombination seems to be the driving force in the major genetic diversity and evolution of this species.[4–7]

The first nosocomial outbreak caused by vancomycin-resistant enterococci (VRE) detected in Portugal was associated with a single VanA *E. faecalis* clone that was widespread in different hospitals.[8] Several VRE and vancomycin-susceptible enterococci (VSE) strains persistently recovered or widely disseminated in different ecological niches have been reported.[9–11] The main purposes of our study were to determine the genetic relationships among diverse and representative *E. faecalis* strains collected in Portugal during the last decade and to gain insights into antibiotic resistance, virulence traits and plasmid content among persistent and sporadic *E. faecalis* clones from Portugal.

Material and methods

Bacterial strains

Forty *E. faecalis* isolates (22 VSE and 18 VRE), representing disseminated, persistent and/or multiresistant strains recovered during 1996–2007 in different settings, were selected for multilocus sequence typing (MLST). They included: (i) 21 clinical VRE and VSE isolates from hospitalized patients in different cities (Oporto, Coimbra and Viseu) representative of all PFGE types detected to date in our collection, including the arbitrarily designated strains B and N disseminated in Portuguese hospitals[8] and strains isolated from single patients; (ii) 4 isolates from swine excrement and 8 from environmental samples recently collected from 6 piggeries in rural areas of different regions of the country and selected as representative of multiresistant phenotypes, including 1 VRE and PFGE types disseminated in more than 1 piggery; (iii) 3 isolates from retail poultry of different commercial brands, including the only VRE detected among *E. faecalis* poultry isolates and 2 of the few vancomycin-susceptible *E. faecalis* recovered from this host;[10] (iv) 3 faecal VSE isolates from healthy volunteers living in three different cities, comprising 1 strain recovered also from poultry (the same PFGE type) and 2 strains representative of clones expressing high-level resistance to gentamicin, as no vancomycin-resistant *E. faecalis* was detected in our collection of 124 *E. faecalis* faecal isolates from human volunteers during 1999–2001;[11] and (v) 1 VRE isolate collected from hospital waste water corresponding to the hospital widespread strain B.[9]

Species identification and vancomycin resistance gene detection were performed by a multiplex PCR assay.[12] Antimicrobial susceptibility to 14 antibiotics (vancomycin, teicoplanin, erythromycin, tetracycline, ampicillin, ciprofloxacin, gentamicin, kanamycin, streptomycin, chloramphenicol, nitrofurantoin, daptomycin, linezolid and tigecycline) was determined by the standard agar dilution method.[13] High-level resistance was determined for all aminoglycosides according to CLSI guidelines. A multidrug resistance phenotype was considered for isolates non-susceptible to three or more antimicrobials of different chemical groups.

Virulence traits

We searched for the presence of genes coding for the virulence markers enterococcal surface protein (*esp*), cytolysin/haemolysin (*cyl*), gelatinase (*gelE*), hyaluronidase (*hyl*) and aggregation substance (*agg*) by a multiplex PCR described previously.[14]

Clonal relationship

The clonal relationship among isolates was established by PFGE and MLST as described previously.[4,8,15] Clusters of related STs were grouped into CCs by using eBURST (http://www.mlst.net).[16]

Genetic elements encoding vancomycin resistance

The backbone structure of Tn*1546* was determined by the PCR overlapping assay described by Woodford *et al.*[17] in all VRE isolates, some of which have been recently characterized.[18] Plasmid size and content were determined in the clinical VRE isolates by the S1 nuclease method described by Barton *et al.*,[19] and the plasmid location of the *van* gene was confirmed by hybridization of S1-digested genomic DNA with an intragenic *vanA* probe.[20] Plasmid extraction was performed by an alkaline lysis protocol,[21] and the relationships among plasmids were established by comparisons of the profiles obtained after digestion with *Eco*RI, *Cla*I and *Hin*dIII by standard methods.[20]

Results

E. faecalis population structure

The 40 *E. faecalis* isolates studied were recovered from hospitalized patients ($n=21$) and non-clinical sources ($n=19$) that included 3 from healthy humans, 12 from swine excrements and piggery environments, 3 from poultry carcasses and 1 from hospital wastewater. All isolates were multidrug-resistant, with the exception of one isolate from poultry and one from swine. Vancomycin resistance was encoded by the *vanA* gene in 18 isolates (45%) and it was mainly observed among human clinical isolates ($n=15$). They were divided into 22 PFGE types corresponding to 16 different STs including ST6 ($n=13$), ST16 ($n=7$), ST21 ($n=3$), ST35 ($n=3$), ST159 ($n=3$) and ST19, ST22, ST26, ST30, ST41, ST55, ST59, ST117, ST160, ST200 and ST224 (one isolate each). The largest CCs found among *E. faecalis* isolates were CC2 (ST6) and CC21 (ST21, ST22 and ST224), previously identified as major *E. faecalis* CCs (Figure 1).[4] This is the first description of the STs 159, 160, 200 and 224. Isolates belonging to a PFGE type were associated with a single ST, except for the PFGE type SN205 comprising isolates associated with unrelated genetic lineages (ST16 and ST224) by MLST. Detailed epidemiological data about these strains are provided in Table 1.

(i) CC2 (ST6). Thirteen isolates were classified into four PFGE types belonging to ST6 (11 VRE and 2 VSE) and were recovered

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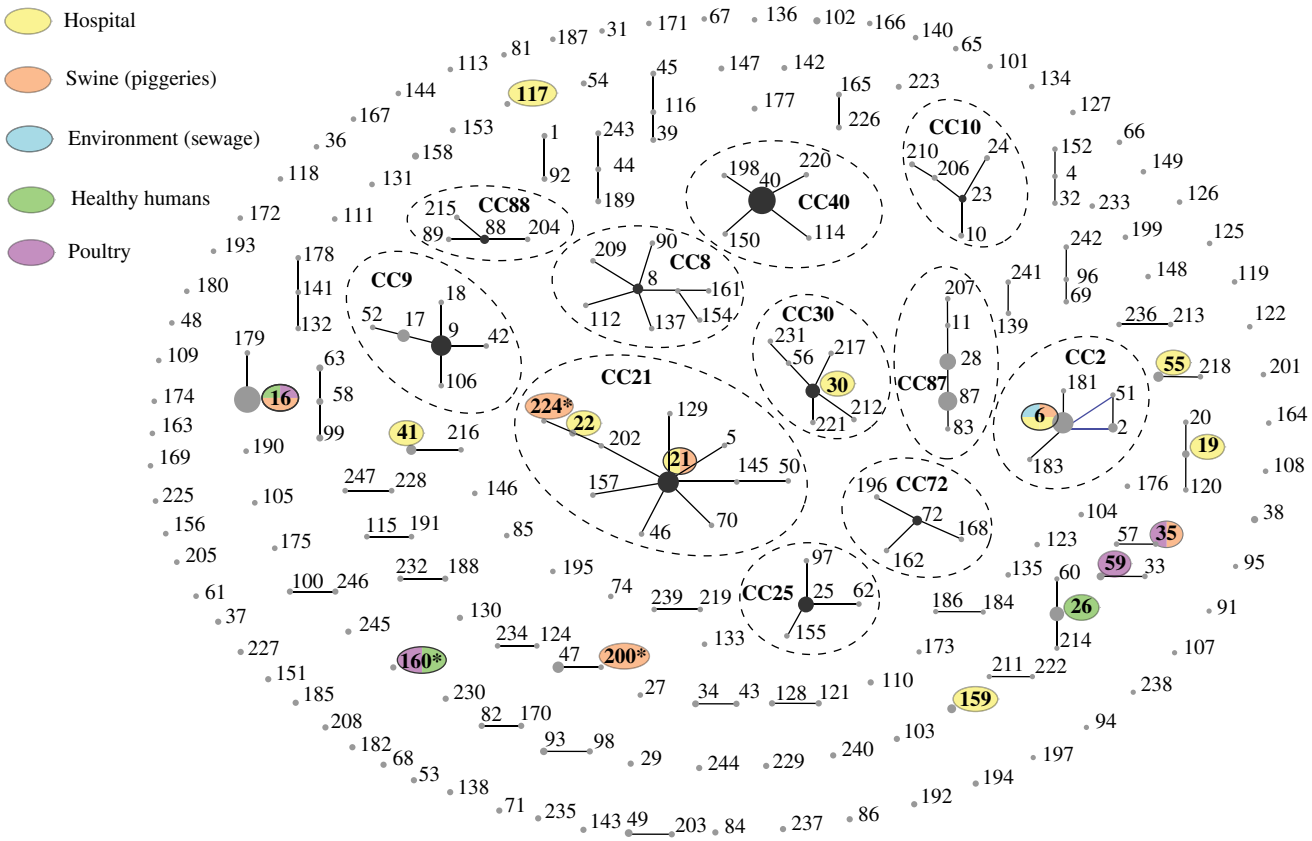


Figure 1. Clustering of 16 *E. faecalis* STs by use of eBURST. STs are indicated by coloured circles, representing 40 isolates from the present study, with 241 MLST profiles representing 350 *E. faecalis* isolates from the database (www.mlst.net). Each ST is represented as a node; the relative size of each node is indicative of its prevalence among the isolates; an asterisk indicates a new sequence type; black lines connect single-locus variants and blue lines connect double-locus variants. Colours indicate isolation sources and CCs are represented by dashed circles (CC2, CC8, CC9, CC10, CC21, CC25, CC30, CC40, CC72, CC87 and CC88). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

from hospitalized patients ($n=11$), swine ($n=1$) and hospital waste water ($n=1$) samples during a 12 year period (1996–2007). Most ST6 isolates ($n=10/13$) corresponded to the arbitrarily designed clone B responsible for different hospital outbreaks throughout Portugal in recent years[8] and were also recovered from swine liquid manure and hospital sewage. All ST6 isolates showed resistance to ciprofloxacin, gentamicin and kanamycin eventually associated with resistance to vancomycin and erythromycin (85% each), tetracycline and chloramphenicol (62% each) or streptomycin (46%). All ST6 isolates possessed the putative virulence *gelE* and *agg* genes, whereas *cyl* and *esp* were detected in 85% and 54% of isolates, respectively. More than half of the ST6 isolates were *agg*+/*gelE*+/*cyl*+/*esp*+ (54%), some were *agg*+/*gelE*+/*cyl*+ (31%) and only two isolates (15%) were *agg*+/*gelE*+. Different Tn*1546* types were identified among ST6-*vanA* clinical isolates (PP-4, PP-5, PP-15 and PP-16) and Tn*1546* type A was detected in the isolate recovered from swine.

(ii) CC21. Five isolates corresponding to three PFGE types recovered from hospital and swine samples were identified as ST21 (one from a hospitalized patient and two from a piggery; PFGE types 'X' and SN206), ST22 (hospital; PFGE type W) and ST224 (swine; PFGE type SN205.4) clustering in CC21. The ST224 strain represents a widely disseminated strain detected in five intensive piggeries. The three ST21 isolates recovered in a hospital and a piggery from 2000 to 2007 were resistant to erythromycin, exhibited variable resistance to glycopeptides and aminoglycosides, and showed highly related PFGE types arbitrarily designated as 'X'. The remaining CC21 isolates were also resistant to erythromycin. All but one harboured *gelE*+ and some also contained *agg* ($n=3$) or *esp* ($n=3$) or *cyl* ($n=1$). The only VRE isolate within CC21 was a clinical strain containing the Tn*1546* variant PP-4.

(iii) ST159. ST159 was assigned as a new ST and was identified in three clinical isolates corresponding to the PFGE type 'N' from hospitals in Coimbra ($n=1$ VRE in 1999) and Viseu ($n=2$, one VSE in 2001 and one VRE in 2002). These ST159 isolates were resistant to at least five antibiotics, and erythromycin and ciprofloxacin resistances were commonly detected. Their virulence profiles were *agg*+/*gelE*+/*cyl*+/*esp*+ ($n=2$) and *agg*+/*gelE*+ ($n=1$). Both *vanA* isolates harboured PP-4.

(iv) ST16. Three PFGE types comprising seven isolates from swine ($n=5$ VSE; one PFGE type), poultry ($n=1$ VRE) and healthy volunteer ($n=1$ VSE) samples collected during 2001–07 in different regions of Portugal were identified as ST16. Isolates corresponding to PFGE types SN205 and 22 were isolated from animals and recovered during 2 years from piggery samples of five scattered farms (2006–07) and poultry meat samples (1999–2001) of one commercial brand distributed at the national level, respectively. Resistances to tetracycline, erythromycin and

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Table 1. Epidemiological features of *E. faecalis* isolates from human, swine, poultry and environmental samples in Portugal

CC[a]	ST	PFGE type[b]	Epidemiology/origin	Source (product)[c]	Region/year of isolation	Antibiotic resistance profile[d,e]	Virulence traits[e]	Tn*1546* type[f]	*vanA* plasmid(s)[g]	Reference
CC2	6	**B-B_5** (n=8)	outbreak strain (n=37 isolates) widespread in six hospitals (1996–2008)	HP_A (blood, n=1; urine, n=2), HP_B (blood, n=1), HP_C (urine, n=3; wound, n=1)	Centre/1996–2001 North/2001–02	(VAN), (TEC), (TET), (ERY), CIP, GEN, KAN, (CHL)	*agg*, *gel*, (*cyl*), (*esp*)	PP-4 (n=3), PP-5 (n=1), PP-15 (n=2), PP-16 (n=1)	75–85 (n=4), 100 (n=3)	7; this study
CC2	6	**B_2**	from different sites of hospital sewage	HW_C (waste water)	North/2002	VAN, TEC, ERY, CIP, GEN, STR, KAN, CHL	*agg*, *gel*, *cyl*	PP-16	100	8
CC2	6	**B_6**	from swine excrements of an intensive piggery	SW_{II} (liquid manure)	South/2007	VAN, TEC, TET, ERY, CIP, GEN, KAN, CHL	*agg*, *gel*	A	100	this study
CC2	6	C	clinical isolate	HP_C (pus)	North/2001	VAN, TEC, ERY, CIP, GEN, STR, KAN, CHL	*agg*, *gel*, *cyl*	PP-15	40+100	this study
CC2	6	G	clinical isolate	HP_A (blood)	Centre/2002	VAN, TEC, TET, ERY, CIP, GEN, KAN	*agg*, *gel*, *cyl*, *esp*	PP-4	75	7
CC2	6	R	clinical isolate	HP_B (blood)	Centre/2002	ERY, CIP, GEN, STR, KAN	*agg*, *gel*, *cyl*, *esp*	—	—	this study
CC21	21	**X**	clinical isolate	HP_A (unknown)	Centre/2000	VAN, TEC, ERY	*agg*, *gel*	PP-4	145	7
CC21	21	**X_6**	from one facility of an intensive piggery	PE_{IV} (air)	North/2007	TET, ERY, GEN, STR, KAN	*gel*, *esp*	—	—	this study
CC21	21	SN206 (X_7)	from an intensive piggery; related to clone X (7 bands)	PE_{IV} (septic tank)	North/2007	TET, ERY, GEN, STR, KAN	*agg*, *gel*, *esp*	—	—	this study
CC21	22	W	clinical isolate	HP_B (unknown)	Centre/2002	ERY, CIP, STR	*gel*	—	—	this study
CC21	224	**SN205.4**	strain disseminated in five piggeries (2006–07)	SW_{II} (solid manure)	South/2006	TET, ERY, GEN, STR, KAN, CHL	*agg*, *cyl*, *esp*	—	—	this study
CS	159	**N-N_5** (n=3)	outbreak strain disseminated in two hospitals (1999–2002)	HP_A (blood, n=1), HP_B (urine, n=2)	Centre/1999–2002	(VAN), (TEC), (TET), ERY, CIP, (GEN), (KAN)	*agg*, *gel*, (*cyl*), (*esp*)	PP-4	85 (n=2)	7; this study
ST	16	62	community surveillance	HV (faecal swab)	North/2001	TET, ERY, GEN, STR, KAN, CHL	*agg*, *gel*, *cyl*, *esp*	—	—	10
ST	16	**22**	from retail poultry samples commercialized at national level (1999–2001)	RP (carcass)	North/2001	VAN, TEC, TET, ERY, CIP, STR	*agg*, *gel*	A	ND	9

Continued

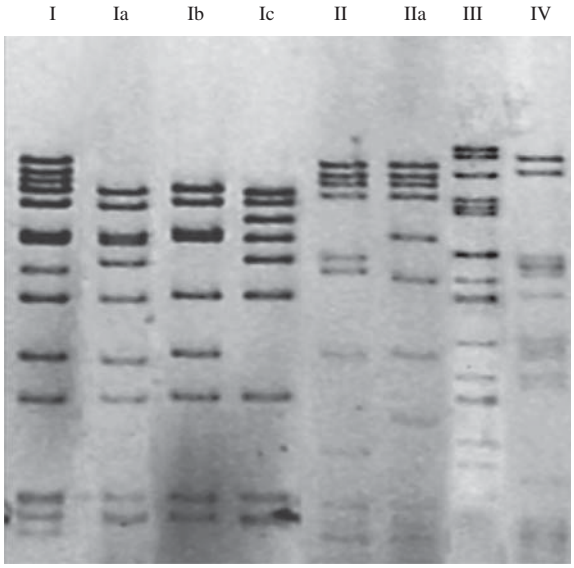
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Table 1. *Continued*

CC[a]	ST	PFGE type[b]	Epidemiology/origin	Source (product)[c]	Region/year of isolation	Antibiotic resistance profile[d,e]	Virulence traits[e]	Tn*1546* type[f]	*vanA* plasmid(s)[g]	Reference
ST	16	**SN205-205.4** ($n=5$)	strain disseminated in five intensive piggeries (2006–07)	PE_I (waste lagoon, $n=1$), SW_{III} (faeces, $n=1$), PE_{IV} (food, $n=1$), PE_V (manure, $n=2$)	South/2006, North/2007	TET, ERY, GEN, (STR), KAN, (CHL)	*agg*, (*gel*), *cyl*, (*esp*)	—	—	this study
ST	35	51	from retail poultry samples	RP (carcass)	North/1999	TET, ERY, GEN, STR, KAN	*agg*, *gel*, *cyl*	—	—	9
ST	35	**SN204** ($n=2$)	strain disseminated in two piggeries (2006–07)	SW_{III} (faeces, $n=1$), PE_{II} (food, $n=1$)	South/2006–07	TET, ERY, CIP, GEN, STR, KAN, CHL	*gel*	—	—	this study
ST	19	I	clinical isolate	HP_B (urine)	Centre/2001	TET, ERY, CIP, GEN, STR, KAN	*agg*, *gel*, *esp*	—	—	this study
ST	26	61	community surveillance	HV (faecal swab)	North/2001	TET, ERY, GEN, STR, KAN, CHL	*agg*, *gel*, *esp*	—	—	10
ST	30	K	clinical isolate	HP_C (pus)	North/2001	VAN, TEC, TET, ERY	*agg*, *gel*, *esp*	A	100	this study
ST	41	D	clinical isolate	HP_B (urine)	Centre/2001	TET	*agg*, *gel*	—	—	this study
ST	55	Q	clinical isolate	HP_A (blood)	Centre/2001	VAN, TEC, ERY, KAN	*esp*	PP-4	75	7
ST	59	45	from retail poultry samples	RP (carcass)	North/2001	TET, GEN, KAN	*agg*, *gel*	—	—	9
CS	117	F	clinical isolate	HP_C (blood)	North/2002	VAN, TEC, ERY	*agg*, *gel*	PP-5	150+300	this study
CS	160	**56**	strain recovered from both HV and poultry during 2001	HV (faecal swab)	North/2001	TET, CIP, GEN, KAN	*gel*	—	—	10
ST	200	SN201	from swine excrements of an intensive piggery	SW_{II} (solid manure)	South/2006	TET, ERY	*agg*, *gel*, *esp*	—	—	this study

[a]CC, clonal complexes are shown according to eBURST clustering; CS, singletons; ST, sequence types not assigned to any particular CC.
[b]Persistent and/or disseminated PFGE types are shown in bold. Strains identified with different PFGE subtypes were included in some cases and their number is designated in parentheses.
[c]HP, hospitalized patients; HV, healthy volunteers; RP, retail poultry; SW, swine; PE, piggery environment; HW, hospital sewage. The different hospitals are designated by capital letters (A, Coimbra; B, Viseu; C, Porto) and the piggeries by Roman numerals (I–V).
[d]VAN, vancomycin; TEC, teicoplanin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; KAN, kanamycin; CHL, chloramphenicol.
[e]The variable presence of a given antibiotic resistance gene or virulence trait among isolates belonging to the same PFGE type is indicated by parentheses.
[f]Tn*1546* designation is based on the results obtained by a PCR overlapping assay described previously.[17,18]
[g]Plasmid size is shown in kilobases (kb); ND, not determined.

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Plasmid type	Size (kb)	PFGE types (ST)	Tn*1546* type	Origin (no. of isolates)	Date
I	85	B (ST6); N (ST159)	PP-4; PP-5	HP ($n=3$)	1999–2001
Ia	75	B and G (ST6)	PP-4	HP ($n=2$)	1996–2002
Ib	75	B (ST6); Q (ST55)	PP-4	HP ($n=2$)	1999–2001
Ic	75	B (ST6)	PP-4	HP ($n=1$)	2001
II	100	B (ST6)	PP-15	HP ($n=2$)	2001
IIa	100	B (ST6)	PP-16	HW ($n=1$)	2002
III	100	B (ST6)	A	SW ($n=1$)	2007
IV	100	K (ST30)	A	HP ($n=1$)	2001

Figure 2. Gel electrophoresis (1% agarose) of *Eco*RI-digested *van*A plasmids isolated from representative VRE isolates. Lanes: lane 1, type I (HP; PFGE type B-ST6; PP-5); lane 2, type Ia (HP; PFGE type G-ST6; PP-4); lane 3, type Ib (HP; PFGE type Q-ST55; PP-4); lane 4, type Ic (HP; PFGE type B-ST6; PP-4); lane 5, type II (HP; PFGE type B-ST6; PP-15); lane 6, type IIa (HW; PFGE type B-ST6; PP-16); lane 7, type III (SW; PFGE type B-ST6; Tn*1546* type A); lane 8, type IV (HP; PFGE type K-ST30; Tn*1546* type A). HP, hospitalized patient; SW, swine; HW, hospital sewage.

aminoglycosides were expressed in all isolates, whereas those to vancomycin and ciprofloxacin were only detected in the poultry isolate. Different virulence profiles were observed: *agg*+/*cyl*+/*esp*+ (four from piggeries), *agg*+/*gelE*+/*cyl*+/*esp*+ (one healthy volunteer), *agg*+/*gelE*+/*cyl*+ (one swine) and *agg*+/*gelE*+ (one poultry). The *vanA* transposon characterized in the poultry isolate corresponded to the variant A.

(v) ST35. ST35 was identified in three VSE animal isolates from poultry, swine and a piggery (one each). The piggery isolate corresponded to a strain persisting in two different piggeries for 2 years. All were resistant to tetracycline, erythromycin, gentamicin, streptomycin and kanamycin, and the piggery isolate additionally expressed resistance to ciprofloxacin and chloramphenicol. Both swine and piggery isolates harboured *gelE*, while the poultry isolate was positive for *agg*, *gelE* and *cyl*.

(vi) Other E. faecalis STs. The STs ST19, ST26, ST30, ST41, ST55, ST59, ST117, ST160 and ST200 were represented by only one strain. They were recovered from hospitalized patients (ST19, ST30, ST41, ST55 and ST117), healthy humans (ST26 and ST160), poultry (ST59) and swine (ST200) from 2001 to 2006. Different antibiotic resistance and virulence marker profiles were observed within these STs (Table 1). Most STs contained *agg* and *gelE* with or without *esp*. The Tn*1546* types A, PP-4 and PP-5 were observed in ST30, ST55 and ST117 isolates, respectively.

Genetic elements encoding vancomycin resistance

Five Tn*1546* types, previously described by our group,[18] were identified among the 18 VRE isolates, either containing IS*Ef1* (PP-4 and PP-5) or IS*1216* (PP-15 and PP-16) or corresponding to the prototype transposon A.[17] The 17 VRE isolates studied carried *vanA* on conjugative plasmids of variable size (40–300 kb), those of medium size (e.g. 80 and 100 kb) being the most frequently observed (Table 1). Four types (I–IV) were associated with the different Tn*1546* types identified (Figure 2). Type I (75–85 kb), comprising four subtypes, was identified in four *E. faecalis* clones clustering in CC2 (ST6; two PFGE types; $n=5$), ST159 ($n=2$) and ST55 ($n=1$), and contained the most disseminated Tn*1546* types (variants PP-4 and PP-5) identified in three hospitals during 1996–2002.[18] Type II (100 kb), two subtypes, was identified in three ST6 isolates (PFGE type B) harbouring the related Tn*1546* variants PP-15 and PP-16, which were recovered from the same hospital and respective hospital waste water in different years. Plasmid types III and IV (100 kb each) were recovered from two *E. faecalis* strains of clinical and animal origins, both harbouring Tn*1546* type A. Megaplasmids hybridizing with the *vanA* gene were identified in clinical isolates of ST21 (145 kb) and ST117 (150 and 300 kb) (Table 1).

Discussion

This study indicates the dominance in the Portuguese nosocomial setting of the major *E. faecalis* clonal lineage CC2 that is globally associated with human infections, reveals the absence in Portugal of particular CCs (CC9, CC40 and CC87) that are widely spread in other EU countries[4,7,22] and describes the role of horizontal transfer in the dissemination and persistence of VRE in Portuguese hospitals.

CC2 was identified among most clinical isolates studied, which mainly corresponded to a widely disseminated multidrug-resistant strain previously designated as 'clone B', recovered from Portuguese hospitals since 1996,[8] but also among *vanA* isolates from swine manure and hospital sewage, indicating a community contamination by this *E. faecalis* high-risk CC. Moreover, the diversity of the antibiogram and virulence gene profiles of CC2 isolates from extra-hospital settings further supports the suggestion that CC2 is particularly proficient at the DNA exchange highlighted by McBride *et al.*[7] A recent study including clinical isolates from a hospital in Southern Portugal

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also described the predominance of *E. faecalis* CC2 mainly associated with a dominant and epidemic clone,[23] confirming the establishment of this clonal lineage in our country. Although we did not identify other high-risk CCs, the new singleton ST159 was persistently obtained from clinical isolates in different Portuguese cities during a 4 year period (1999–2002) and comprised both multiresistant VRE and VSE isolates mainly possessing *esp* and *cyl*. Interestingly, the double-locus variant of ST159 designated as ST103 (differing in two of the seven housekeeping genes) was also identified in clinical VRE isolates from the USA in 2002 (P. Ruiz-Garbajosa, A. R. Freitas, M. Zervos, S. Donabedian, R. Cantón, F. Baquero and T. M. Coque, unpublished results), which might reflect the possible emergence of a new epidemic ST in Portugal with a parallel emergence of related STs in other distant nations. Other STs identified from clinical isolates as ST19, ST30 and ST55 have also been associated with human strains from Spain, the Netherlands, the UK and the USA from the mid-1990s, but their relevance to the whole *E. faecalis* population structure remains to be proved.[4,7]

CC21 and ST16 isolates were mainly associated with widespread persistent clones from swine or poultry samples in Portugal. CC21 encompasses mostly animal and community surveillance isolates and only a few clinical strains.[4] Moreover, an ST21 strain was recovered from human hospital and piggery samples, highlighting the dispersion of this CC in different ecological settings. Although previously characterized CC21 strains usually harboured less antibiotic resistance and fewer virulence traits than isolates of other CCs,[4,7,22] the different genetic content exhibited by isolates of the Portuguese CC21 clones indicates successful acquisition of diverse genetic elements that might facilitate their persistence and spread in environments under selective antibiotic pressure. The presence of ST16 and ST160 strains in both poultry and faecal samples of healthy humans supports the spread of enterococcal strains between animals and humans via the food chain, which has also been observed in Denmark, New Zealand, Japan, Thailand and the USA, sometimes also associated with ST16.[11,24–29] In addition, ST59 identified in one of the few Portuguese isolates from retail poultry included in this study was recently described in healthy animals from Spain,[4] in chickens with arthritis and amyloid arthropathy from France[30] and in human clinical samples from Poland.[22] In our set of *E. faecalis* isolates, *cyl* was frequently associated with specific clonal lineages (CC2, ST159 and ST16), while *esp* was widely disseminated among CC2, CC21 and most singletons, both in human and animal isolates. These observations support previous data from other authors in which the role of *cyl* and *esp* genes in the pathogenicity of enterococci is underscored.[7,29]

The finding of similar *vanA* plasmids among clonally related (ST6-CC2) or unrelated strains (ST6, ST55 and ST159) recovered from different hospitals over long periods of time highlights the role of horizontal gene transfer in the spread of vancomycin resistance among enterococci. The similarity of the restriction fragment length polymorphisms corresponding to plasmids containing similar Tn*1546* variants suggests the evolution of specific plasmids by different genetic events.

In summary, our findings reflect an *E. faecalis* population represented by CC2, CC21, ST159 and five unrelated STs among hospitalized humans, and CC21, ST16 and ST35 in the community setting in Portugal. The high prevalence of the worldwide-disseminated CC2 is associated with clonal expansion of a single strain in our area. In addition, plasmid dissemination seems to have greatly influenced the dissemination of vancomycin resistance among *E. faecalis* in Portugal.

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Transparency declarations

None to declare.

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Human and Swine Hosts Share *Enterococcus faecium* CC17, CC5, and *Enterococcus faecalis* CC2 Clonal Clusters with Common Plasmids Harboring VanA-Tn*1546*

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Human and Swine Hosts Share Vancomycin-Resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 Clonal Clusters Harboring Tn*1546* on Indistinguishable Plasmids▽†

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VRE isolates from pigs ($n = 29$) and healthy persons ($n = 12$) recovered during wide surveillance studies performed in Portugal, Denmark, Spain, Switzerland, and the United States (1995 to 2008) were compared with outbreak/prevalent VRE clinical strains ($n = 190$; 23 countries; 1986 to 2009). Thirty clonally related *Enterococcus faecium* clonal complex 5 (CC5) isolates (17 sequence type 6 [ST6], 6 ST5, 5 ST185, 1 ST147, and 1 ST493) were obtained from feces of swine and healthy humans. This collection included isolates widespread among pigs of European Union (EU) countries since the mid-1990s. Each ST comprised isolates showing similar pulsed-field gel electrophoresis (PFGE) patterns (≤6 bands difference; >82% similarity). Some CC5 PFGE subtype strains from swine were indistinguishable from hospital vancomycin-resistant enterococci (VRE) causing infections. A truncated variant of Tn*1546* (encoding resistance to vancomycin) and *tcrB* (coding for resistance to copper) were consistently located on 150- to 190-kb plasmids (rep_{pLG1}). *E. faecium* CC17 (ST132) isolates from pig manure and two clinical samples showed identical PFGE profiles and contained a 60-kb mosaic plasmid (rep_{Inc18} plus rep_{pRUM}) carrying diverse Tn*1546*-IS*1216* variants. The only *Enterococcus faecalis* isolate obtained from pigs (CC2-ST6) corresponded to a multidrug-resistant clone widely disseminated in hospitals in Italy, Portugal, and Spain, and both animal and human isolates harbored an indistinguishable 100-kb mosaic plasmid (rep_{pRE25} plus rep_{pCF10}) containing the whole Tn*1546* backbone. The results indicate a current intra- and international spread of *E. faecium* and *E. faecalis* clones and their plasmids among swine and humans.

Vancomycin-resistant enterococci (VRE) are among the most common nosocomial pathogens in the United States and in several European Union (EU) countries (23, 50). They have frequently been isolated from farm animals, pets, and retail food products in Europe, but until very recently, the detection of VRE from either processing or production food animal environments in the United States was infrequent (11, 20, 42, 53). There is limited evidence as to the direct role of the food chain in the dissemination of VRE among humans. Despite this, the potential hazard has been widely recognized and led to the adoption of intervention measures, such as the ban on the growth-promoting use of antimicrobials in the EU. A remarkable reduction in the prevalence of VRE among animals and humans has been observed after the EU withdrawal (see reference 42 and references therein). However, the role of nonhuman hosts as reservoirs of highly transmissible clones, the transient or permanent human fecal carriage of VRE of animal origin, and the consequent risk of gene transfer to resident human flora are issues still discussed and not fully addressed (41, 42).

Both *Enterococcus faecium* and *Enterococcus faecalis* are opportunistic pathogens comprising some host-specific lineages (30, 53). Strains from human-adapted clonal complexes (CCs) causing most enterococcal infections may eventually be recovered from farm and companion animals (e.g., *E. faecium* clonal complex 17 [CC17] and *E. faecalis* CC2), and strains from CCs commonly found among animals have also been isolated from humans (*E. faecium* CC5, *E. faecalis* sequence type 16 [ST16], or *E. faecalis* CC21) (4, 9, 13, 14, 28, 53). Documented cases of animal-human VRE transmission frequently involve healthy humans in close interaction (farming or petting) with animals, but most of these studies do not provide molecular characterization of either clones or their

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TABLE 1. Origins of VRE isolated from swine and healthy humans during national surveillance studies in Denmark, Portugal, Spain, Switzerland, and the United States (1995 to 2008)

Species	Country	No. isolated from:		Origin	Reference(s)
		Swine	Healthy humans		
E. faecium (n = 40)	Denmark	15	3	15 VREfm from healthy pigs among 1,594 *E. faecium* fecal isolates (DANMAP[a], 1995–2006)	17, 26; this study
				3 VREfm recovered from 525 community-dwelling human samples (2002–2006)	17; this study
	Portugal	4	9	4 VREfm from 84 fecal or environmental samples in different production piggeries (1997; 2006–2007); one isolate from 1997 belongs to the CC5 widespread clone recovered in different countries over years	14, 35
				9 VREfm fecal isolates from 99 healthy volunteers living in different Portuguese cities (2001–2004)	37
	United States	6	0	6 VREfm recently isolated from 55 swine (10.9%) in three Michigan counties (2008)	11
	Spain	2	0	2 VREfm recovered from 900 pig fecal samples at slaughterhouses (9.7% of all pigs slaughtered in 1998) in Valencia and Murcia (1998–2000)	22, 35
	Switzerland	1	0	1 VREfm recovered from samples of pig feces among 155 *Enterococcus* isolates obtained in 16 Swiss farms (1999–2000)	5, 35
E. faecalis (n = 1)	Portugal	1	0	1 VREfs from 84 fecal or environmental samples in different production piggeries (2006–2007)	13

[a] DANMAP, Danish Integrated Antimicrobial Resistance Monitoring and Research Programme.

subcellular genetic elements (1, 3, 10, 17, 26, 28, 31, 33), despite the comprehensive epidemiological studies of Tn*1546* (*vanA*) and Tn*5382* (*vanB*) (8, 24, 38, 52, 54).

In this work, a comparative multilayered molecular analysis of representative VRE strains from swine, healthy humans, and clinical isolates recovered from wide surveillance studies was carried out with the aim of identifying and characterizing epidemic VRE clones and plasmids shared by human and swine hosts.

MATERIALS AND METHODS

Bacterial strains and epidemiological background. The epidemiological background of the 41 isolates analyzed in this study is shown in Table 1. It includes representative isolates of VRE recovered from swine and healthy humans in national surveillance studies conducted in Portugal and Denmark (1995 to 2008) (references 17, 26, 35, 37, and 38 and this study), strains widespread among swine from Switzerland and Spain (5, 22), and the first VRE isolates recently recovered from animals in the United States (11). For comparison, we included a large and well-typed collection of clinical VRE isolates (140 *E. faecium* and 50 *E. faecalis* isolates) recovered from 23 countries, including Portugal, Spain, Denmark, and the United States, during the last 3 decades, most of which had caused hospital outbreaks (12, 16). Testing of susceptibility to 12 antibiotics was performed either by E strip (bioMérieux, Solna, Sweden) or by a standard agar dilution method following recommended guidelines of the manufacturer or CLSI (6). The presence of genes coding for antimicrobial (glycopeptides, macrolides, tetracyclines, and aminoglycosides) and copper (*tcrB*) resistance and putative virulence traits (*agg*, *gel*, *cyl*, *esp*, and hyl_{Efm}) was analyzed by using different PCR schemes (18, 36).

Clonal relatedness. Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), as described previously (36, 48; http://www.mlst.net). Computer analysis of the PFGE banding patterns was performed with the Fingerprinting II Informatix software package (Bio-Rad Laboratories, Hercules, CA). The similarity of the PFGE banding patterns was analyzed by the Dice coefficient, and cluster analysis was performed by the unweighted pair group method with arithmetic average (UPGMA). We applied a cutoff equivalent to 82% to group possibly genetically related isolates (34). Different PFGE types were designated by capital letters or numbers (35, 36, 38). Subtypes were designated by a number (indicating the number of bands that differed from the index strain) and primes when necessary (to distinguish among subtypes with the same number of bands but showing different banding patterns) (48).

Characterization of glycopeptide resistance. The Tn*1546* backbone was analyzed by PCR mapping, as previously described (38, 54). Conjugation experiments were performed by filter mating at a 1:1 donor-recipient ratio using *E. faecium* GE-1 and/or 64/3 and *E. faecalis* JH2-2 as recipient strains and vancomycin (6 mg/liter), rifampin (30 mg/liter), and fusidic acid (20 mg/liter) as antimicrobial selective markers. The genomic locations of *vanA* and *tcrB* were assessed by hybridization of I-CeuI and S1 nuclease (Takara Bio Inc., Shiga, Japan)-digested genomic DNA using intragenic *vanA*, *tcrB*, and 23S rRNA gene probes (16). Plasmid analysis included determination of size and content by PFGE of S1 nuclease-digested genomic DNA as previously described (16). Also, the identification of replication initiator proteins (rep) and maintenance systems (the toxin-antitoxin systems Axe-Txe and ω-ε-ζ and the partition module par_{pAD1}) was performed using recently developed PCR plasmid-typing methods, sequencing, and hybridization (25, 44). Restriction fragment length polymorphism (RFLP) analysis using EcoRI or ClaI enzyme was performed for plasmids of <150 kb (13). The plasmids were designated with Roman numerals (*E. faecalis*) or capital letters (*E. faecium*), following the nomenclature of previous studies in which some of the strains were initially reported (13). Hybridization experiments were performed by using the Gene Images AlkPhos Direct labeling and detection system (Amersham GB, GE Healthcare Life Sciences UK Limited). We referred to the rep sequences according to the plasmid type in which they were identified, as well by as the numeric nomenclature used by Jensen et al. (25).

RESULTS

Thirty-two (31 *E. faecium* [VREfm]; 1 *E. faecalis* [VREfs]) of the 41 VRE isolates studied were grouped in PFGE types highly similar to those of strains causing infection in hospitalized patients. They were identified as *E. faecium* CC5 (n = 30), *E. faecium* CC17 (n = 1), and *E. faecalis* CC2 (n = 1). Five other fecal isolates from healthy humans were classified as *E. faecium* ST18 (CC17), which is one of the predominant STs of the polyclonal subcluster CC17, although no similar PFGE types were observed among hospital VRE. The other four isolates were recovered from healthy humans, but their PFGE

or MLST profiles were not related to animal or hospital VRE. A detailed analysis of the isolates found in swine and healthy and hospitalized humans is provided in Table 2.

CC5 *E. faecium* carrying *vanA* on large plasmids (>150 kb) spread among human and swine hosts from the EU and the United States. Thirty *E. faecium* isolates clustering in CC5 (17 ST6, 6 ST5, 5 ST185, 1 ST147, and 1 ST493) were obtained from swine samples from Denmark, Portugal, Spain, Switzerland, and the United States, as well as from fecal samples from healthy Danes (1995 to 2008) (Table 2). Each ST comprised isolates recovered during a wide temporal and geographical frameshift that showed similar PFGE banding patterns (≤6 bands difference, >82% similarity [Fig. 1]): ST6 (1995 to 2008; 5 countries; subtypes A, A1, A1′, A1″, A1‴, and A2), ST5 (2003 to 2008; 2 countries; A1⁗, A3″, A3‴, and A5), ST147 (2003; 1 country; A5), ST185 (2001 to 2008; 3 countries; A3, A3′, A4, and A4′), and ST493 (2005; 1 country; A6). The isolates were classified as clonally related following interpretive criteria using PFGE (48) and taking into account the similarity among MLST profiles (all STs were single- or double-locus variants [SLVs or DLVs, respectively] of ST6, with the exception of ST493, which differs in three alleles). These strains were similar to a CC5 epidemic VRE clone widespread among swine from different EU countries since the mid-1990s, and they were considered to be the same clone (35). Comparison with large collections of hospital VRE revealed two clonally related isolates causing urinary tract infections in patients from different Portuguese hospitals (ST5, subtype A3⁗). These clinical isolates were not associated with a nosocomial outbreak.

All human and animal isolates expressed *tetM*-mediated tetracycline resistance, and most isolates (25/30) were also resistant to erythromycin (*ermB*), while none contained the putative virulence gene *esp* or *hyl*. They all carried a deleted variant of Tn*1546* previously designated type "D" and largely linked to swine hosts in different studies (26, 38, 54) and the gene *tcrB* coding for copper resistance, both located on large conjugative plasmids ranging from ca. 150 kb to 190 kb (see Fig. S1 in the supplemental material). All *vanA* plasmids carried a protein homologous to RepA of the recently sequenced megaplasmid pLG1 (GenBank accession number HM565183), which seems to be widespread among hospital *E. faecium* strains (29; A. R. Freitas and T. M. Coque, unpublished data).

CC17-ST132 *E. faecium* carrying Inc18-like *vanA* plasmids spread among human and swine hosts from Portugal. One ST132-VREfm isolate from swine ($n = 1$) recovered in Portugal in 2007 showed a PFGE type identical to that of two Portuguese isolates causing urinary tract infections recovered in 2002 (14) (Table 2 and Fig. 1). ST132 is an SLV of ST18, a predominant clone of the CC17 polyclonal subcluster. Although ST132 is not a predominant CC17 clone, isolates belonging to the ST have been recovered from unrelated patients hospitalized in Portugal and Tanzania, and it has been associated with a nosocomial outbreak described in Spain during an 18-month period, suggesting potential transmissibility (14, 39, 43, 53; http://efaecium.mlst.net).

All three isolates expressed resistance to ampicillin, erythromycin, and glycopeptides and high levels of resistance (HLR) to gentamicin and kanamycin due to the presence of *ermB*, *vanA*, *aac(6′)-aph(2″)*, and *aph(3′)-IIIa* genes. They also contained the *esp* gene, usually associated with a pathogenicity island of *E. faecium* (Table 2). Similar Tn*1546* backbones identified in these isolates (all carried IS*1216* within the *vanX-vanY* region and differed only upstream of *vanR*) were located on ca. 60-kb plasmids showing highly similar RFLP patterns (Fig. 2). Plasmid DNA hybridized with probes specific for homologues of replicases from the Inc18 plasmid pRE25 ($rep2_{pRE25}$; GenBank accession no. X92945) and pRUM ($rep17_{pRUM}$; GenBank accession no. AF507977). The *vanA* plasmid recovered from the clinical isolate contained a third rep highly homologous to pIP501 and other Inc18-like plasmids ($rep1_{pIP501}$; GenBank accession no. AJ505823) and was the only transferable vancomycin-resistant plasmid harbored by the clone.

Plasmids showing an RFLP pattern identical to that identified in ST132 strains have also been observed among strains isolated from hospital sewage and the Douro river in Portugal over a long time. Although they belong to ST368 and ST369, which are SLVs of ST132, they showed different PFGE types (data not shown).

Although no clonal relationships with clinical or animal isolates were observed for the two PFGE types corresponding to the five *E. faecium* ST18 (CC17) isolates recovered from a healthy human during a 5-year period, it is of interest to highlight the relationship of the genetic elements of these strains with others described above. Both clones expressed resistance to ampicillin, erythromycin, and tetracycline, while they differed in susceptibility to ciprofloxacin and resistance to high levels of gentamicin and streptomycin (Table 2). Two Tn*1546* variants, one containing an IS*Ef1* insertion, which is usually recovered from hospital VRE (previously designated PP5) (39), and the other associated with swine (type D), were linked to each PFGE type (Table 2), probably reflecting different acquisition events. The rep gene content was similar to that described above for other *E. faecium* CC17 isolates.

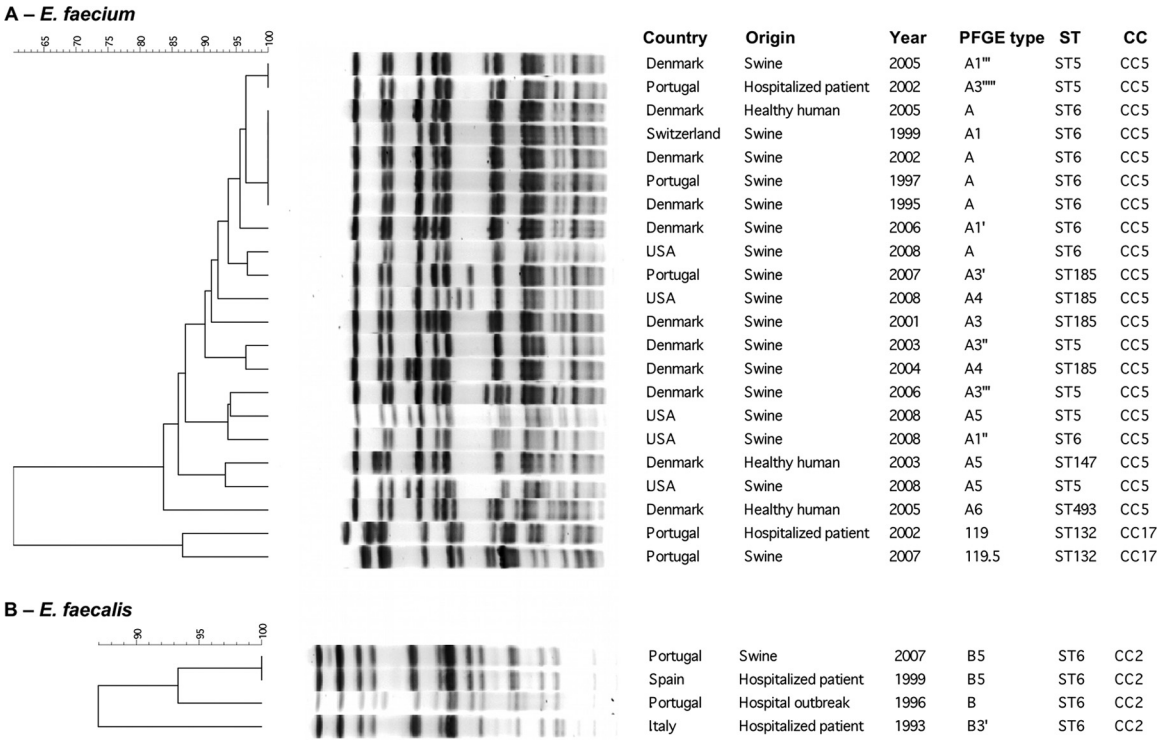
CC2-ST6 *E. faecalis* strains carrying pheromone-like *vanA* plasmids are disseminated among human and swine hosts in Europe. The VREfs isolate recovered from Portuguese swine showed a PFGE type identical to that of a multidrug-resistant ST6-CC2 *vanA E. faecalis* strain isolated from Portuguese, Italian, and Spanish hospitals since at least 1993 (15) (Fig. 1). They commonly expressed resistance to tetracycline, erythromycin, ciprofloxacin, and HLR to gentamicin, while HLR to kanamycin were seen and resistance to chloramphenicol was variable. The *vanA*, *tetM*, and *aac(6′)-aph(2″)* genes were detected in all four isolates, with the exception of the pig isolate, which lacked *ermB* but still contained *aph(3′)-IIIa* (Table 2). Like the majority of VREfs from Portuguese hospitals, the swine isolate did not contain *esp* (unpublished results).

A complete Tn*1546* backbone was identified in all CC2-ST6 isolates analyzed except the Portuguese clinical isolate, in which an IS*Ef1* insertion was identified (36). Plasmids carrying Tn*1546* ranged from 85 kb to 100 kb. The same ClaI-digested DNA pattern was observed among plasmids of ca. 100 kb from the Spanish clinical isolate (1999) and the Portuguese swine isolate (Fig. 2), which contained sequences homologous to those of replicases linked to pRE25 ($rep2_{pRE25}$) and to pheromone-responsive plasmids pBEE99 and pTEF2 ($rep9_{pCF10}$) (GenBank accession numbers NC_013533 and NC_004671, respectively).

TABLE 2. Epidemiological and genetic backgrounds of clonally related enterococcal isolates from humans and swine recovered in Denmark, Portugal, Spain, Switzerland, and the United States

Species and CC	ST	PFGE type	No. of isolates	Source	Country	Yr of isolation	Antibiotic resistance profile[g]	Antibiotic resistance genes	*tcrB*[h]	Putative virulence traits	Tn*1546* type[f]	Mating[h]	*vanA* plasmid	
													Size (kb)	RFLP[i]
E. faecalis CC2	ST6	B3	1	Hospitalized patient (blood)	Italy	1993	VAN, TEC, TET, ERY, CIP, GEN, CHL	*vanA*, *tetM*, *ermB*, *aac(6)-aph(2)*		*agg*, *gel*	A		100	VI
		B[a]	1	Hospitalized patient (blood)	Portugal	1996	VAN, TEC, TET, ERY, CIP, GEN, KAN, CHL	*vanA*, *tetM*, *ermB*, *aac(6)-aph(2)*		*agg*, *gel*, *cyl*, *esp*	PP-4		85	I
		B5	1	Hospitalized patient (blood)	Spain	1999	VAN, TEC, TET, ERY, CIP, GEN, CHL	*vanA*, *tetM*, *ermB*, *aac(6)-aph(2)*		*agg*, *gel*	A		100	III
		B5	1	Piggery (manure)	Portugal	2007	VAN, TEC, TET, ERY, CIP, GEN, KAN, CHL	*vanA*, *tetM*, *aac(6)-aph(2)*, *aph(3)-IIIa*		*agg*, *gel*	A		100	III
E. faecium CC17	ST132[b]	119[c]	2	Hospitalized patient (UTI[j])	Portugal	2002	VAN, TEC, AMP, ERY, CIP, GEN, KAN, Q-D	*vanA*, *ermB*, *aac(6)-aph(2)*, *aph(3)-IIIa*		*esp*	PP-13		65	P
		119.5	1	Piggery (manure)	Portugal	2007	VAN, TEC, AMP, ERY, GEN, KAN	*vanA*, *aac(6)-aph(2)*, *aph(3)-IIIa*		*esp*	PP-31		60	P1
E. faecium CC5	ST5	A3 [d]	2	Hospitalized patient (UTI)	Portugal	2001–2002	VC, TEC, TET, ERY	*vanA*, *tetM*, *ermB*			D		150	ND
		A1 , A3 , A3	4	Slaughterhouse (feces)	Denmark	2003–2006	VAN, TEC, TET, ERY	*vanA*, *tetM*, *ermB*			D (*n* 2)		150–170	ND
		A5	2	Piggery (animal feces)	United States	2008	VAN, TEC, TET, ERY	*vanA*, *tetM*, *ermB*			D		170	ND
	ST6	A, A1 , 1 , 2	9	Slaughterhouse (feces)	Denmark	1995–2006	VAN, TEC, TET, (ERY)	*vanA*, *tetM*, *(ermB)*			D (*n* 3)		145–180	ND
		A	1	Healthy humans	Denmark	2005	VAN, TEC, TET, ERY	*vanA*, *tetM*, *ermB*			D		175	ND
		A[e]	1	Slaughterhouse (feces)	Portugal	1997	VAN, TEC, TET, ERY	*vanA*, *tetM*, *ermB*			D		175	ND
		A1	1	Piggery (feces)	Switzerland	1999	VAN, TEC, TET, ERY	*vanA*, *tetM*, *ermB*			D		150	ND
		A1	2	Slaughterhouse (feces)	Spain	1998–2000	VAN, TEC, TET, ERY, KAN, STR	*vanA*, *tetM*, *ermB*, *aph(3)-IIIa*			D		150	ND
		A, A1	3	Piggery (animal feces)	United States	2008	VAN, TEC, TET, ERY	*vanA*, *tetM*, *ermB*			D (*n* 2)		170	ND
	ST147	A5	1	Healthy humans	Denmark	2003	VAN, TEC, TET	*vanA*, *tetM*			D		175	ND
	ST185	A3, A4	2	Slaughterhouse (feces)	Denmark	2001–2004	VAN, TEC, TET, (ERY)	*vanA*, *tetM*, *(ermB)*			D		160–180	ND
		A3	2	Piggery (soil)	Portugal	2007	VAN, TEC, TET	*van*, *tetM*			D		150	ND
		A4	1	Piggery (animal feces)	United States	2008	VAN, TEC, TET, ERY	*vanA*, *tetM*, *ermB*			D		170	ND
	ST493	A6	1	Healthy humans	Denmark	2005	VAN, TEC, TET, ERY, KAN	*vanA*, *tetM*, *ermB*, *aph(3)-IIIa*			D		190	ND

[a] Representative isolate from a hospital outbreak strain widespread in six Portuguese hospitals in different cities (1996–2008).
[b] ST132 is a single-locus variant of ST18.
[c] Representative strain of two isolates causing single urinary tract infections in unrelated patients in one Porto hospital during 2002; only one was characterized.
[d] Clinical strain causing single urinary tract infections and disseminated in two hospitals during 2001–2002.
[e] Representative isolates of a strain widespread in Europe since the mid-1990s (35).
[f] The Tn*1546* designation is based on PCR mapping as described previously (38, 54). Briefly, type A corresponds to the whole Tn*1546* backbone; type D contains alterations within *orf1* and a point mutation at position 8234 in *vanX*; PP-4 contains an IS*Ef1* insertion within the *vanX-vanY* region; PP-13 and PP-31 have an IS*1216* insertion within the *vanX-vanY* region (PP-31 differs in the region upstream of *vanR*). The number of isolates included in the Tn*1546* typing is indicated when appropriate.
[g] VAN, vancomycin; TEC, teicoplanin; AMP, ampicillin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; GEN, high-level resistance to gentamicin; KAN, high-level resistance to kanamycin; STR, high-level resistance to streptomycin; CHL, chloramphenicol; Q-D, quinupristin-dalfupristin. Antibiotic designations in parentheses represent variable resistance to the indicated antibiotic.
[h] , negative; , positive.
[i] ND, not done.
[j] UTI, urinary tract infection.



Country	Origin	Year	PFGE type	ST	CC
Denmark	Swine	2005	A1'''	ST5	CC5
Portugal	Hospitalized patient	2002	A3''''	ST5	CC5
Denmark	Healthy human	2005	A	ST6	CC5
Switzerland	Swine	1999	A1	ST6	CC5
Denmark	Swine	2002	A	ST6	CC5
Portugal	Swine	1997	A	ST6	CC5
Denmark	Swine	1995	A	ST6	CC5
Denmark	Swine	2006	A1'	ST6	CC5
USA	Swine	2008	A	ST6	CC5
Portugal	Swine	2007	A3'	ST185	CC5
USA	Swine	2008	A4	ST185	CC5
Denmark	Swine	2001	A3	ST185	CC5
Denmark	Swine	2003	A3''	ST5	CC5
Denmark	Swine	2004	A4	ST185	CC5
Denmark	Swine	2006	A3'''	ST5	CC5
USA	Swine	2008	A5	ST5	CC5
USA	Swine	2008	A1''	ST6	CC5
Denmark	Healthy human	2003	A5	ST147	CC5
USA	Swine	2008	A5	ST5	CC5
Denmark	Healthy human	2005	A6	ST493	CC5
Portugal	Hospitalized patient	2002	119	ST132	CC17
Portugal	Swine	2007	119.5	ST132	CC17
Portugal	Swine	2007	B5	ST6	CC2
Spain	Hospitalized patient	1999	B5	ST6	CC2
Portugal	Hospital outbreak	1996	B	ST6	CC2
Italy	Hospitalized patient	1993	B3'	ST6	CC2

FIG. 1. Computer analysis of SmaI-digested genomic DNAs of representative vancomycin-resistant *E. faecium* (A) and *E. faecalis* (B) isolates from humans and swine. PFGE subtypes were established by visual analysis following the criteria of Tenover et al. (48). Computer analysis was performed with the Fingerprinting II Informatix software package (Bio-Rad Laboratories, Hercules, CA). The acquisition of the image was performed by using a Gel Doc XR camera (Bio-Rad Laboratories Inc.). The image was normalized by using four reference lanes of the low-range PFGE marker (0.13 kb to 1,018.5 kb; New England BioLabs, La Jolla, CA) as an external DNA size marker. The phylogenetic tree was subsequently constructed by use of the Dice coefficient and UPGMA clustering (optimization, 0.5%; band tolerance, 1.5%; threshold cutoff value set at 82%) (36). Year, year of isolation.

DISCUSSION

The present study suggests interhost transmission of particular VRE strains belonging to predominant enterococcal clonal complexes associated with hospital-acquired human infections (*E. faecium* CC17 and *E. faecalis* CC2) or swine colonization (*E. faecium* CC5) in several European countries. The recovery from swine in Europe and the United States of an *E. faecium* CC5 clone with the ability to either colonize humans or cause human infections is of concern, and it might be added to the list of clonal lineages of Gram-positive organisms, such as methicillin-resistant *Staphylococcus aureus* ST398 or *Staphylococcus pseudintermedius* ST71, which are increasingly reported among animals and humans (46, 47). Although the origin and transmission routes of swine colonized by clonally related *E. faecium* CC5 isolates could not be established, inter- and intracountry food trade or dispersal of contaminated animals for food production cannot be ruled out. Contaminated imported chickens were suggested as the cause for clonal expansion of VREfs among poultry and pets in Japan and New Zealand during the last decade, although the isolates were not studied in detail at the genetic level (33, 40). Besides dispersal of animals in global markets, the selective pressure exerted by antibiotics (e.g., tetracyclines and β-lactams) or metals (e.g., copper) heavily used in veterinary medicine and husbandry may have contributed to the maintenance of VRE among swine farms and facilitated the horizontal transfer of conjugative plasmids to other enterococcal hosts (or other Gram-positive species serving as intermediates in the processes of horizontal gene transfer). Similarly to that described for *E. faecium* CC5, widespread clones of the predominant human enterococcal lineage *E. faecium* CC17 or *E. faecalis* CC2 have been recovered from companion and farm animals (4, 9, 13, 14).

Although some of the most common STs associated with *E. faecium* CC5 (ST5 and ST6), *E. faecium* CC17 (ST18), and *E. faecalis* CC2 (ST6) were detected, an unexpected diversity of STs and PFGE subtypes was observed within closely related *E. faecium* isolates belonging to CC5 and CC17. Examples of isolates showing the same or similar PFGE types but clustering in different STs have previously been described for both *E. faecalis* (13, 27) and *E. faecium* (7). It is known that large plasmids or integrative conjugative elements (ICEs), which are frequent in *E. faecium* (16, 21, 29, 55), can affect digested genomic DNA banding patterns (34, 49). In addition, a recent paper shows that mobilization of ICEs mediated by plasmids may also contribute to the diversity of housekeeping genes included in the MLST scheme (32). All these observations highlight not only the plasticity of enterococcal clones, which are able to evolve by diverse lateral transfer or mutational events, but also the potential difficulties in establishing epidemiological links among strains in some instances.

Confirming previous observations, specific Tn*1546* variants

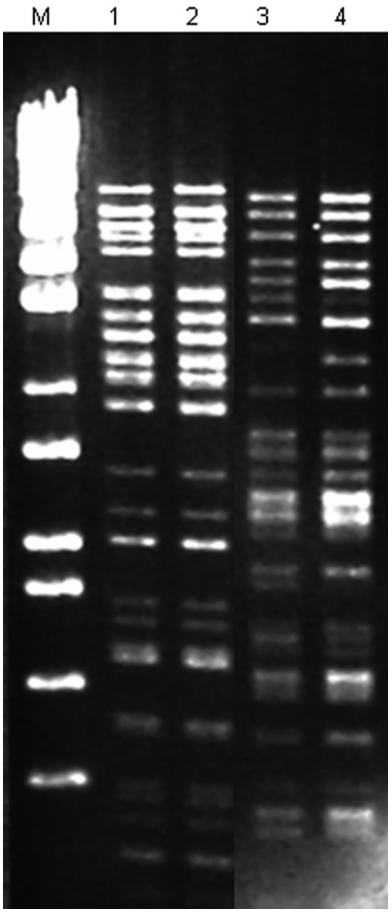


FIG. 2. ClaI-digested plasmid DNAs of VRE isolates recovered from hospitals and swine from different origins. Lane M, molecular marker ladder EcoT 14 I/BglII (Takara Bio Inc., Otsu, Japan); lane 1, *E. faecalis* ST6-CC2 (PFGE B5) from an outbreak strain recovered in Spain in 1999; lane 2, *E. faecalis* ST6-CC2 (PFGE B5) from a swine isolate recovered in Portugal in 2007; lane 3, *E. faecium* ST132-CC17 (PFGE 119.5) from a swine isolate recovered in Portugal in 2007; lane 4, *E. faecium* ST132-CC17 (PFGE 119) from a clinical isolate recovered in Portugal in 2002 that is representative of strains causing urinary tract infections in two unrelated patients during 2002.

were associated with VRE from humans or animals (10, 24, 38, 52, 54). The linking of such Tn*1546* variants with particular plasmid types from *E. faecium* (megaplasmids and mosaic plasmids containing replication proteins belonging to the RepA_N family, such as those of pLG1 or pRUM, and Inc18) or *E. faecalis* (pheromone-responsive plasmids) is also in agreement with some recent studies (12, 25, 29, 44, 56). Rosvoll et al. recently described the presence of *vanA*-Inc18 plasmids containing one or two rep (rep_1 and/or rep_2) among *E. faecium* strains from poultry and farmers in Norway and Italy (44), some of them closely related to the first *vanA*-Inc18 plasmid recovered in France in 1986 from a clinical isolate (45). Other studies have also described the presence of large plasmids carrying Tn*1546* among *E. faecium* isolates from hospitalized humans (2, 51) and swine (19). The recovery of resistant plasmids from clonally unrelated isolates from different sources over an extended period of time, such as that carried by the ST132 clone, is of concern, since it would reflect a wide spread and maintenance in both human and nonhuman hosts.

In summary, this study documents that enterococcal clones belonging to host-adapted clonal complexes of *E. faecium* (CC5 and CC17) and *E. faecalis* (CC2) are shared by swine and humans. The fact that these clones are able to colonize and cause human infections (and, in some instances, nosocomial outbreaks, such as *E. faecium* ST132 or *E. faecalis* ST6) confirms the relevance of reverse and alternative routes for dissemination of commensal and opportunistic bacteria. Multilayered molecular epidemiology studies will be required to understand the spread and evolution of clones and genetic elements encoding vancomycin resistance and overcoming the species barriers between humans and swine.

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3.2. Analysis of the *plasmidome* of enterococci from different environments with special emphasis on that encoding antibiotic resistance or adaptive traits. Characterization of *vanA*-Tn*1546* and *vanB*-Tn*1549*/Tn*5382* transposons responsible for the global spread of vancomycin-resistance among *Enterococcus* from humans and animals

3.2.1. Analysis of plasmids responsible for the virulence/epidemicity of specific clonal lineages highly adapted to the hospital environment

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Global spread of the *hyl*$_{Efm}$ colonization-virulence gene in megaplasmids of the *Enterococcus faecium* CC17 polyclonal subcluster

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Global Spread of the hyl_{Efm} Colonization-Virulence Gene in Megaplasmids of the *Enterococcus faecium* CC17 Polyclonal Subcluster[▽]

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Enterococcus faecium **has increasingly been reported as a nosocomial pathogen since the early 1990s, presumptively associated with the expansion of a human-associated *Enterococcus faecium* polyclonal subcluster known as clonal complex 17 (CC17) that has progressively acquired different antibiotic resistance (ampicillin and vancomycin) and virulence (*esp_{Efm}*, *hyl_{Efm}*, and *fms*) traits. We analyzed the presence and the location of a putative glycoside hydrolase *hyl_{Efm}* gene among *E. faecium* strains obtained from hospitalized patients (255 patients; outbreak, bacteremic, and/or disseminated isolates from 23 countries and five continents; 1986 to 2009) and from nonclinical origins (isolates obtained from healthy humans [25 isolates], poultry [30], swine [90], and the environment [55]; 1999 to 2007). Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Plasmid analysis included determination of content and size (S1-PFGE), transferability (filter mating), screening of Rep initiator proteins (PCR), and location of *vanA*, *vanB*, *ermB*, and *hyl_{Efm}* genes (S1/I-CeuI hybridization). Most *E. faecium* isolates contained large plasmids (>150 kb) and showed variable contents of *van*, *hyl_{Efm}*, or *esp_{Efm}*. The *hyl_{Efm}* gene was associated with megaplasmids (170 to 375 kb) of worldwide spread (ST16, ST17, and ST18) or locally predominant (ST192, ST203, ST280, and ST412) ampicillin-resistant CC17 clones collected in the five continents since the early 1990s. All but one *hyl_{Efm}*-positive isolate belonged to the CC17 polyclonal subcluster. The presence of *hyl_{Efm}* megaplasmids among CC17 from Europe, Australia, Asia, and Africa since at least the mid-1990s was documented. This study further demonstrates the pandemic expansion of particular CC17 clones before acquisition of vancomycin resistance and putative virulence traits and describes the presence of megaplasmids in most of the contemporary *E. faecium* isolates with different origins.**

The number of enterococcal nosocomial infections caused by *Enterococcus faecium* has increasingly been reported since the early 1990s worldwide (18, 47, 55). By using multilocus sequence typing (MLST) and the eBURST algorithm, the majority of contemporary isolates recovered from the hospital environment were initially grouped within a lineage designated clonal complex 17 (CC17), which comprised different sequence types (STs) apparently related and evolved from an ST17 founder (27, 55). However, recent studies using goeBURST and ClonalFrame algorithms indicate that *E. faecium* CC17 clinical isolates identified to date group in a subcluster composed of different clones that have evolved independently, although they still might be genetically linked based on previous studies (33, 48, 55). Interestingly, half of the STs within this CC17 polyclonal subpopulation have also been identified from samples obtained from healthy humans, swine, poultry, and pets (55).

Although the traits that might have played a role in the recent transition of *E. faecium* from commensal to nosocomial pathogen have not been identified (55), it seems that the clones able to infect or persistently colonize human hosts have progressively acquired genetic elements which confer selective advantages for different genogroups (18). They include traits coding for resistance to antibiotics (ampicillin and vancomycin) or enhancing colonization and/or virulence such as the *fms* genes (encoding microbial surface components recognizing adhesive matrix molecules [MSCRAMM]), *esp_{Efm}* genes (encoding a surface protein responsible for biofilm formation), or *hyl_{Efm}* genes (encoding a putative glycoside hydrolase which seems to facilitate intestinal colonization and peritoneal invasion) (2, 18, 39, 40, 55).

Hyl_{Efm} shows homology to proteins such as glucosaminidases or hyaluronidases, which are considered virulence factors in other Gram-positive human pathogens like *Streptococcus pyogenes*, *Listeria monocytogenes*, or *Clostridium perfringens* (7, 22, 39). Hyl_{Efm} was initially described as a hyaluronidase but recently was annotated as a putative glycoside hydrolase after *in silico* analysis comparing this gene to the *spy1600* gene from *Streptococcus pyogenes* (45). Analysis of complete ge-

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nomes has shown that glycosyl hydrolases facilitate intestinal colonization in many bacterial organisms (15).

The hyl_{Efm} gene has been detected in ampicillin-resistant vancomycin-susceptible *E. faecium* (AREF) CC17 recovered in the United States in 1986 and has been lately identified among AREF CC17 and vancomycin-resistant *E. faecium* (VREF) strains obtained from hospitals all over the world (10, 18, 39, 54, 55). Other authors have recently demonstrated the location of hyl_{Efm} on large plasmids that can also carry genes conferring resistance to glycopeptides in three strains from the United States and Colombia recovered in the mid-1990s and 2005, respectively, and showed that some of these plasmids enhanced gastrointestinal colonization and increased mortality in an experimental peritonitis mouse model (2, 40).

An increase in the number of *E. faecium* CC17 strains carrying hyl_{Efm} in hospitals in different countries has been documented during the last decade, although we lack information about the genetic background associated with this trait (2, 10, 39, 54, 55). The presence and the location of hyl_{Efm} among a comprehensive collection of well-characterized ampicillin-susceptible *E. faecium* (ASEF), AREF, and VREF strains obtained from different countries over the last 2 decades (1986 to 2009) are analyzed in this study.

MATERIALS AND METHODS

Bacterial epidemiological background. We studied 455 *E. faecium* isolates with clinical (n = 255) and nonclinical (n = 200) origins. They included 172 VREF (152 *vanA* and 20 *vanB*) strains causing documented hospital outbreaks (only one single isolate per outbreak and phenotype) or disseminated in more than one health care institution in 23 countries in Europe (Denmark, Finland, France, Germany, Greece, Hungary, Italy, Netherlands, Poland, Portugal, Serbia, Spain, and United Kingdom), North and South America (Argentina, Brazil, Canada, Chile, Paraguay, and United States), Asia (Saudi Arabia and Singapore), Africa (Tunisia), and Australia during a 24-year period (1986 to 2009). We also analyzed 52 AREF and 31 ASEF clonally unrelated isolates causing bacteremia in different patients (1995 to 2008) in a teaching hospital (1,200 beds; serving a population of 550,000) in Spain, a country where VREF rates are still under 3% (10; this study). The remaining 200 *E. faecium* isolates were recovered from farm animals (swine and poultry), healthy humans, and environmental sources (hospital wastewaters and rivers), and they were representative of strain collections obtained in Portugal from 1999 to 2007 (16, 34, 36; this study). Clonal relatedness among all strains containing hyl_{Efm} and almost all the isolates lacking hyl_{Efm} was established by pulsed-field gel electrophoresis (PFGE) and MLST (10, 21; http://efaecium.mlst.net).

Characterization and location of genes encoding putative virulence factors and antibiotic resistance. The presence of hyl_{Efm}, esp_{Efm}, *vanA*, *vanB*, and *ermB* was screened by different multiplex PCR schemes as described elsewhere (14, 29, 49). The chromosomal or plasmid location of these genes was assessed by hybridization of I-CeuI-digested genomic DNA with specific hyl_{Efm}, esp_{Efm}, *vanA*, *vanB*, *ermB*, and 23S rRNA probes for all strains included in the study (30). Genomic DNAs from *E. faecium* strain RC714 (*vanA* and *ermB*), *E. faecalis* strain V583 (*vanB*), and *E. faecium* strain C68 (hyl_{Efm} and esp_{Efm}) were used as positive controls and the template for generating specific probes by PCR (8, 12, 37).

Transferability of genes and plasmid characterization. Transferability of acquired antibiotic resistance genes frequently associated with CC17 isolates, vancomycin, and erythromycin was addressed by filter mating at a 1:1 donor/recipient ratio, using *E. faecium* strains GE-1 and BM4105RF as recipients and brain heart infusion (BHI) agar supplemented with 6 mg/liter of vancomycin and 20 mg/liter of erythromycin as selective media (20, 38). The sizes of plasmids containing hyl_{Efm} were determined by using a modified protocol of the technique described by Barton et al., followed by hybridization with specific probes in wild-type strains and transconjugants selected with antibiotics (4) (see below). In order to further characterize the enterococcal hyl_{Efm} plasmids, screening of replication initiator proteins corresponding to Gram-positive plasmids was assessed by a multiplex PCR method recently described by Jensen et al. (23).

Molecular techniques. Genomic DNA from isolates, wild types, and transconjugants was extracted using a QIAamp DNA minikit (Qiagen, Hilden, Germany). PCR assays were performed in volumes of 25 μl under the following conditions: 1.5 mM $MgCl_2$, 1× reaction buffer, 0.2 mM each of deoxynucleoside triphosphate, 1 μM each of primer, and 1.5 units of *Taq* DNA polymerase (GoTaq Flexi DNA polymerase; Promega, Madison, WI), with amplification programs of 10 min at 94°C, followed by 30 cycles of 30 s to 60 s at 94°C, 30 s to 60 s at 45 to 70°C, and 30 s to 2 min at 72°C, and a final extension step of 10 min at 72°C. PCR products were purified with the Wizard SV PCR purification kit (Promega, Madison, WI) and sequenced on an ABI Prism 377 automated sequencer (Applied Biosystems PE, Foster City, CA). DNA Southern transfer and hybridization were performed by standard procedures (43). Labeling and detection were carried out using the Gene Images Alkphos direct labeling system kit by following the manufacturer's instructions (Amersham GB/GE Healthcare Life Sciences UK Limited). A modification of the Barton et al. method was used (4). Briefly, genomic DNA was digested with 14 U of S1 nuclease (Takara Bio Inc., Shiga, Japan) for 15 min at 37°C, and migration of DNA fragments was carried out in a Chef-DR III device (Bio-Rad, La Jolla, CA) using the following settings: switch time of 5 to 25 s for 6 h, followed by 30 to 45 s for 18 h. PFGE was performed as described previously (24) using the following electrophoresis conditions: 5 to 30 s for 22 h, 14°C, and 6 V/cm^2 (I-CeuI) and 1 to 20 s for 26 h, 14°C, and 6 V/cm^2 (SmaI).

Nucleotide sequence accession numbers. The sequences corresponding to those for hyl_{Efm} from representative isolates were assigned the following NCBI accession numbers: HM043740, HM043741, HM043742, HM043743, and HM043744.

RESULTS

***hyl_{Efm}* is globally distributed among genotypically diverse *E. faecium* isolates obtained from humans.** The hyl_{Efm} gene was detected in 51 isolates (11.2%) which were frequently resistant to vancomycin (n = 34/51; 67%) and contained esp_{Efm} (n = 30/51; 59%). These hyl_{Efm}-positive strains were collected from clinical samples obtained from hospitalized patients (n = 45) and hospital sewage (n = 6).

The hyl_{Efm} gene was detected among clinical VREF strains (n = 28/172, 16%) from 13 countries in Europe (Germany, Greece, Hungary, Netherlands, Poland, Portugal, Serbia, and Spain), North America (Canada and United States), Asia (Saudi Arabia), Africa (Tunisia), and Australia from 1992 to 2009 (Table 1). They belonged mostly to different STs considered within the CC17 population (n = 27/28). The STs identified comprise predominantly ST16, ST17, and ST18 (n = 19), followed by ST80, ST125, ST192, and ST412 (n = 1 each) and ST173 and ST280 (n = 2 each). It is of interest to note that the *vanA* VREF ST280 isolate recovered in Portugal in 2003 showed an identical PFGE type to that of another *vanB* VREF clinical isolate collected in Singapore in 2004. They differed in the presence of hyl_{Efm} and the sizes of their plasmids.

The hyl_{Efm} gene was also detected in 33% of AREF CC17 isolates causing nosocomial bacteremia (n = 17/52) from 1997 to 2007 (4 ST16 isolates, 9 ST18 isolates, and 1 isolate each from ST17, ST103, ST203, and ST419) and among 6 VREF strains collected from sewage downstream of health care institutions (4 ST18 and 2 nonanalyzed isolates). As with the ST280 isolates, we also identified AREF isolates showing the same PFGE types but with variable content in hyl_{Efm}, which corresponded to ST16. The only non-CC17 isolate (ST65) carrying hyl_{Efm} caused a hospital outbreak in Greece in 1999 (42). Although in this study all human isolates containing hyl_{Efm} caused infections, some of the outbreak clones were also recovered from colonized patients during extended periods of time (data not shown). The remaining 31 clinical ASEF (3

TABLE 1. Characteristics of *Enterococcus faecium* strains containing hyl_{Efm} megaplasmids isolated from different continents from 1992 to 2009[g]

ST	*E. faecium* group	No. of isolates	Approx size (kb) of hyl_{Efm} plasmid (no. of isolates)	No. of isolates containing esp_{Efm}	Epidemiology (no. of isolates)		Country (yr) of isolation	Reference(s)
					Sample source(s)	Hospital outbreak		
16	VREF	5	170 (1), 220 (4)	5	Urine (1), wound (1), and feces (3)	Yes	NLD (2002), USA (1996–2001), AUS (1996–1999)	3, 8
	AREF	4	275 (1), 300 (2), 340 (1)	4	Blood (4)	Yes	ESP (2001–2004)	10; this study
17	VREF	5	220 (2), 240 (1), 260 (1), 300 (1)	3	Blood (1), urine (1), and unknown (3)	Yes	ESP (2009), USA (1992), BRA (1996), TUN (2003), SAR (2000–2003)	1, 26, 32
	AREF	1	275	1	Blood	No	ESP (2008)	This study
18	VREF	9	200 (2), 220 (4), 240 (1) 260 (1), 280 (1)	4	Feces (2), blood (1), and fluid drainage (1), Catheter (1) and unknown (4)	Yes (5)	PRT (2001–2008), POL (2005), SER (2005), SAR (1999–2003)	17, 25, 26, 28, 35
		4	200 (1), 260 (1), 300 (2)	0	Hospital sewage	No	PRT (2001–2002)	36
	AREF	9	270 (1), 280 (4), 300 (4)	5	Blood	No[f]	ESP (1997–2007)	10; this study
65[a]	VREF	1	230	1	Blood	Yes	GRC (1999)	42
80	VREF	1	230	1	Blood	Yes	HUN (2004)	6
103	AREF	1	280	1	Blood	No[f]	ESP (2001)	10
125	VREF	1	220	1	Urine	No	PRT (2007)	17
173	VREF	2	280 (2)	0	Blood (1) and unknown (1)	Yes	AUS (2000–2001)	9
192	VREF[b]	1	200	1	Blood	Yes	DEU (2004)	53
203	AREF[c]	1	375	1	Blood	Yes	ESP (2006)	This study
280	VREF[d]	2	200 (1), 300 (1)	0	Blood (1) and urine (1)	Yes	PRT (2002–2003)	17, 35
412	VREF[e]	1	200	1	Unknown	Yes	CAN (2005)	13
419	AREF	1	300	1	Blood	Yes	ESP (2005)	This study

[a] Non-CC17 clone.
[b] CC78 ST disseminated in hospitals in Germany and Korea.
[c] ST widely disseminated in Germany and Spain presently.
[d] ST comprising widely disseminated VREF and AREF strains from Portugal (2001 to 2003), Singapore (2004), and early American nosocomial isolates (18).
[e] This clone is widely disseminated in Greece (11).
[f] Some isolates were persistently recovered in the hospital during these years, although a clinical outbreak could not be documented.
[g] All VREF strains were resistant to ampicillin. AUS, Australia; CAN, Canada; DEU, Germany; ESP, Spain; GRC, Greece; HUN, Hungary; NLD, Netherlands; POL, Poland; PRT, Portugal; SAR, Saudi Arabia; SER, Serbia; TUN, Tunisia; USA, United States.

CC17 and 28 non-CC17) and 194 VREF, AREF, and ASEF (mostly non-CC17) isolates obtained from healthy human and animal samples did not contain hyl_{Efm}.

Hyl_{Efm} is a member of family 84 of the glycoside hydrolases, which is conserved among enterococci (http://www.uniprot.org/uniprot/Q8GMZ8). The sequenced hyl_{Efm} genes displayed ≥99% identity with those from *E. faecium* genomes (GenBank accession numbers AF544400, ZP_00604207, ZP_05663180, ZP_05671579, and EFF30024). Analysis of translated Hyl_{Efm} amino acid sequences showed homology to those of proteins previously described as hyaluronidases which have more recently been classified as members of family 84 of the glycoside hydrolases with β-*N*-acetylglucosaminidase activity, since it is not clear whether this family does genuinely process hyaluronic acid (45; http://www.cazy.org/fam/GH84.html; http://www.uniprot.org/uniprot/Q8GMZ8; J. A. Laverde-Gomez and G. Werner, unpublished results). Family 84 of the glycoside hydrolases comprises a diverse group of enzymes found in higher eukaryotes and pathogenic or symbiotic bacteria (http://www.cazy.org/Glycoside-Hydrolases.html).

hyl_{Efm} is consistently located on *E. faecium* megaplasmids. All CC17 isolates studied contained a variable number of plasmids of different sizes, but megaplasmids ranging from 150 to 450 kb were identified in most of them. The hyl_{Efm} gene was always located on plasmids ranging from 170 kb to 375 kb (Fig. 1) which did not contain replication proteins of the known Gram-positive plasmids described among *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Lactococcus*, or *Bacillus* spp. to date (23). However, other modules from widely spread enterococcal plasmids such as Inc18 or pAD1 (50) were identified in some megaplasmids from AREF and VREF isolates (A. R. Freitas, A. P. Tedim, and T. M. Coque, unpublished data). Under antibiotic selection with vancomycin, hyl_{Efm} from four out of 34 VREF isolates producing Hyl_{Efm} was cotransferred with genetic elements encoding resistance to glycopeptides (3 *vanA* plasmids of ca. 50 to 60 kb and 1 *vanB* Tn*5382*-like chromosomal element). The *vanB* isolate corresponded to the *E. faecium* strain C68, which was one of the first hyl_{Efm}-positive *E. faecium* isolates identified (39). The *van* and hyl_{Efm} genes were located on different plasmids in all isolates, while the *ermB*

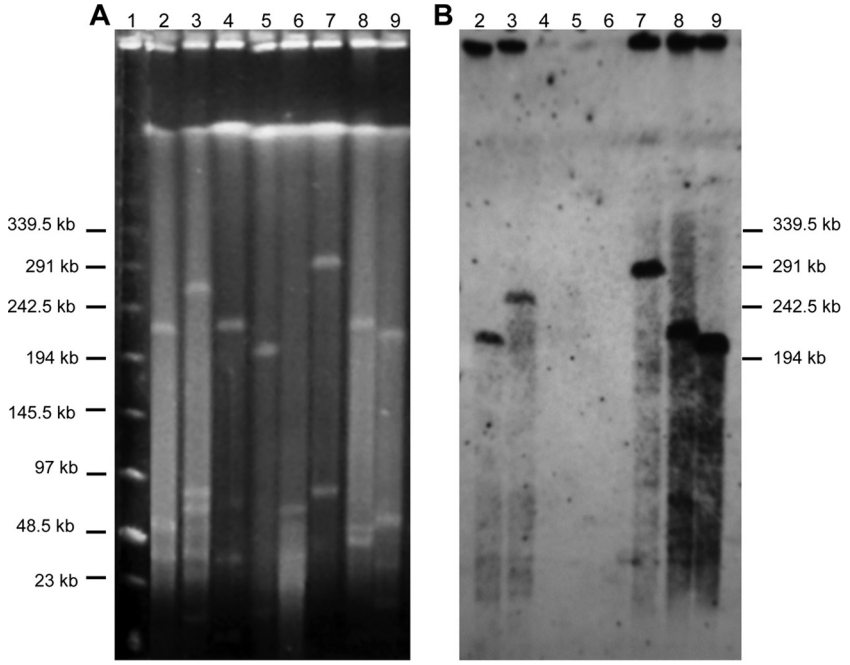


FIG. 1. S1-PFGE of AREF and VREF strains (A) and hybridization of S1 genomic digested *E. faecium* DNA with a hyl_{Efm} probe (B). Lane 1, low-range PFGE marker (0.13 kb to 1,018.5 kb; New England Biolabs, La Jolla, CA); lane 2, VREF ST16 (United States, 2001); lane 3, VREF ST18 (Serbia, 2005); lane 4, VREF ST17 (Brazil, 1996); lane 5, VREF ST64 (Chile, 2004); lane 6, vancomycin-resistant *E. faecalis* (negative control for hyl_{Efm}); lane 7, VREF ST280 (Portugal, 2003); lane 8, VREF ST80 (Hungary, 2004); lane 9, VREF ST17 (United States, 1992).

gene was often detected on hyl_{Efm} plasmids which were often transferable (data not shown). The esp_{Efm} gene was always chromosomally located.

DISCUSSION

The global spread of hyl_{Efm} associated mainly with megaplasmids of *E. faecium* CC17 isolates recovered in the last 2 decades is reported. Galloway-Peña et al. have recently suggested that the acquisition of ampicillin resistance, hyl_{Efm}, and esp_{Efm} by ancestral American human *E. faecium* isolates carrying different MSCRAMM genes might have contributed to the selection and expansion of the CC17 population in the United States since the early 1980s (18). We describe the variable content in genetic elements containing the *van*, hyl_{Efm}, or esp_{Efm} genes for the worldwide spreading STs, such as ST16, ST17, ST18, and also locally predominant ST192 (Germany and Korea), ST203 (Germany and Spain), ST280 (Portugal and also identified in early CC17 from the United States), or ST412 (Greece) AREF clones (this study and references herein; data obtained from the MLST website [http://efaecium.mlst.net]). Since the above-mentioned STs seem to represent different subgroup founders within CC17 (55), our results indicate the occurrence of a global spread of the CC17 polyclonal population before the acquisition of different traits associated with antibiotic resistance or pathogenicity.

The hyl_{Efm} gene in three *E. faecium* strains obtained from the United States and Colombia has recently been identified in large conjugative plasmids (>145 kb) containing antibiotic resistance genes and expressing increased virulence in a mouse peritonitis model (2, 40). In the present study, we document for the first time that the hyl_{Efm} gene from clonally unrelated *E. faecium* strains recovered from hospitals in Europe, Australia, Asia, and Africa has been consistently located on *E. faecium* plasmids of variable size (175 kb to 375 kb) since at least the mid-1990s, which is almost a decade after the first hyl_{Efm}-positive isolates were recovered in the United States (18). Moreover, plasmids larger than 150 kb were present in all *E. faecium* CC17 and non-CC17 strains with human and animal origins that we have analyzed, with the exception of the first VREF non-CC17 and VREF CC17 strains described in France in 1988 and in the United Kingdom in 1992, respectively.

The existence of large plasmids (>150 kb) among vancomycin-resistant *E. faecium* strains with human and animal origins recovered from the mid- to late 1990s may be inferred from different recent studies (5, 16, 19, 41, 52). Their presence in most isolates analyzed in this work suggests that they encode traits essential for the adaptation of *E. faecium* to particular hosts, and thus, they might be defined as megaplasmids in the classical way (31). We should mention that large plasmids had not been recognized among enterococci before the 1990s (51), although the lack of studies analyzing these genetic elements by suitable methods precludes conclusions about their recent acquisition. However, it is of interest to highlight that Teixeira et al. in 1995 documented a great variability in the phenotypic expression of sugar metabolism (mannitol, glycerol, sorbitol, and raffinose) among VREF and VSEF strains from collections of the Center for Diseases Control and Prevention in the United States, although the association of these traits with the plasmid content of the isolates was not analyzed (46). Megaplasmids encoding α-galactosidases isolated from *E. faecium* clones of different origins have recently been communi-

cated (56); however, the locations of both a α-galactosidase gene(s) and hyl_{Efm} on the same plasmid was not addressed. Megaplasmids are common among *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* and confer on these microorganisms the ability to utilize complex carbohydrates from vegetables in the diet, host-derived glycoproteins and glycoconjugates which could enhance their adaptation and persistence in the colon (44).

The origin of these megaplasmids remains undetermined since the plasmid classification based on Rep proteins was designed upon the sequences of small- or medium-size plasmids from known families of Gram-positive bacteria (23). However, their remarkable variation in the size and content of the genes encoding resistance to gentamicin, erythromycin, or vancomycin or modules originated from different plasmids which are widespread among enterococci or streptococci, suggesting that the hyl_{Efm} megaplasmids might result from the cumulative effect of lateral transfer events (2; this study). Although we did not identify Tn*1546* and hyl_{Efm} genes within the same plasmid in this study, the hyl_{Efm} megaplasmids often cotransfer with that encoding vancomycin resistance, which is of concern as it could further facilitate the success of the *E. faecium* CC17 subcluster in hospitals.

In summary, we describe the global distribution of megaplasmids among CC17 and non-CC17 isolates which may contain a glycoside hydrolase, Hyl_{Efm}. The encoding hyl_{Efm} gene seems to have spread in Europe, Africa, Asia, and Australia isolates by at least the mid-1990s. The common presence of large plasmids in human *E. faecium* CC17 that are able to collect and convey different traits, enhancing antibiotic resistance and/or virulence, might have contributed to the recent emergence and ramping success of *E. faecium* as a human-adapted, leading nosocomial pathogen. Further characterization of *E. faecium* large plasmids will contribute to the identification of traits responsible for the epidemicity of CC17 populations and toward a further understanding of the recent changes in the ecology and evolution of this species.

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A multiresistance megaplasmid pLG1 bearing a *hyl*$_{Efm}$ genomic island in hospital *Enterococcus faecium* isolates

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a b s t r a c t

Enterococcus faecium is considered to be a nosocomial pathogen with increasing medical importance. The putative virulence factor, hyl$_{Efm}$, encoding a putative hyaluronidase, is enriched among the hospital-associated polyclonal subpopulation of E. faecium.. The hyl$_{Efm}$ gene is described to be part of a genomic island and was recently identified to be plasmid-located. Here, we present a description of the structure, localization, and distribution of the putative pathogenicity factor hyl$_{Efm}$ and its putative island among 39 clinical isolates and elucidate the composition and host range of pLG1, a hyl$_{Efm}$ multiresistance plasmid of approximately 281.02 kb.

The hyl$_{Efm}$ gene was located within a 17,824-bp element highly similar to the putative genomic island (GI) structure that had been previously described. This genomic region was conserved among 39 hyl$_{Efm}$-positive strains with variation in a specific region downstream of hyl$_{Efm}$ in 18 strains. The putative hyl$_{Efm}$ was located on large plasmids (150–350 kb) in 37 strains. pLG1 could be horizontally transferred into four different E. faecium recipient strains (n = 4) but not into E. faecalis (n = 3). Sequencing of pLG1 resolved putative plasmid replication, conjugation, and maintenance determinants as well as a pilin gene cluster, carbon uptake and utilization genes, heavy metal and antibiotic resistance clusters.

The hyl$_{Efm}$ transferable plasmid pLG1 bears additional putative pathogenicity factors and antibiotic resistance genes. These findings suggest horizontal gene transfer of virulence factors and antibiotic resistance gene clusters by a single genetic event (conjugative transfer) which might be triggered by heavy antibiotic use common in health care units where E. faecium is increasingly prevalent.

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Introduction

Enterococcus faecium is a commensal of the animal and human intestines but may serve as a facultative pathogen under specific conditions and in patients with severe underlying diseases. In addition, results from recent molecular typing and characterization experiments revealed the existence of a specific polyclonal hospital-associated population of E. faecium strains which differ from commensal human and animal isolates, (Willems and van Schaik, 2009). The numbers of invasive E. faecium infections are growing in many countries owing to the increasing prevalence of these hospital-associated strains (Top et al., 2008; Tenover, 2001). These hospital-associated E. faecium clones are usually resistant to ampicillin and ciprofloxacin (Lester et al., 2009; Leavis et al., 2006; Werner et al., 2010). Their accessory genome also appear to be enriched in genetic determinants that enhance colonization or virulence such as the collagen–adhesin genes acm and scm (Nallapareddy et al., 2007; Sillanpaa et al., 2008), genes encoding other matrix-binding or pili-associated proteins (Sillanpaa et al., 2009; Hendrickx et al., 2009a), the esp gene encoding an enterococcal surface protein (Heikens et al., 2007), a putative hyaluronidase, hyl$_{Efm}$ (Rice et al., 2003), and a genomic island (GI) of unknown function (Lester et al., 2009; Heikens et al., 2008). The esp and hyl$_{Efm}$ genes are part of GIs, most probably acquired by horizontal spread. The recently described gene hyl$_{Efm}$ is supposed to encode a putative hyaluronidase (Rice et al., 2003). Hyaluronidases are puta-

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Table 1
Investigated strains. Approximate hyl_{Efm} and vanA plasmid (pl) sizes were calculated by S1-nuclease PFGE analysis.

Strain	Year	MSLT	MLVA	hyl_{Efm}-pl. (kb)	van	vanA-pl. (kb)	hyl_{Efm} genomic island[a]					
							1	2	3			
									a	b	c	d
DO	1991	18	–	C			+	+	+	+	+	+
UW1952	1998	18	1	252	vanA	59	+	+	+	+	–	+
UW2774	1999	18	281	281	vanA	281	+	+	+	+	–	+
UW2460	1999	17	1	214	vanA	28	+	+	+	+	+	+
UW2457	1999	80	1	205	vanA	205	+	+	+	+	+	+
UW3056	2000	65	5	228	0		+[b]	+	+	+	+	+
UW3488	2001	18	1	205	vanA	205/50	+	+	+	+	+	+
UW3183	2001	18	1	214	vanA	104	+[b]	+	+	+	+	+
UW3308	2001	65	–	206	vanA	44	+[b]	+	+	+	+	+
UW5905	2004	192	159	188	vanA	68	+	+	+	+	–	+
UW5352	2004	17	7	296	vanB		+	+	+	+	+	+
UW5275	2004	376	1	180	vanB		+	+	+	+	+	+
U0317	2005	78	–	195			+	+	+	+	+	+
UW6379	2005	18	1	C	vanA	101	+	+	+	+	–	–
UW6352	2005	18	1	316	vanA	316	+	+	+	+	+	+
UW6151	2005	17	1	263	vanA	98	+	+	+	+	+	+
UW6112	2005	378	5	216	0		+	+	+	+	+	+
UW6993	2006	192	159	188	0		+	+	+	+	–	+
UW6990	2006	18	7	216	0		+	+	+	+	+	+
UW6985	2006	81	7	223	0		+	+	+	+	–	+
UW6982	2006	192	159	185	0		+	+	+	+	–	+
UW6951	2006	192	159	190	0		+	+	+	+	–	+
UW6947	2006	17	7	187	0		+	+	+	+	–	+
UW6943	2006	192	159	189	0		+	+	+	+	–	+
UW6941	2006	192	12	164	0		+	+	+	+	–	+
UW6929	2006	192	159	266	0		+	+	+	+	–	+
UW6893	2006	192	159	169	0		+	+	+	+	–	+
UW6883	2006	378	new	217	0		+	+	+	+	–	+
UW6882	2006	117	282	255	0		+	+	+	+	+	+
UW6717	2006	317	–	187	0		+	+	+	+	–	+
UW6715	2006	192	159	166	0		+	+	+	+	–	+[b]
UW6711	2006	192	159	190	0		+	+	+	+	–	+
UW6498	2006	18	7	223	vanA	101	+	+	+	+	+	+
UW6923	2007	202	1	168	0		+	+	+	+	–	+
UW6920	2007	117	282	250	0		+	+	+	+	+	+
UW6919	2007	192	159	168	0		+	+	+	+	–	+
UW6918	2007	17	7	187	vanB		+	+	+	+	–	+
UW6917	2007	17	1	168	0		+	+	+	+	–	+
UW6916	2007	378	new	217	0		+	+	+	+	+	+

[a] hyl_{Efm} GI (hylGI) presence corresponds to PCR amplification of overlapping regions as shown in Fig. 1.
[b] Product larger than expected. C: Our results indicate chromosomal localization.

tive virulence factors that play a role in adhesion or colonization (Hynes et al., 2000), tissue degradation, and spread (Pecharki et al., 2008). Recent studies indicate that acquisition of hyl_{Efm}-containing plasmids by E. faecium is associated with increased colonization and enhanced virulence in a mouse peritonitis model (Arias et al., 2009; Rice et al., 2009). An increase in the numbers of CC17-E. faecium-carrying hyl_{Efm} in hospitals in different countries has been documented during the last decade (Freitas et al., 2010). In the present study, we specified the structure of the putative GI harbouring the hyl_{Efm} gene and investigated its prevalence among different E. faecium strains recovered from hospitalized patients in Germany during the last decade. We also present the sequence analysis of a multiresistance megaplasmid, pLG1, bearing the hyl_{Efm} genomic region.

Materials and methods

Bacterial strains

Our unit serves as a focal laboratory for enterococci in Germany. In general, enterococcal isolates sent to us are preselected for being vancomycin- and multiresistant or with the suspicion of clonal spread (outbreak). For the investigation of the hyl_{Efm} GI and its environment, a subset of 39 hyl_{Efm}-positive E. faecium clinical isolates (15 VRE and 24 VSE) was selected originating from 11 federal states (1998–2007) and from different clinical laboratories or university medical centres, with different sequence types (MLST) and MLVA types to exclude copy isolates (listed in Table 1). Multilocus sequence typing (MLST) and multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) were done as described elsewhere (see Werner et al., 2010; Klare et al., 2005). hyl_{Efm} GI structure as well as the plasmid content of all strains and localization of hyl_{Efm} and vanA markers were evaluated as described below.

PFGE and Southern hybridization

Preparation of samples for subsequent macrorestriction was done as previously described (Werner et al., 2003). PFGE and analysis of the resulting band patterns were done as described elsewhere (Werner et al., 2010). A CHEF III apparatus (BIO-RAD, Munich, Germany) was used. The sizes of the resolved macrorestriction fragments were predicted according to an external size standard (SmaI-digested Staphylococcus aureus NCTC8325) and calculated using software BioNumerics v. 5.1 (Applied Maths, Sint-Martens-Latem, Belgium) (Murchan et al., 2003). Southern hybridization was done using DIG-probe synthesis and CDP-Star detection substrate following the manufacturer's recommendations (Roche Biochem-

Table 2
Primers used in this study for gene (hyl_{Efm}, vanA, vanB) or hyl_{Efm} GI region PCR determination.

Primer	Sequence	Reference
hyl1	GAGTAGAGGAATATCTTAGC	Rice et al. (2003)
hyl2	AGGCTCCAATTCTGT	Rice et al. (2003)
hylGI1-F	CATTTAGAAGGCGAGATGTCTTCAGATAA	This study
hylGI1-R	TCTCAATGATCGAGGTTTCTTCAATGTAT	This study
hylGI2-F	TGCCTTCCACGTTTTTTAGCTCAAGGAGGAGAG	This study
hylGI2-R	GGCCGTTGATAGACAGAAGCCATTTCCTGC	This study
hylGI3a-F	ACAGTTAGAAGAAGTCTGGAAACCGGTAA	This study
hylGI3a-R	GGCCGTTGATAGACAGAAGCCATTTCCTGC	This study
hylGI3b-F	CAATTACCGTTCTTTTGGACTG	This study
hylGI3b-F	ATCGTTTCAACAGAGCTTGC	This study
hylGI3c-F	ACGGTTGCTAATACTCTTCCAC	This study
hylGI3c-R	ATTGGAGATGCTGGATACGG	This study
hylGI3d-F	TGTGCGACTTCATTTACGATACGGAC	This study
hylGI3d-R	AAGGTCAAGTACCTAGTGATGGACATGAA	This study
vanA1	TCTGCAATAGAGATAGCCGC	Klare et al. (2005)
vanA2	GGAGTAGCTATCCCAGCATT	Klare et al. (2005)
vanB1	CATCGCCGTCCCCGAATTTCAAA	Klare et al. (2005)
vanB2	GATGCGGAAGATACCGTGGCT	Klare et al. (2005)

icals, Mannheim, Germany). hyl_{Efm} probe was generated using primers hyl1 and hyl2 (Table 2). For vanA-positive strains (n = 12), also vanA localization was analysed. vanA probe was generated using primers vanA1 and vanA2 (Table 2). Other probes used in Southern hybridization are listed in Supplementary Table 1.

I-CeuI macrorestriction

Chromosomal location of hyl_{Efm} was evaluated by Southern hybridization of I-CeuI macrorestriction analysis as previously described (Novais et al., 2004).

S1-nuclease macrorestriction

Plasmid content of the strains and plasmid localization of hyl_{Efm} was evaluated by S1 nuclease macrorestriction (and later Southern hybridization) analysis (Barton et al., 1995; Freitas et al., 2009). Briefly, genomic DNA was digested with 14 U of S1 nuclease (Takara, Bio Inc., Shiga, Japan) for 15 min at 37 C. DNA was separated in PFGE using ramped pulse-field times as follows: 5.0–35.0 s for 22 h at 14 C. This method is based in the specific digestion of single-stranded DNA or single-stranded regions of double-stranded DNA allowing the linearisation of plasmids and resolution in PFGE as detectable bands on a faint genomic background (Barton et al., 1995). A band running at an apparent high molecular size contains undigested chromosomal and plasmid DNA.

hyl_{Efm} genomic environment

Detection of hyl_{Efm} and hyl_{Efm} GI

Presence of the hyl_{Efm} gene was determined by PCR using the primers hyl1 and hyl2 as previously described (Rice et al., 2003) using Illustra PuRe-Taq Ready-To-Go PCR Beads (GE Healthcare Europe GmbH) following the recommendations of the manufacturer. The presence of resistance genes vanA and vanB was tested in a multiplex PCR (Klare et al., 2005). The presence of the whole putative hyl_{Efm} PAI was tested by amplification of overlapping regions (hylGI1, hylGI2, and hylGI3) of approximately 5.5 kb using an Expand long template PCR (Roche Biochemicals, Mannheim, Germany) under the conditions recommended by the manufacturer. Region hylGI3 was analysed by amplification of smaller regions (hylGI3a, hylGI3b, hylGI3c, hylGI3d) for further detailed analysis. Specificity of the PCR products was confirmed by HindIII digestion following the recommendations of the manufacturer (New England Biolabs, Ipswich USA). hyl_{Efm} plasmid acquisition was analysed in the transconjugants by SmaI, and S1 nuclease macrorestriction and Southern hybridization as described above. All the primers used are listed in Table 2.

Sequencing of PCR products

Sequencing reactions were performed according to the manufacturer's recommendations for cycle sequencing of PCR products (Applied Biosystems Germany, Darmstadt, Germany). Sequence files were read, evaluated, aligned and compared to the reference set of alleles using software LaserGene 8.0 from DNA-STAR (Madison, WI, USA).

Plasmid DNA isolation

Plasmids were isolated using the previously described method by Woodford et al. (1993) with the following modifications: 50 g/ml RNAase were added to the first lysis step; extraction with phenol–chloroform was done three times following a final extraction with chloroform; final DNA precipitation with 100% ethanol was done overnight at –20 C following centrifugation for 10 min at 15,000 rpm. DNA was washed again using 70% ethanol and spun for additional 10 min before continuing with drying and the resuspension of the samples in double-distilled water. The isolated plasmid samples were loaded on 0.8% (w/v) agarose gel electrophoresis for visual inspection. Subsequently, DNA concentration was determined using Quant-iT® PicoGreen® dsDNA Quantitation Reagent following the instructions of the manufacturer Invitrogen® (Molecular Probes Inc., Paisley, UK).

Plasmid sequencing

Isolated plasmid DNA (approximately 5 g) from strain 64/3xUW2774 was subjected to 454 sequencing at GATC Biotech (Konstanz, Germany). This strain acquired the hyl_{Efm}-vanA ermB megaplasmid denominated pLG1 by horizontal transfer on filter mating experiments. pLG1 was selected for sequencing since it is the only plasmid present in this strain, it is transferable, and it bears the antibiotic resistance marker vanA. The plasmid sequencing at GATC Biotech was done according to standard procedures for sequencing on the Genome Sequencing (GS) FLX system (Roche). For preparation of the single-read library, the double-stranded DNA (dsDNA) was column-purified and sheared on a Covaris ATA to fragments in a size of approximately 700 bp. The A and B adapters for sequencing of the Roche technology were ligated to the ends of the sheared DNA fragments. The samples were run on a 2% agarose gel, and the band in a size range of 700–900 bp was excised and column-purified. After concentration measurement, the resulting library was immobilized onto DNA capture beads and the amplicon beads obtained were amplified through emPCR according to the manufacturer's recommendations. Following amplification, the emulsion was chemically broken and the beads carrying the amplified DNA library were recovered and washed by filtration. The sample was sequenced on a GS FLX Pico-Titer plate device. The preassembled contigs were analysed using DNA-Star® software SeqMan Engine and DS Gene software packages. Coding sequence (CDS) prediction and annotation were done with Kodon® (Applied Maths, Texas, USA) and with the help of BlastX and BlastN [http://blast.ncbi.nlm.nih.gov/Blast.cgi (Zhang et al., 2000)]. Southern hybridization allowed confirmation of plasmid localization of contigs that contained chromosomal-like sequences. The primers used for probe synthesis are listed in Supplementary Table 1. Gap closure was done when possible by PCR, long PCR, primer walking, and cycle sequencing. The primer pairs used for gap closure are listed in Supplementary Table 2.

Filter mating assays

Filter mating experiments that allow tight cell-to-cell contact were done as described elsewhere (Werner et al., 2003) to investi-

Table 3
Strains used for filter mating experiments.

Strain	Species	MLST	Resistance	hyl_{Efm} present	Size of plasmids present (kb)[a]
• Recipients					
64/3	Efm	ST21	FUS/RAM	No	None
BM4105RF	Efm	ST172	FUS/RAM	No	None
64SS	Efm	ST21	STR/SPCM	No	None
GE-1	Efm	ST515	FUS RAM	No	None
OG1RF	Efs	ST1	FUS/RAM	No	None
JH2-2	Efs	ST8	FUS/RAM	No	None
UV202	Efs	ST8	FUS/RAM	No	None
• Donors					
UW2774	Efm	ST18	VAN/ERY/GEN/PEN	Yes	**281; 75**
• Transconjugants					
64/3xUW2774[b]	Efm	ST18	VAN/ERY/FUS/RAM	Yes	**281**

ST, sequence type; ST-n, St new; Efm, E. faecium; Efs, E. faecalis; FUS, fusidic acid; RAM, rifampicin; VAN, vancomycin; ERY, erythromycin; GEN, gentamycin (high level); AMP, ampicillin; STR, streptomycin; SPCM, spectinomycin. Only antibiotic resistances relevant for this study are presented.

[a] Approximate plasmid size resolved by S1-nuclease PFGE analysis.

[b] Strain used for plasmid sequencing, also used as donor in filter mating experiments. Plasmid sizes in bold type correspond to hyl_{Efm} plasmids.

gate the transfer of hyl_{Efm} plasmids while selecting for vancomycin resistance transfer. For the first conjugation, three E. faecium and three E. faecalis strains were used as recipients and one E. faecium strain performed as donor that carried the vanA and hyl_{Efm} genes on the same plasmid. A second conjugation was done using the transconjugant strain 64/3xUW2774 as donor and strain 64SS as recipient. All strains are listed in Table 3. The mating pairs used are presented in Table 4. Transconjugants that had acquired vancomycin resistance were selected on BHI agar (Difco Labs., Detroit, USA) containing the selective antibiotic for the donor (vancomycin, 5 mg/l) and the selective antibiotics for the corresponding recipient. Presence of the hyl_{Efm} gene, tested by PCR, was indicative of hyl_{Efm} horizontal acquisition. Transconjugants were tested for clonality by antibiotic resistance pattern determination and by SmaI macrorestriction. Horizontally transferred hyl_{Efm} was analysed by SmaI, I-CeuI, and S1 nuclease macrorestriction and Southern hybridization.

Hyaluronidase activity

Two different tests for evaluation of hyaluronidase activity were used. The first method uses a strain of Streptococcus equi which produces an external capsule made mainly of hyaluronic acid and does not produce hyaluronidase. Test strains were inoculated in crossed streaks with the S. equi strain and were incubated for 48 h at 37 C and 40 C. Degradation of the mucous capsule produced by S. equi was observed in the region where a hyaluronidase-positive strain had grown (van der Linden and Nicklas, personal communication). The second is a plate method using brain heart infusion agar (BHI), BSA (1% w/v), and hyaluronic acid sodium salt from S. equi (0.04% w/v) (Sigma–Aldrich, Saint Louis, USA), revealing clear zones after hyaluronic acid degradation (Smith and Willett, 1968; Farrell et al., 1995). For the establishment of the tests, S. aureus strains NCTC8325 and NCTC8325-1 were used as positive controls, and E. faecium strain 64/3 was used as a negative control.

Table 4
Transfer of hyl_{Efm}-vanA plasmid under vancomycin selection. Notice that only transfer into E. faecium and not into E. faecalis strains could be detected.

Donor strain	Recipient strain	Transfer rate[a]
E. faecalis		
UW2774	OG1RF	–(<5.0E–07)[b]
UW2774	JH2-2	–(<3.7E–06)[b]
UW2774	UV202	–(<2.9E–06)[b]
E. faecium		
UW2774	64/3	2.8×10^{6}
UW2774	BM4105RF	5.5×10^{-5}
UW2774	GE-1	7.5×10^{-5}
64/3xUW2774[c]	64SS	4.4×10^{-2}

[a] Expressed as the number of transconjugants (vancomycin-resistant) per recipient cell.

[b] In E. faecalis, no transconjugants were found (detection limit given in parentheses).

[c] Second conjugation using transconjugant strain 64/3xUW2774 as donor.

Transmission electron microscopy (TEM)

Briefly, 200-mesh Formvar-carbon-coated copper grids were floated on the surface of bacterial suspensions (1×10^{9} CFU/ml) for 10 min. Grids were washed and blocked with 1% bovine serum albumin in PBSb. PilA was labelled by floating the grids for 1 h on drops containing a 1/100 dilution of anti-PilA rabbit immune serum in PBSb. Antibodies were labelled with protein A-Gold (15 nm) in PBS. Grids were washed four times, fixed, and bacteria were stained with 1.8% methylcellulose (25 centipoises, Sigma–Aldrich) and 0.4% uranyl acetate (pH 4) and subsequently air-dried for 10 min. Grids were examined by using a Jeol 1010 transmission electron microscope (Jeol-Europe, Amsterdam, The Netherlands) (Hendrickx et al., 2008; van Wamel et al., 2007).

Results and discussion

hyl_{Efm} genomic environment

hyl_{Efm} genomic island structure

Recent advances in genomic research projects prompted us to compare the reannotated putative GI with data in public gene databases. The hyl_{Efm} GI containing hyl_{Efm} previously suggested by Rice et al. (2003) was searched among the available E. faecium genome sequences of strain DO (NZ_AAAK00000000) and U0317 (NZ_ABSW00000000). The hyl_{Efm} GI was reannotated into a 17,817-bp structure that was also found identical (by microbial BlastN) in six other E. faecium microbial genomic contigs (ACIY01000535.1, ACOB01000003.1, ACJQ01000003.1, ACAX01000152.1, ACBA01000164.1, and ACAS01000191.1). Later sequencing of hyl_{Efm} plasmid pLG1 allowed confirmation of the reannotated hyl_{Efm} genomic region (Fig. 1 and Table 5). Concerning the putative GI structure in the investigated clinical E. faecium isolates, long-template PCR and subsequent HindIII digestion of corresponding products revealed a conserved structure with a difference downstream of hyl_{Efm} (Table 1). Twenty out of the 38 strains investigated yielded no product in region hylGI3c. The prevalence of this event among the strains analysed can be biased by overrepresentation of hyl_{Efm}-positive isolates recovered during 2006. The insertion into region hylGI3c in pLG1 was found to be a 2344-bp–IS66 remnant. It was located downstream of hyl_{Efm} in the region

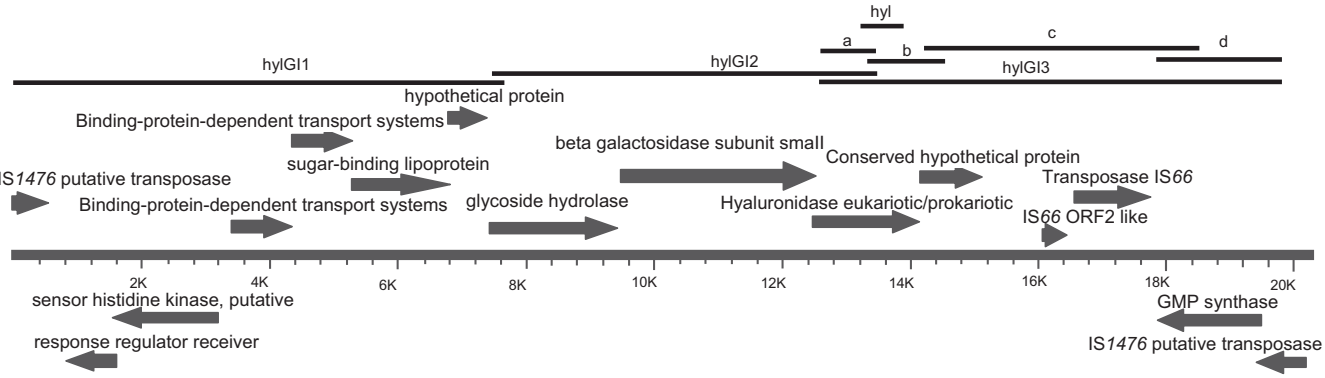


Fig. 1. Structure of the genomic environment of hyl_{Efm} in pLG1. Solid bars show fragments amplified by PCR. hylGI1, hylGI2, and hylGI3 correspond to the regions amplified by long-template PCR (hylGI3-) a, b, c, d, and hyl are regions amplified by regular PCR.

Table 5
Annotation of some of the identified ORFs in pLG1 (accession numbers HM565168–HM565236). For each ORF, the corresponding contig (and its accession number) with the exact location of the ORF within the contig are presented. repA-pLG1 bearing the oriV was arbitrarily taken as pLG1-0001.

Locus tag	Contig	Accession number	Coordinates	Product
hyl_{Efm} genomic island				
pLG1-0203	65	HM565216	347:1075	Response regulator receiver [E. faecium 1,230,933]
pLG1-0204	65		1053:2639	Sensor histidine kinase, putative [E. faecium U0317]
pLG1-0205	65		2836:3792	Binding-protein-dependent transport systems [E. faecium DO]
pLG1-0206	65		3804:4715	Binding-protein-dependent transport systems [E. faecium DO]
pLG1-0207	65		4739:6211	Sugar-binding lipoprotein [E. faecium DO]
pLG1-0208	65		6262:6873	Hypothetical protein EfaeDRAFT_1158 [E. faecium DO]
pLG1-0209	65		6852:8816	Glycoside hydrolase [E. faecium 1,230,933]
pLG1-0210	65		9093:11924	Beta galactosidase subunit small [E. faecium 1,230,933]
pLG1-0211	65		11921:13582	Hyaluronidase eukaryotic/prokaryotic [E. faecium DO]
pLG1-0212	65		13622:14554	Conserved hypothetical protein [E. faecium DO]
pLG1-0213	65		14885:15127	Hypothetical protein EfaeDRAFT_2591 [E. faecium DO]
pLG1-0214	65		15117:15470	IS66 Orf2 like [E. faecium DO]
pLG1-0215	65		15572:17119	Transposase IS66 [E. faecium DO]
pLG1-0216	65		17267:18832	GMP synthase [E. faecium U0317]
Replication and mobilization				
pLG1-0001	21	HM565183	40769:41809	Replication-associated protein RepA [E. faecium 1,230,933]
pLG1-0015	21		3411:3896	Single-strand binding protein [E. faecium DO]
pLG1-0019	21		5746:5997	Topoisomerase [E. faecium DO]
pLG1-0020	21		6023:7747	DNA topoisomerase [E. faecium DO]
pLG1-0054	21		39614:39946	Positive regulator for conjugation [E. faecium DO]
pLG1-0055	1	HM565168	458:4825	Type III restriction endonuclease [E. faecium 1,230,933]
pLG1-0056	1		5756:6199	Putative DNA repair protein RadC [E. faecalis PC1.1]
pLG1-0059	1		7169:7792	Resolvase, N-terminal:helix-turn-helix region [E. faecium DO]
pLG1-0146	17	HM565181	394:1017	MobC filamentation induced by cAMP- Fic [E. faecium 1,231,410]
pLG1-0162	26	HM565186	236:871	Resolvase, N-terminal: helix-turn-helix region [E. faecium DO]
pLG1-0165	29	HM565187	1168:1782	Site-specific recombinase, resolvase family [E. faecium E1071]
pLG1-0236	78	HM565222	1084:1875	repB replication-associated protein [E. faecium 1,231,410]
pLG1-0277	94	HM565234	3880:4443	Resolvase [E. faecium 1,230,933]
pLG1-0250	86	HM565227	9139:9693	Resolvase [S. pneumoniae CGSP14]
pLG1-0177	35	HM565192	2109:3716	Putative relaxase
Maintenance				
pLG1-0164	29	HM565187	14:1117	Abortive infection bacteriophage resistance protein [E. faecium E1071]
pLG1-0004	21	HM565183	44100:44354	Transcriptional regulator/antitoxin, MazE [Mesorhizobium sp. BNC1]
pLG1-0005	21		44355:44672	Plasmid stabilization system protein [E. faecium 1,141,733]
pLG1-0006	21		2:145	PemI family protein [E. faecalis V583]
pLG1-0017	21		4814:5407	Abortive infection protein AbiGI [E. faecium 1,230,933]
pLG1-0163	26	HM565186	895:1245	PemK family protein [E. faecium DO]
pLG1-0110	12	HM565176	7743:8012	Plasmid stabilization system [E. faecium DO]
T4SS-like components				
pLG1-0034	21	HM565183	18203:19408	(VirB1-like) peptidase M23B [E. faecium D344SRF]
pLG1-0035	21		19408:21438	(VirB4-like) ATPase [E. faecium 1,231,410]
pLG1-0040	21		25576:28188	(VirD4-like) conjugation protein [E. faecium 1,230,933]
• pilA cluster				
pLG1-0044	21	HM565183	31362:33452	pilF conserved hypothetical protein
pLG1-0045	21		33449:33709	Conserved hypothetical protein [E. faecium 1,231,501]
pLG1-0046	21		33722:34483	pilE conserved hypothetical protein
pLG1-0047	21		34499:35251	Sortase family protein [E. faecium E1071]
pLG1-0048	21		35266:37242	PilA [E. faecium]
pLG1-0049	21		37295:37993	Sortase [E. faecium 1,141,733]

Table 5 (Continued)

Locus tag	Contig	Accession number	Coordinates	Product
Carbohydrate transport and metabolism				
pLG1-0063	3	HM565170	3013:4488	Sulfatase [E. faecium DO]
pLG1-0064	3		4789:5610	PTS system mannose/fructose/sorbose family IID [E. faecium DO]
pLG1-0067	3		7423:7902	PTS system sorbose subfamily IIB component [E. faecium DO]
pLG1-0068	3		7906:9684	Beta-galactosidase [E. faecium DO]
pLG1-0070	3		10565:11701	Radical SAM [E. faecium DO]
pLG1-0080	4	HM565171	4564:5424	Oxidoreductase domain protein [E. faecalis TX0104]
pLG1-0081	4		5436:6263	3-methyl-2-oxobutanoate hydroxymethyltransferase [E. faecalis V583]
pLG1-0082	4		6278:7126	Pantoate-beta-alanine ligase [E. faecium DO]
pLG1-0083	4		7136:7531	Aspartate decarboxylase [E. faecium DO]
pLG1-0087	4		11004:11654	ABC transporter related protein [E. faecium DO]
pLG1-0101	8	HM565173	123:1373	PTS lactose/cellobiose IIC component [E. faecium DO]
pLG1-0105	12	HM565176	1641:2942	PTS lactose/cellobiose IIC component [E. faecium DO]
pLG1-0108	12		5395:6087	Sugar isomerase [E. faecium 1,141,733]
pLG1-0119	12		15704:16579	Metallophosphoesterase [E. faecium DO]
pLG1-0120	12		16751:17698	Aldo/keto reductase [E. faecium DO]
pLG1-0122	12		18485:19738	Extracellular solute-binding protein, family 1 [E. faecium DO]
pLG1-0123	12		19821:20744	Binding-protein-dependent transport systems [E. faecium DO]
pLG1-0126	12		22897:24585	Phosphoenolpyruvate-dependent sugar phosphotransferase system, EIIA 2 [E. faecium DO]
pLG1-0127	12		24740:25885	Phosphoenolpyruvate-dependent sugar phosphotransferase system, EIIA 2 [E. faecium DO]
pLG1-0128	12		25990:26283	Phosphotransferase system PTS, lactose/cellobiose-specific IIA subunit [E. faecium DO]
pLG1-0129	12		26285:26596	Phosphotransferase system, EIIC [E. faecium DO]
pLG1-0132	12		28886:29839	Alpha-mannosidase [E. faecium DO]
pLG1-0133	12		32931:34307	Bleomycin hydrolase [E. faecium DO]
pLG1-0134	12		34382:35200	Myo-inositol catabolism IolB [E. faecium DO]
pLG1-0135	12		35250:36227	Carbohydrate kinase, PfkB [E. faecium DO]
pLG1-0136	12		36178:38136	Pyruvate decarboxylase [E. faecium DO]
pLG1-0137	12		38158:39312	Iron-containing alcohol dehydrogenase [E. faecium DO]
pLG1-0138	12		39335:39766	GCN5-related N-acetyltransferase [E. faecium DO]
pLG1-0149	17	HM565181	2754:3806	Membrane protein, putative [E. faecium DO]
pLG1-0150	17		3819:4757	Ketopantoate reductase ApbA/PanE [E. faecium DO]
pLG1-0151	18	HM565182	85:561	Sugar-specific permease, EIIA 1 domain [E. faecium DO]
pLG1-0152	18		574:2076	PTS, EIIB: EIIC [E. faecium DO]
pLG1-0153	18		2090:2779	N-acylglucosamine-6-phosphate 2-epimerase [E. faecium 1,231,502]
pLG1-0154	18		2939:3757	Helix-turn-helix protein RpiR:Sugar isomerase (SIS) [E. faecium DO]
pLG1-0157	18		4710:5519	ROK [E. faecium DO]
pLG1-0187	49	HM565202	1:174	Hydrolase, HAD superfamily [E. faecium E1071]
pLG1-0193	55	HM565208	1:177	Binding-protein-dependent transport system [E. faecium 1,231,502]
pLG1-0199	63	HM565214	21:248	Glucose-6-phosphate isomerase [E. faecium TX1330]
pLG1-0200	64	HM565215	3:236	NAD-dependent epimerase/dehydratase [E. faecium E1071]
pLG1-0225	73	HM565219	2569:2796	ABC transporter, ATP-binding protein [E. faecalis V583]
pLG1-0253	87	HM565228	2860:4029	Sugar isomerase (SIS) [E. faecium DO]
pLG1-0254	87		4045:5040	DeoC/LacD family aldolase [E. faecium 1,230,933]
Phage-related				
pLG1-0181	42	HM565196	87:248	Head-tail joining protein [Enterococcus phage EFAP-1]
pLG1-0184	45	HM565199	2:331	Bacteriophage terminase large subunit [Lactobacillus phage phiPYB5]
pLG1-0186	47	HM565201	84:194	Prophage pi3 protein 45 [E. faecium 1,230,933]
pLG1-0142	13	HM565177	322:939	Phage integrase [E. faecium 1,231,502]
pLG1-0062	3	HM565170	55:1149	Phage integrase:Phage integrase, N-terminal SAM-like [E. faecium DO]
pLG1-0164	29	HM565187	14:1117	Abortive infection bacteriophage resistance protein [E. faecium E1071]
Resistance				
• Heavy metal				
pLG1-0167	32	HM565189	11:424	Integral membrane protein [E. faecium 1,231,501]
pLG1-0168	32		771:3239	Copper-translocating P-type ATPase [E. faecium E1071]
pLG1-0221	70	HM565218	3201:3434	Putative copper chaperone [E. faecium E1071]
pLG1-0222	70		3587:5536	Cadmium-translocating P-type ATPase [E. faecium E1071]
pLG1-0259	91	HM565231	827:1282	Copper transport repressor, CopY/TcrY family [E. faecium E1071]
pLG1-0260	91		1295:3760	TcrA [E. faecium]
pLG1-0261	91		3845:4051	TcrZ [E. faecium]
pLG1-0262	91		4190:6352	TcrB [E. faecium]
pLG1-0263	92	HM565232	112:1077	CtpA [Listeria monocytogenes]
pLG1-0090	6	HM565172	990:1319	Cadmium efflux system accessory protein [E. faecium 1,230,933]
• Antibiotic				
pLG1-0091	6	HM565172	1606:2091	Teicoplanin resistance protein [E. faecium]
pLG1-0092	6		2244:3161	VanY protein [E. faecium]
pLG1-0093	6		3338:4633	Transposase [E. gallinarum EG2]
pLG1-0094	6		5106:5714	D-ala-D-ala dipeptidase [E. faecium 1,230,933]
pLG1-0095	6		5720:6799	Vancomycin/teicoplanin A-type resistance protein VanA [S. aureus]
pLG1-0096	6		6744:7724	Vancomycin resistance protein VanH [S. aureus]
pLG1-0097	6		7927:9081	Sensor histidine kinase VanS [E. faecium E1071]
pLG1-0061	2	HM565169	605:1342	ErmB [Lactobacillus yplantarum]

between the GMP synthase (pLG1-0216) and a conserved hypothetical protein (pLG1-0213) putative genes (Table 5 and Fig. 1). Recently, Gourbeyre et al. (2010) described that IS66 is a family of ISs largely limited to the Proteobacteria, which normally include three ORFs (orfA, orfB, and orfC), well-conserved terminal inverted repeats of about 20–30 bp, and are flanked by an 8-bp direct repeat. IS66 of pLG1 consists of the transposase (orfC) and what seems to be the orfA component. pLG1 bears the 25-bp inverted repeats but the 8-bp direct repeats at the insertion site are not present. This insertion is absent from the hyl_{Efm} GI of strain DO and from the six E. faecium genomic contigs where the hyl_{Efm} genomic region is conserved (see above).

The putative GI is flanked by two incomplete inverted copies of the E. faecium insertion sequence (IS) IS1476 (U63997) putative transposase gene. The regions flanking the putative GI outside of IS1476 could not be determined neither by primer walking (Seegene, Seoul, Korea) nor by 454 sequencing and data assembly.

The reannotated first two genes of the putative hyl_{Efm} GI are predicted to encode a 2-component system transcription regulation operon consisting of a sensor histidine kinase (pLG1-0203) and a response regulator (pLG1-0204). Two-component systems are the most frequent surface-exposed signal transduction systems in bacteria allowing to sense and to respond to changes in many different environmental conditions (Mascher et al., 2006). They are commonly used by pathogenic bacteria to control the expression of virulence factors required for infectivity (Stock et al., 2000).

Concerning the hyaluronidase activity tested in this study, none of the 39 enterococcal strains tested were able to lyse hyaluronic acid despite designated as hyl_{Efm}-positive. Original annotation of hyl_{Efm} was based on translated nucleotide similarity (BlastX) to a hyaluronidase from Streptococcus pyogenes known as Spy1600 (accession numbers NP_269657 and NP_607664) and to a hyaluronidase from Clostridium perfringens (Rice et al., 2003). A recent report demonstrates that Spy1600 despite its original annotation as a hyaluronidase does not have detectable hyaluronidase activity but does remove -O-linked N-acetylglucosamine from mammalian glycoproteins (Sheldon et al., 2006). This might also be the case of hyl_{Efm}, however no biochemical evidence for the actual enzymatic activity of hyl_{Efm} has been reported so far. The predicted hyl_{Efm} protein sequence was searched for protein domains and functional sites stored in the Prosite database (http://expasy.org/prosite/). We did not find any motifs that could predict an alternative function. ORFs adjacent to spy1600, as those adjacent to hyl_{Efm}, encode a transcriptional regulator, other glycosidases, and sugar-binding proteins supporting the idea that these genes are involved in simple carbohydrate metabolism although a role in deglycosylation of human O-glcNAc proteins for compromising the host cell machinery cannot be ruled out (Sheldon et al., 2006). Blast comparison of the hyl_{Efm} GI and a 20-kb region-spanning spy1600 showed no similarity between these two regions except for the hyl_{Efm} gene and the region-encoding two binding protein-dependent transport systems (pLG1-037 and pLG1-038) (64%identity). As in the original putative hyl_{Efm} GI description of Rice et al. (2003), this genomic region showed the presence of genes related to carbon metabolism (Fig. 1 and Table 5). It is then possible that the association of hyl_{Efm} to pathogenicity is due to a metabolic advantage of strains conferred by the hyl_{Efm} GI genes.

hyl_{Efm} localization in E. faecium isolates

hyl_{Efm} was localized on megaplasmids ranging from 150 to 350 kb in 37 of 39 clonally different strains analysed (Fig. 2). Among the vanA-positive strains (n = 12), vanA and hyl_{Efm} were localized on the same plasmid in four isolates (including pLG1) while eight carry these genes on different plasmids (online Supplementary Fig. 1). The colocalization of vanA and hyl_{Efm} in the same plasmid has also been reported by Arias et al. (2009) and Rice et al. (2009). Freitas et al. (2010) analysed a set of 51 hyl_{Efm}-positive isolates from a worldwide collection of vancomycin-resistant and vancomycin-susceptible enterococci belonging to the polyclonal hospital genogroup termed clonal complex (CC) 17 (CC17). They found that hyl_{Efm} was located on plasmids ranging from 170 to 375 kb and vanA and hyl_{Efm} genes were never identified on identical plasmids contrasting the results presented here. The different sizes of the hyl_{Efm} megaplasmids suggest differences on their structure and composition.

We did not see hybridization of hyl_{Efm} to any of the plasmid bands of strains DO and UW6379 (Fig. 2). Instead, Southern hybridization of I-CeuI PFGE showed hybridization to a chromosomal band (data not shown) suggesting that in these two strains hyl_{Efm} is integrated in the chromosome, although in previous studies it has been reported that hyl_{Efm} is located on a plasmid in strain DO (Arias et al., 2009; Rice et al., 2009).

Transfer of hyl_{Efm} plasmids

In this study, we demonstrate in vitro transfer of hyl_{Efm} plasmids from a clinical E. faecium strain (UW2774) into non-clinical E. faecium recipient strains of different MLST types (Table 4), although a number of molecular and epidemiological studies have revealed the exclusive prevalence of hyl_{Efm} among clinical strains (Rice et al., 2003; Top et al., 2008). Transfer of hyl_{Efm} plasmids into E. faecalis could not be achieved, which is in accordance to the fact that this marker is exclusively found among E. faecium (Rice et al., 2003) suggesting a limited host range of hyl_{Efm} plasmids as a reason for this exclusive distribution. Conjugation experiments yielded the transconjugant strain 64/3xUW2774 bearing only pLG1. Further transfer of pLG1 from this transconjugant into the recipient strain 64SS was also confirmed (Table 4). Transfer of hyl_{Efm} plasmids without transfer of other plasmids and later sequence analysis (see below) suggest that hyl_{Efm} plasmids are self-transferable. Horizontal transmission of hyl_{Efm} plasmids has been previously described both when hyl_{Efm} and vanA are genetically linked (Arias et al., 2009; Rice et al., 2009) and when they are not (Freitas et al., 2010).

hyl_{Efm} plasmid sequencing

Large plasmids have not been previously described in enterococci (Weaver et al., 2002), however, according to recent reports, it seems like they are widely disseminated (Freitas et al., 2010; van Schaik et al., 2010). This study is the first to document the draft sequence of a megaplasmid (pLG1) in E. faecium. Sequencing led to findings regarding the backbone and the classification of this plasmid as well as its possible contribution to metabolism, pathogenicity, and resistance, by determinants that are encoded on this conjugative element.

454-Sequencing of hyl_{Efm} vanA megaplasmid pLG1 yielded a total of 99 contigs, 35 of them smaller than 500 bp 13 of which did not contain any predicted CDS and were excluded from the analysis. The 64 contigs larger than 500 bp comprised a total of 256.16 kb. The average contig size was 4002 bp, and the largest contig was 40,663 bp. Given the calculated plasmid size of 281.02 kb and the total number of aligned bases of 2,853,150 bp, the covery was about 10.1 reads per bp. Difficulties in the assembly were due to the presence of several insertion sequences, transposons, and other repetitive sequences.

Southern hybridization was positive for all the 32 probes confirming location of the tested contigs on the sequenced plasmid and not in the chromosome (data not shown). Eighteen PCR reactions were performed to close gaps in the draft assembly. This resulted in a final total of 69 sequencing contigs of pLG1 (accession numbers HM565168–HM565236). The identified homologies

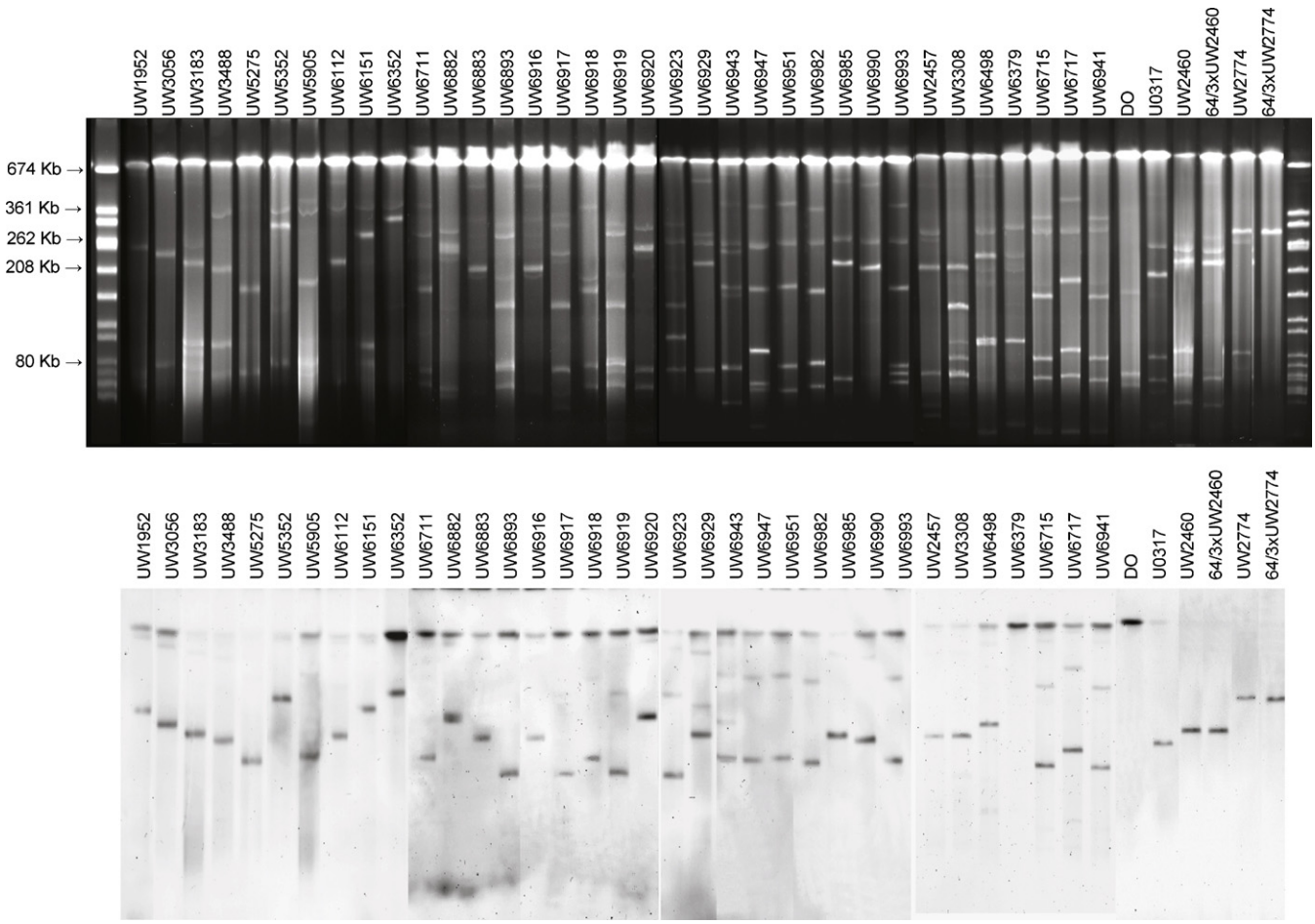


Fig. 2. S1-nuclease PFGE of 39 hyl_{Efm}-positive isolates (top) and corresponding Southern hybridization membranes using hyl_{Efm} probe (bottom). Characteristics of the strains and the calculated hyl_{Efm} plasmid sizes are described in Table 1. Linearised plasmids are resolved in the gel on a background of degraded chromosomal DNA. The upper band (migrating at approximately 674 kb) visible in all lanes correspond to undigested chromosomal and plasmid DNA.

of predicted open reading frames (ORFs) can be grouped into replication, mobilization/conjugation, resistance, and metabolism as presented below. An overview of the identified ORFs and their predicted functions is presented in Table 5.

Replication and mobilization

A 1038-bp ORF was identified as a putative replicase gene (repA-pLG1 pLG1-0001) based on similarity at the protein level to the RepA of pAD1 (gb|AAB00503.1) and is expected to encode a replication initiator protein, originally from E. faecalis.

Francia et al. (2004) have demonstrated that the replication initiator protein of pAD1, RepA, is necessary and sufficient for the initiation of plasmid DNA replication at an oriV located within its own coding sequence. A putative oriV was also identified in the conserved C-terminal sequence of repA-pLG1 with high conservation of the direct and inverted repeats which are described to be essential in RepA binding to oriV.

Most members of the RepA-pAD1-related family are similar in size (Francia et al., 2004). The identified RepA-pLG1 consists of 436 amino acids (AA), remarkably larger than RepA-pAD1 (336 AA) and other annotated enterococcal RepA (326–346 AA). Francia et al. (2004) compared 38 RepA homologues and found that five amino acids of the N-terminal domain are absolutely conserved. This motif is also conserved in RepA-pLG1. Weaver et al. (2009) constructed an unrooted phylogenetic tree of 43 RepA homologues associated with mobile genetic elements to demonstrate the broad distribution and narrow host range of RepA_N plasmids. This phylogenetic analysis applied to the RepA-pLG1 confirmed that it is a RepA_N family replicon and revealed that it fits squarely in the enterococcal sequences but constitutes a single branch.

The C-terminal sequence of RepA_N homologues is highly variable and provides the host specificity (Weaver et al., 2009). The C-terminal sequence of RepA_pLG1 has a low similarity (30%) to that of RepA_pAD1. The specificity provided by the C-terminal sequence of RepA_pLG1 could explain why hyl_{Efm} is never found among E. faecalis as well as the negative results in hyl_{Efm} plasmid transfer into E. faecalis.

An ORF (pLG1-0236) with 100% similarity to the replication-associated gene repB of pTEF3 (ref|ZP_00602517.1|) was found. RepB proteins are ATPases that, together with a DNA-binding protein and a centromere-like site, comprise an 'active partition locus' which facilitates plasmid distribution at cell division (Weaver et al., 2009). No genetic link of repB and the identified repA could be established.

A putative relaxase gene rel-pLG1 (pLG1-0177) was also identified. Analysis of the translated sequence of this ORF showed that the relaxase domain was contained in the first 150 amino acids of the N terminus. Relaxases are the proteins that initiate and terminate conjugative DNA processing and are the only essential protein of the mobilization (Mob) machinery of mobilised plasmids (Garcillan-Barcia et al., 2009). Garcillan-Barcia et al. (2009) have described a classification scheme of conjugative transfer systems based on the comparison of the sequences and properties of the relaxase proteins contained in gene sequence databases. This

analysis was applied to Rel-pLG1, and it was seen that it belongs to the MobP family, the largest relaxase family, MobP7 subfamily, and it clusters among lactococcal and E. faecalis relaxases. MobP7 relaxases have been found encoded by RepA_N plasmids before (e.g. pCF10) (Garcillan-Barcia et al., 2009).

Further replication and segregation of related genes present in pLG1 are presented in Table 5.

Pili and conjugation

Hendrickx et al. (2009b) have previously described four different pilin gene clusters (PGC) in E. faecium. Pili are supposed to be implicated in adhesion to multiple types of human cells and in biofilm formation (reviewed in Sava et al., 2010). A copy of the entire PGC1 (EU909697) is present in pLG1 (99% identity on the DNA level) (from pLG1-0044 to pLG1-0049).

All the conserved motifs of the described pilin subunits (Hendrickx et al., 2008) were found intact in the PilF, PilE, and PilA of pLG1. A recent study demonstrated the plasmid colocalization of pilA (fms21) and hyl_{Efm} in three of seven infectious E. faecium isolates and speculated that ancestral E. faecium isolates carried the pilA gene cluster and subsequently acquired antibiotic and virulence determinants (Kim et al., 2010).

Transmission electron microscopy imaging using anti-PilA immunogold particles, showed that among the population of the donor strain UW2774, cells expressed PilA pili, however, this phenotype could not be confirmed in any of the transconjugants due to low adherence of the strains to carbon-coated copper grids (unpublished).

Conjugation systems appear to be a subgroup of T4SS that have evolved the additional capacity to translocate DNA–protein complexes (Christie, 2001). In Gram-positive plasmids, proteins homologous to most of the protein families involved in DNA transfer and in Gram-negative bacterial plasmid transfer have been described (reviewed in Grohmann et al., 2003). These should provide (i) substrate presentation (coupling proteins or VirD4), (ii) energetics of the translocation process (ATPases or VirB4, VirB11 homologues), and (iii) formation of the mating channel (lytic transglycosylases or VirB1 homologues and transmembrane proteins or VirB6 homologues). In Gram-positive bacteria, the close contact between donor and recipient cells preceding conjugative transfer is thought to be established without the help of pili (Abajy et al., 2007) and is supposed to involve the role of adhesins, aggregation substance or proteins with cell wall anchor motifs. To our knowledge, no association between pili and DNA/protein secretion or transfer has been assessed in Gram-positive bacteria. Nevertheless, type IV pilin-like protein genes have been demonstrated to be involved in genetic transformation of Gram-positive bacteria (reviewed in Averhoff, 2004), and secretion channel-like genes have been described for several Gram-positive conjugative plasmids (reviewed in Alvarez Martinez and Christie, 2009). Hence, participation of pilus-like structures in the conjugative transfer processes of Gram-positive bacteria cannot be excluded (Abajy et al., 2007).

In pLG1, a set of genes arranged in an operon-like way are colocated upstream the previously described pilin gene cluster (Table 5) resembling a complete set of transfer genes related to T4SS. Presence of genes required for mobilization as well as a T4SS allowing assembly and functionality of the mating channel are hallmarks of self-transmissible plasmids (Garcillan-Barcia et al., 2009). In the vicinity of this region, an ORF (pLG1-0015) with similarity to a single-stranded DNA (ssDNA)-binding protein (SSBP) was found which is normally encoded in the conjugation regions of some sex pheromone plasmids such as pAD1.

Maintenance

The plasmid pLG1 has several putative coding sequences that potentially contribute to its segregational stability (Table 5). Among them pLG1-0004 and pLG1-0005 seem to constitute a complete plasmid stabilization module. The predicted proteins have small sizes (9.3 kDa and 12.2 kDa, respectively) and show an overlapping organization, characteristics of proteic TA modules found in Gram-negative bacteria (Grady and Hayes, 2003). TA systems have been also described among Gram-positive bacteria, e.g. in E. faecalis pAD1 plasmid (Weaver et al., 2003), in E. faecium in the pRUM (Grady and Hayes, 2003), and in the pVEF plasmids (Sletvold et al., 2008). Despite homology (37% identity) of pLG1-002 to a MazE component, search of the complete mazEF region of E. coli among pLG1 sequences did not retrieve matches that indicated the presence of the entire mazEF system. Functionality tests of these and other putative plasmid maintenance systems should be performed in order to determine the genes that are responsible for the stability of hyl_{Efm} and van plasmids among the enterococcal population.

Resistance

Several genes encoding heavy metal resistance were found on pLG1 (Table 5), including a complete tcrYAZB operon conferring copper resistance. Bacteria have developed homoeostatic mechanisms to ensure adequate levels of copper within the cell. In response to toxic copper concentrations, plasmid-borne copper resistance mechanisms such as the tcrYAZB operon of E. faecalis and E. faecium are often employed (Hasman, 2005). Our data corroborate previous studies describing the presence of copper resistance, glycopeptide and macrolide resistance gene clusters on the same conjugative plasmid (Hasman and Aarestrup, 2002) and additionally demonstrate the presence of hyl_{Efm} on the same conjugative element.

A complete vanA operon was identified in pLG1 (Table 5). Presence of a transposase (IS204/IS1001/IS1096/IS1165) between vanY and vanX was partially resolved. This vanA cluster constitutes a subtype of Tn1546-like elements which often bear IS elements like ISEf1, IS1678, or IS1216 V, ISEfa5, IS19-like between vanX and vanY (Werner et al., 2008; Jung et al., 2007; Camargo et al., 2005; Lopez et al., 2010).

ermB, which encodes resistance to macrolides, lincosamides, and streptogramin B antibiotics was also identified on pLG1. The acquired antibiotic resistance determinants observed by plasmid sequencing was congruent with the observed antibiotic resistance of the transconjugants, which also acquired the selection marker vancomycin resistance, and macrolide resistance conferred by ermB.

Metabolism

As previously mentioned, the genes neighbouring the putative hyl_{Efm} gene seem to be involved in carbohydrate metabolism. In the same way, several of the identified ORFs in pLG1 are putative metabolic genes that might be involved in uptake and utilization of different carbohydrates (Table 5). Several of the identified ORFs show homology to phosphotransferase systems (PTS) which constitute the major carbohydrate transport system in bacteria by active translocation across the cell membrane. Neighbouring all the identified PTS components, putative CDSs related to sugar carbon metabolism were found. Changes in the metabolism of strains that have acquired pLG1 are being currently investigated.

Conclusions

The hyl_{Efm} gene is part of a highly conserved genetic structure that is mostly plasmid-located among a wide set of clinical E. faecium isolates. We characterized for the first time a hyl_{Efm} -containing megaplasmid and describe the presence of resistance

and metabolic genes as well as putative pathogenicity factors on the same conjugative element. hyl_{Efm} megaplasmids are widely distributed among clinically associated E. faecium and seem to be restricted to this species. Colocalization of hyl_{Efm} with other genetic elements on the same conjugative plasmid suggests coselection of potential pathogenicity factors under the selective conditions imposed within the clinical environment, which might explain the rapid adaptation and increased success of hospital-associated subpopulations of E. faecium.

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Nucleotide sequence accession number: The E. faecium plasmid pLG1 sequences have been deposited in the GenBank database under accession numbers HM565168–HM565236.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijmm.2010.08.015.

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3.2. Analysis of the *plasmidome* of enterococci from different environments with special emphasis on that encoding antibiotic resistance or adaptive traits. Characterization of *vanA*-Tn*1546* and *vanB*-Tn*1549*/Tn*5382* transposons responsible for the global spread of vancomycin-resistance among *Enterococcus* from humans and animals

3.2.2. Analysis of plasmids and transposons in antibiotic resistant enterococci from humans and animals.

3.2.2.1. The spread of vancomycin resistant *Enterococcus faecium* (1986-2009): a historic view from the plasmid side

3.2.2.2. Influence of pAD1 and Inc18 plasmids in the worldwide spread of vancomycin-resistant *Enterococcus faecalis* outbreak strains (1987-2005)

3.2.2.3. Spread and persistence of Inc18::Tn*1546* plasmids among enterococci from poultry food products in Portugal

3.2.2.4. Spread and diversification of pRUM and Inc18-like plasmids carrying Tn*1546* among CC17 *Enterococcus faecium* from hospitals in Portugal (1996-2008)

3.2.2.5. Host range of enterococcal *vanA* plasmids among Gram-positive intestinal bacteria

3.2.2.6. Diversity of Tn*1546* and its role in the dissemination of vancomycin-resistant enterococci in Portugal

The spread of vancomycin-resistant *Enterococcus faecium* (1986-2009): A historic view from the plasmid side

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Presentation of these data at international conferences:

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ABSTRACT

CC17 *Enterococcus faecium* (Efm) has increasingly been reported as a nosocomial pathogen and it has been suggested as the substrate for the acquisition of vancomycin resistance (VR) in this species. Our goal was to analyze the plasmid diversity among CC17 recovered from the last two decades and to further characterize the plasmids associated with vancomycin resistance in this lineage.

Seventy-two VREfm (52 vanA, 20 vanB) causing outbreaks from 28 countries of 5 continents (1986-2009) and 82 vancomycin-susceptible (VSEfm; 53 ampicillin-resistant, AREfm; 29 ampicillin-susceptible, ASEfm) causing bacteremia in Spain (1995-2008) were analyzed. Clonality was established by PFGE and MLST. Van-Tns (*1546/1549/5382*) were characterized by PCR mapping, RFLP and sequencing. Plasmid characterization included determination of size (S1-PFGE), comparison of ClaI-RFLP patterns and identification of relaxases (rel), rep initiation proteins (rep), and toxin-antitoxin systems (TA) by PCR, hybridization and sequencing.

Most VREfm isolates belonged to CC17 (88%: 58 PFGE types, 26 STs, mainly ST16, ST17, ST18) and non-CC17 isolates (12%: CC5, CC9 and 4 singletons) included the three first VRE strains described worldwide in Europe and the USA. All AREfm belonged to CC17 (10 STs, mainly ST18) and ASEfm were clonally diverse (15 STs, CC22 and CC94 being predominant). *vanA* (25 Tn*1546* types) was always located on plasmids while *vanB* (4 Tn*1549*/Tn*5382*-types) was mostly located on chromosome (16/20). A higher diversity of plasmids was observed among VR/AREfm than among ASEfm (1-10 /cell vs ≤2 /cell; 2-445 kb). A diversity of plasmids replicating by rolling-circle and theta (Inc18, RepA_N, pHTβ-like) mechanisms and containing relaxases belonging to $MOBP_7$ (pEF1, pHTβ, pLG1, pAD1), $MOBQ_3$ (Inc18) and $MOBV_1$ (RCR) families were detected among enterococci of different CCs. Small plasmids and TA systems were rare among ASEfm. VanA plasmids mainly correspond to Inc18 and pRUM (with Axe-Txe) derivatives often containing rel-pEF1; pHTβ-like and megaplasmids being identified in a few isolates.

The worldwide spread of vancomycin resistance is driven by CC17-Efm and mosaic plasmids derivatives of Inc18 and/or pRUM. The similarity of plasmid types among CC17 AREfm and VREfm reflects a common genetic background and it confirms the role of this clonal complex as substrate for vancomycin resistance in *E. faecium*.

INTRODUCTION

Vancomycin resistant enterococci (VRE) causing human infections have received an outstanding interest among clinicians since they are usually resistant to most available therapeutical options (2) and the glycopeptide resistance may be transferred to more pathogenic organisms as *Staphylococcus aureus* (57). The first VRE strains identified were two *Enterococcus faecium* (VREfm) isolates recovered in France and in the United Kingdom in 1986-1988 (46, 77) and a *E. faecalis* (VREfs) clinical isolate identified in the northeast of the USA in 1989 (63). Since then, VRE has globally been spread although the dissemination pathways differ among geographical areas in time and genetic background, *Enterococcus faecium* being the predominant VRE species identified worldwide (82, 88).

Acquired resistance to glycopeptides in enterococci may be caused by different operons commonly designated as Van followed by a capital letter (VanA/B/D/E/G/L/M) although VanA and VanB are the most commonly found (11, 86, 92). The *vanA* gene is part of the Tn*3* derivative designated as Tn*1546*, which has been found within conjugative plasmids of different families (RepA_N, Inc18-type, pMG1-like and untypeable megaplasmids) (18, 43, 61, 68, 76, 81, 95). The *vanB* gene is linked to Tn*1547* or CTn*1549*/Tn*5382* which can be transferred either by integrative chromosomal elements (7, 23, 58) or plasmids although they have not been fully characterized (59, 91, 93). Vancomycin-resistant epidemic plasmids from *E. faecium* have been only identified among a few clinical isolates in America and Asia (mostly pMG1-like) (75, 94) and from farm animals (mosaic Inc18 plasmids related to the firstly described pIP186 *vanA* plasmid) (67, 68). Two recent analysis of VREfm from different origins revealed they frequently carry sequences of genes involved in the replication of Inc18, pRUM, rolling circle plasmids and small theta replicating plasmids although further details about these genetic elements were not provided (32, 61). Recently, the plasmid conferring vancomycin resistance in *E. faecalis* and transferred to *Staphylococcus aureus* was identified as belonging to the Inc18-type (96). Knowledge about the plasmid content of VRE strains and the regulatory processes involved in gene transfer of conjugative enterococci plasmids still remains scarce, highlighting the need to better understand the plasmid biology of these species.

From the ecological point of view, different questions remains to be explained as the overrepresentation of *van* genetic determinants among *E. faecium* in comparison with *E. faecalis* which represents the predominant species causing human infections; the apparent predominance of isolates belonging to the CC17 polyclonal subcluster, the unfrequent transfer to other gram positive microorganisms belonging to the same exchange communities or the global dissemination of a few types of *van* transposons. In

this work, the population structure of VREfm causing outbreaks worldwide since 1986 and the characterization of the mobile genetic elements they contain based on the presence of particular modules for basic plasmid functions as replication, mobilization (relaxases) and stability (toxin-antitoxin systems and bacteriocins) is provided. A comprehensive characterization of plasmids conferring resistance to glycopeptides was also performed.

MATERIAL AND METHODS

Bacterial strains and epidemiological background. We analyzed 154 *E. faecium* isolates (72 VREF and 82 VSEF) recovered from different geographical locations (1986-2009). They included 72 vancomycin-resistant *E. faecium* strains isolated from hospitalized patients (VRE; 52 *vanA* and 20 *vanB*), most of them causing documented hospital outbreaks (only one single isolate per outbreak and phenotype), or disseminated in more than one healthcare institution within 28 countries in Europe (Denmark, Finland, France, Greece, Germany, Hungary, Italy, Norway, Poland, Portugal, Serbia, Spain, The Netherlands, and United Kingdom,), America (Argentina, Brazil, Canada, Chile, Colombia, Ecuador, Paraguay, Peru, Venezuela, and the USA), Asia (Singapore and Saudi Arabia), Africa (Tunisia) and Australia, during a 24-year period (1986-2009). They have been previously documented and represent an important subset of the global VRE population since the first description of these isolates in 1988 (1, 4, 6, 7, 10, 15, 16, 29, 34, 35, 38-42, 46-50, 53, 56, 62, 65, 69, 71, 73, 78, 85). As a control group, 82 VSEF were also studied. They include 53 AREF (ampicillin-resistant) and 29 ASEF (ampicillin-susceptible) clonally unrelated isolates causing bacteremia in different patients (1995-2008) in a teaching hospital (1,200 beds; serving a population of 600,000) in Spain and two other AREF strains causing hospital outbreaks in Norway and Denmark in late 1990's (9, 35, 65). Note that Spain, Norway and Denmark are countries with a VREF rate still under 3%. Epidemiological features of the studied isolates are shown in Table 1. Antimicrobial susceptibility to 14 antibiotics (vancomycin, teicoplanin, ampicillin, erythromycin, ciprofloxacin, tetracycline, gentamicin, streptomycin, chloramphenicol, daptomycin, linezolid, tigecycline, rifampicin and fusidic acid) was performed by the disk diffusion method following CLSI guidelines (8).

Putative virulence factors. The presence of enterococcal surface protein (*esp*), hyaluronidase (hyl_{Efm}), aggregation substance (*agg*), a collagen-binding adhesin gene

(*acm*) and chromosomal enterocins A and B was investigated by PCR and further sequencing as described (52, 70, 80).

Clonal relatedness*.* It was established by pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested genomic DNA (New England Biolabs, Ipswich, MA, USA) and MLST following standard procedures (http://www.mlst.net) (29, 72).

Characterization of transposons encoding resistance to glycopeptides. Genes coding for resistance to vancomycin were confirmed by PCR (17). The backbone structure of Tn*1546* was determined by PCR mapping and further sequencing of fragments of unusual size (55, 90). The transposons containing the *vanB* alleles were preliminary discriminated by comparison of the DNA profiles generated by digestion of the 5,959-bp *van*RSYWHBX amplicons using *Bsp*HI/*Dra*I enzymes (New England Biolabs, USA) (14). Distinction between Tn*1547* (*vanB1*) and Tn*1549*/Tn*5382* (*vanB2*) was established by analysing specific sequences corresponding to the right junction of Tn*5382*, the $vanS_B$-$vanY_B$ intergenic region flanking coding sequences and the relaxase of Tn*1549* (13, 14); this study). The link between *pbp5* and Tn*5382* was investigated according to Carias *et al*. (7).

Transferabiliy of *van* transposons was analyzed by using the filter mating method at 1:1 donor-recipient ratio and *E. faecium* strains GE-1 and BM4105RF as recipients (28). Transconjugants were selected on Brain Heart Infusion agar plates containing vancomycin (6 mg/L) and fusidic acid (20 mg/L), with or without rifampicin (30 mg/L) after incubation at 37ºC for 24h-48h. Chromosomal and/or plasmid location of *van* transposons was assessed by hybridization of I-*Ceu*I-digested genomic DNA hybridization with *van*A, *vanB* and 23S rRNA probes as described (19). Genomic DNA from *E. faecium* strain UA2-1 and *E. faecalis* strain V583 was used as positive controls for *vanA* and *vanB* hybridizations, respectively.

Plasmid characterization*.* The plasmid content was determined using the methods described by Kado & Liu (for plasmids from 2 kb to 10 kb) (36) and Barton *et al.* (for plasmids from 10 kb to >300 kb) (5) in both wild type and transconjugant isolates. Preliminary classification of *E. faecium* plasmids was inferred by the presence of sequences associated with replication (replicases), mobilization (relaxases) and stability (toxin-antitoxin and bacteriocins) identified by using different PCR typing schemes (24, 32, 33, 51) (see Table 3 of section 6.1.7.3. of material and methods of this thesis). The search of bacteriocins (Bac) previously identified on enterococcal plasmids (P, Q, L50A, L50B, Bac32 and Bac43) was performed by PCR using primers shown in Table 3 of section 6.1.7.3. mentioned before (12, 30, 70, 74). PCR products of each rep/rel/TA/Bac gene

type were sequenced in order to confirm the specificity of the method and to analyze similarities with available sequences. Correlation of particular genes with different plasmid bands was established by hybridization of S1- and/or I-*Ceu*I-digested genomic DNA with specific probes obtained by PCR using DNA from reference plasmids. Relationship among vancomycin-resistant plasmids of similar size and containing similar replicon-relaxase genes was established by comparison of patterns generated after enzymatic digestion with *Eco*RI and *Cla*I (Promega, Woods Hollow Road-Madison, WI, USA). Restriction fragment length polymorphism (RFLP) profiles corresponding to plasmids identified in this study were compared with those of other sequenced plasmids of the same families virtually generated by using the NEBcutter V2.0 tool (http://tools.neb.com/NEBcutter2/index.php). Data of representative RFLP patterns were exported into Fingerprinting II Informatix version 3.0 software (Bio-Rad Laboratories, Hercules, CA, USA) for further interpretation. Cluster analysis was conducted by using the unweighted pair group method with arithmetic averages (optimization 0.5%, tolerance 1.00%).

Molecular techniques/DNA methodology. PCR assays were performed in volumes of 25 µl under the following conditions: 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 1 µM of each primer, and 1.5 units of *Taq* DNA polymerase (GoTaq® Flexi DNA polymerase; Promega, Woods Hollow Road-Madison, WI, USA) with amplification programs of 10 min at 94°C; 30 cycles of 30 sec. to 1 min at 94°C, 30 sec. to 1 min at 45 to 70°C, and 30 sec. to 2 min at 72°C, and a final extension step of 10 min at 72°C. PCR products were purified with the Wizard^{SV} PCR purification kit (Promega, Madison, WI, USA) and sequenced on an ABI Prism 377 automated sequencer (Applied Biosystems PE, Foster City, CA). All probes were generated by PCR using the DNA control templates and the primers listed in Table 3 of section 6.1.7.3. of the appendix of this thesis. DNA southern transfer and hybridization were performed by standard procedures (64). Hybridization experiments were performed by using the Gene Images AlkPhos Direct labeling and detection system (Amersham GB, GE Healthcare Life Sciences UK Limited). Labeling of DNA probes was performed with alkaline phosphatase enzyme by complete denaturation; hybridizations were performed overnight under stringent temperatures (60-70°C), estimation being based on the DNA probe size and G+C content. Chemiluminescence detection was carried out using CDP-Star^{TM} reagent following the manufacturer's instructions (http://www.gehealthcare.com/lifesciences). Plasmid DNA was obtained by using a modification of the alkaline lysis protocol (26) which is detailed described in section 6.3. of material and methods (protocol 2) of this thesis. A modification of the Barton method was also used as described (19) (protocol 4 of section 6.3.). PFGE

was performed as described previously (37) using the following electrophoresis conditions: 5- to 25-s pulses for 6 h and 30- to 45-s pulses for 18 h, at 14°C, and 6 V/cm^2 (S1), 5-to 30-s pulses for 22 h at 14°C and 6 V/cm^2 (I-*Ceu*-I), and 1- to 20-s pulses for 26 h at 14°C, and 6 V/cm^2 (*Sma*I).

RESULTS

Clonal expansion of the polyclonal subcluster CC17 *E. faecium* favours the spread of glycopeptide resistance among enterococci. The 72 VREF included in the study were classified into 58 different PFGE patterns and clustered into 26 different sequence types (ST). Most of them belong to the polyclonal subcluster CC17 (n=63/72, 88%; 18 CC17-STs) and included the widespread ST17 (8 VanA and 7 VanB, 1992-2006), ST16 (7 VanA and 1 VanB, 1995-2002), ST18 (8 VanA, 1992-2008), ST78 (5 VanA, 1998-2005), ST203 (3 VanA and 1 VanB, 2004-2008), ST280 (2 VanB and 1 VanA, 2002-04), ST412 (n=3 VanA, 2005-06), ST117 (n=2 VanA, 1998-2008) and ST202 (n=2 VanA, 2005-06). Other CC17-STs were represented by single strains that were involved in clonal outbreaks and/or persisted in a particular country over years: ST64 (Chile; 2003-04), ST80 (Hungary; 2004), ST192 (Germany; 1996-2004), ST132 (Portugal; 2001-07), ST173 (Australia; 2000-01), ST182 (USA; 1992), ST209 (Italy; 2002), ST438 (Paraguay; 2006) and ST484 (Finland; 1996-98) (Table 1). Some isolates showing identical PFGE patterns were identified over years in different countries and belonged to ST16 (USA, The Netherlands and one VSEF from Spain, 2000-04), ST17 (USA and Argentina; 1992-98), ST78 (Italy, Spain, and Germany; 2001-06), ST280 (Portugal and Singapore; 2002-04) and ST412 (Canada and Venezuela, 2005). Twelve percent of the VRE isolates clustered into CC5 (ST5), CC9 (ST25, ST50, ST358) and four singletons (ST20, ST65, ST114, ST265). It is of interest to highlight that they included the first VRE strains described in France in 1986-87 (ST25), one of the earliest VRE strains described in the USA in 1992 (ST20), and the first VRE outbreak strain from Brazil in 1998 (ST114).

The 52 AREF isolates were classified in 30 PFGE types and 10 STs which mostly belonged to CC17 (94%), and to a lesser extent to CC9 (ST266; 2%) and the singletons ST102 (2%) and ST419 (2%). Among CC17 strains, ST18 was predominant (66%), others as ST16, ST17, ST63, ST103, ST125 and ST203 being infrequently detected or temporarily limited (they represent <10% each). The 30 ASEF isolates were assigned to 30 PFGE types and 15 STs clustering into CC22 (27%; mostly ST22 and ST214), CC94 (23%; mostly ST69 and ST178), CC17 (13%; ST18, ST442), the singleton ST102 (10%),

and CC1 (ST4), CC9 (ST29) and the singleton ST474 (3% each).

Most CC17 isolates analyzed, both VREF and VSEF were resistant to erythromycin (93% vs 92%), ampicillin (95% vs 92%) and fluoroquinolones (98% vs 77%), showing variable rate of resistance to tetracycline (40% vs 23%), and high levels of streptomycin (57% vs 79%) or gentamicin (43% vs 11%). Non-CC17 VREF and VSEF isolates were mostly resistant to erythromycin (79% vs. 69%) and less frequently than CC17 to high-levels of streptomycin (35% vs 24%) or gentamicin (7% vs 0%), ampicillin (26% vs 10%) or tetracycline (23% vs 14%).

Vancomycin-resistant *E. faecium* strains are enriched in putative virulence traits and bacteriocins. Most data related to the presence of putative virulence traits *esp* and *hyl* have been included in a recent publication from our group (19). Briefly, they were mainly found in isolates of the CC17 subcluster since 1992. Only one VRE (ST65) and thee VRE (ST20, ST65, ST114) isolates carrying *hyl* and *esp*, respectively, did not belong to the CC17 subcluster and corresponded to some of the first VRE strains causing hospital outbreaks in Brazil, Greece, and the USA. The *esp* (69%) and *acm* (67%) genes were located in the chromosome while the hyl_{Efm} (30%) gene was always located on megaplasmids (170-375 kb).

Bacteriocins linked to chromosome (enterocin A, enterocin B) or plasmids (enterocin P, Bac32, Bac43) were identified. The pediocin-like enterocin A, confined to *E. faecium* to date (3), was more common than EntB (82% vs 7%). Sequences of both *entA* and *entB* were identical to homologs in different *E. faecium* genomes (GenBank accession numbers GQ182975, ZP_00603732, ZP_05660017, ZP_05665063, ACU65112 and AAD28234).
Plasmid encoded bacteriocins were found in a minority of CC17-VREF isolates (7-15%) recovered since 1992. While genes coding for Bac32 (*bacA*) or Bac43 (*bacA* and *bacB*) were located on small theta replicating plasmid of 12-18kb, the gene encoding enterocin P was linked to large plasmids of ca. 150-200 kb (see below). Sequences were 98-100% identical to homologs at the GenBank databases ($bacA_{32}$, GenBank acc. no. AB158402/FJ618565; Bac43, AB178871; enterocin P, GQ369522). Bac32 (15%) was mostly identified in CC17 STs (all but ST114) with predominance of the ST17 (and its SLVs ST182, ST202 and ST280) and ST18 (and its DLV ST173) major clones. Bac43 (7%) was identified in ST117, ST202, ST203 and ST412 (SLVs of ST203), which correspond to more recent and geographically limited STs. EntP (7%) was distributed among the major subclusters ST16, ST17, ST18 and ST78 from CC17, and ST265.

***vanA* is located on different Tn*1546* derivatives associated with conjugative plasmids.** We identified 25 Tn*1546* types, most of them interrupted by insertion

sequences as IS*1251* (IS*L3* family), IS*Ef1* (IS*30* family) and IS*1216V* (IS*6* family) (Table 2).

Tn*1546* variants containing IS*1251* within *vanS-vanH* region were the most frequently detected (n=21/52 isolates from Europe and America). They correspond to "type F" or "US hospital type" firstly described in the USA during the 1990's (27, 87) and six related variants designated with a numeric subindex (F_{1-6}).

Tn*1546* variants containing IS*1216* within *vanX-vanY* (n=10), *vanS-vanH* (n=3) or upstream *vanR* (n=1) regions were identified in 12/52 isolates, mainly from Europe (Table 2) (41, 55, 90).

Tn*1546* variants carrying IS*Ef1* within *vanX-vanY* region were confined to a few isolates (n=5/52) from Germany, Portugal and Spain (55, 84).

Two new Tn*1546* variants carrying IS*Ef1* and IS*1216* within *vanS-vanH* and *vanX-vanY* regions, respectively, were identified in 2/52 isolates from South America (Table 2).

The whole backbone of Tn*1546* known as "type A" (n=6/52), and types "M" and "D", containing alterations upstream *vanR*, (n=6/52) were identified in isolates from Europe, Brazil or Australia (90).

The Tn*1546* was successfully transferred by conjugation in 79% (41/52) of the strains and located on plasmids of different families (see below) in all but one isolate from Germany. The size of *van*A plasmids varied after conjugation in three isolates containing IS*1216*-carrying Tn*1546* types (PP-13, PP-23) and IS*Ef1*-carrying type PP-5.

***vanB* is mainly located in chromosomal CTn*1549*/Tn*5382*.** We grouped isolates containing *vanB1* (n=3) and *vanB2* (n=17) in four transposable elements on the basis of their RFLP patterns and partial sequences (13, 14) (Table 3).

The *vanB1* platforms showed RFLP-1 (n=2) and RFLP-3 (n=1) patterns which are associated with Tn*1547* or a variant containing a C—T at 2,293 position within $vanS_B$-$vanY_B$ (*sequence submitted to GenBank*). They were identified in 2 CC17 U.S. isolates from early 1990's and one ST209 Italian isolate from 2002, respectively.

The *vanB2* was linked to CTn*1549*/Tn*5382* platforms showing RFLP-2 characterized by an additional *Bsp*HI site within the 1,086-bp $vanS_B$-$vanY_B$ intergenic region; it was predominant in our study and widespread in different continents (Table 3)(14, 78). Variants of this platform caused by insertions of ISEnfa*110* at the left inverted repeat of Tn*5382* in isolates from Chile (49), insertions of ISEnfa*200* within $vanS_B$-$vanY_B$ and the presence of *pbp5* in isolates from Finland, Australia and the USA (13) were observed.

The *vanB* transposons were transferred by conjugation in 30% (6/20) of the strains. Most of them were located on chromosome (n=16) although one isolate from Singapore (ST280) and three from Spain (ST17, ST265) carried *vanB2*-conjugative plasmids ranging

from 50 to 60 kb.

VRE strains contain numerous plasmids belonging to different plasmid families. All studied isolates contained plasmids (1-10/cell) of variable size (2-300 kb) (Tables 5 and 6). Only two non-CC17 *vanA* strains (ST25 and ST5 from France and Portugal in 1986 and 2002) carried a single plasmid and only five CC17 outbreak isolates (1 ST16, 3 ST18, 1 ST202 from Denmark, The Netherlands, Poland and Serbia; 2000-2005) harbored more than 8 plasmids. CC5 or CC9 isolates did not contain > 4 plasmids (Figure 3).

A diversity of plasmids replicating by rolling-circle and theta (Inc18, RepA_N, pHTβ-like) mechanisms and containing relaxases belonging to $MOBP_7$ (pEF1, pHTβ, pLG1, pAD1), $MOBQ_3$ (Inc18) and $MOBV_1$ (RCR) families were detected among enterococci of different CCs (Figure 4). Plasmids with more than one replication or conjugation modules or lacking the rep and rel included in the plasmid typing schemes were frequently found (Tables 5 and 6). All these plasmid types were identified through the entire period of the study. Large plasmids >150 kb were absent in the first VRE strains from Europe and small theta-replicating plasmids related to pB82 were detected after 2005.

vanA-Tns were located on plasmids containing replication proteins of the families RepA_N (pRUM, 40-90 kb; pLG1-like, 150-240 kb), Inc18 (25-175 kb), and pHTβ-like (50-85 kb). The different plasmid types observed will be discussed separately.

(i) RCR plasmids (2-4 kb)

Plasmids of 2-4 kb were frequently detected among VRE (64%, 44/63 CC17 and 2/9 non-CC17) and VSE (29%, 20/53 CC17 and 4/29 non-CC17) from all continents from 1992 to date, CC17 being the predominant host background. They correspond to a family of RCR plasmids only detected in *E. faecium* to date which is represented by pRI1, pJS42, pKQ10 and pEFNP1 (21, 60) (GenBank acc. no. EU370688, AB038522).

RCR plasmids were divided in four groups according their Rep sequences: i) Rep identical to that of pEFNP1; ii) Rep identical to those of pJS42 and pRI1 (these plasmids only differ in an insertion sequence); and iii) Rep showing variable homology (72%-82%) with those of pEFNP1, pRI1, pJS42 and pKQ10 plasmids. Their relaxases exhibited 89%-99% homology with those of plasmids pRI1 and pJS42 (the sequences of relaxases from pKQ10, pEFNP1 are not available at GenBank databases), and 96-100% homology with sequences of different *E. faecium* genomes (GenBank acc. no. ZP_05674581, ZP_05830822, ZP_05671713, and EFF28814). Plasmids within this group were rep^+, rel^+ (n=43; 14 CC17-STs and 2 non-CC17 STs), rep^+, rel^- (n=4; 4 CC17-STs) and they were

co-transferred with genetic elements encoding vancomycin-resistance in 38% of the cases (18/47).

(ii) Small theta-replicating plasmids (5-7 kb, 8-18 kb)

Theta replicating plasmids of low molecular weight seem to be frequent among lactic bacteria, often associated with the production of bacteriocins.

Small plasmids (5-18kb) with sequences highly similar to replication regions of theta-replicating plasmids and relaxases of the $MOBP_7$ family were identified at variable occurrence. They often contain bacteriocins Bac32 or Bac43 (Tables 5 and 6).

Plasmids ranging from 5 to 7 kb with Rep proteins highly similar to pEF418 or pB82 (also designated as pDT1) were detected among VRE (11/72, 15% or 7/72, 10%) and VSE (22/82, 27% or 5/82, 10%), respectively, mostly associated with CC17 (Figure 4). Some pB82-like plasmids carried exclusively by VRE isolates also harboured genes coding for Bac43 similarly to plasmids found among clinical VRE from the USA (74).

Plasmids ranging from 8 to 18 kb contained replicases displaying 99% identity with that of pCIZ2 among VRE (37/72, 51%) and VSE isolates (45/82, 55%), and genes coding for Bac32 or EntQ. pCIZ2 is linked to EntQ and pTI1 is linked to Bac32 (12, 30).

Relaxases showed variable identity (92-98%) to the bacteriocin-encoded plasmids pCIZ2, p200B and pB82. These small plasmids were frequently mobilized by vancomycin-resistant genetic elements (14/47, 30%).

(iii) RepA_N plasmids

The RepA_N family was represented in the collections analyzed by derivatives of pRUM and pLG1 (43, 81). pRUM is a 25kb-mosaic plasmid initially identified in a VRE isolate from the USA in mid-90s which is characterized by the presence of the toxin-antitoxin system Axe-Txe and a truncated relaxase identical to that of pC223 of *Staphylococcus aureus,* a member of the $MOBP_7$ clade (GenBank accession no. AY355285) (22, 25).

A diversity of plasmids (20-260kb) containing rep_{pRUM} (with or without the toxin-antitoxin system) were detected among VRE (83%, 55/63 CC17 and 5/9 non-CC17) and VSE (66%; 38/53 CC17 and 16/29 non-CC17) isolates. Most of them did not contain any of the rel sequences included in the typing scheme used although the presence of rel_{pEF1} (n=18), and rel_{pEF1} plus sequences (rel_{Inc18}) highly homologous (>97%) to nickase proteins of pIP501 and pRE25 (n=1) were detected in some VRE isolates. A few VRE (3/72, 4%) and VSE (5/82, 6%) isolates contained rel_{pRUM} (Figure 4). All rep, rel and Axe-Txe sequences identified were identical with the exception of rep_{pRUM} from Portuguese VRE

isolates and an ASE Spanish isolate. pRUM-like plasmids from Portugal differed from others analyzed in this study in the presence of slightly different RepA (96% homologous to rep_{pRUM}) and the absence of the Axe-Txe system which might represent closely related members of this family related to pRUM.

Van transposons were located in pRUM derivatives ranging from 30 to 60 kb which contained only $rep_{pRUM,}$ (n=8) or rep_{pRUM} and Axe-Txe (n=17), and were frequently transferable (76%). Comparison of the *Cla*I digestion patterns of 20 pRUM::Tn*1546* plasmids revealed different RFLP types. The pRUM-like plasmid from Portugal is representative of a widespread *vanA*-plasmid persistently identified over the years in this country (data not shown).

Large plasmids (60-295 kb, mainly >150 kb) containing rep_{pLG1} were frequently detected among both VRE (71%, 44/63 CC17 and 7/9 non-CC17) and VSE (93%; 47/53 CC17 and 29/29 non-CC17) isolates, while rel_{pLG1} was only identified in one ASE Spanish isolate. VanA was occasionally located in large plasmids containing rep_{pLG1} plus rel_{pEF1} (Table 5). Rep_{pLG1} showed 100% homology with RepA-pLG1 (GenBank accession no. HM565183) in most cases, with others (n=3 VRE, 2 CC17 and one CC5) displaying variable homology (90-96%).

iv) Inc18-like (20-280 kb)

The Inc18 family comprises a wide diversity of broad-host range streptococcal plasmids (20-60kb) characterized by a replication regulated by an antisense RNA mediated transcriptional mechanism, different maintenance systems including a multimer resolution system (Seg A containing abg), a toxin-antitoxin and a partition systems (both part of the SegB region composed by two transcriptional units, δ-ω-ε-ζ), and a T4SL transfer machinery comprising a relaxase of the MOB_{Q3} subfamily (Table S1). Known plasmids carrying Tn*1546* from *E. faecium* (pIP186, pVEF1, pVEF2, pVEF3) from both human and animals are Inc18 derivatives possessing two functional replication proteins, an additional partition system (*prgO-prgN*) and deletions in several core regions, thus greatly complicating their classification (Table S1) (66-68).

VRE isolates recovered since 1986 ranging from 20 to 200 kb contained only rep_{pRE25} (49%, 32/63 CC17 and 3/9 non-CC17), only rep_{Inc18} (7%, 3/63 CC17 and 2/9 non-CC17), or both rep_{pRE25} and rep_{Inc18} (18%; 11/63 CC17 and 2/9 non-CC17). Genes coding for the expression of the postsegregational killing system ε-ζ, the partition system ω-δ, and the global regulator ω, were variably detected among these 72 VRE (18%, 13/63 CC17; 19%, 12/63 CC17 and 2/9 non-CC17; and 50%, 33/63 CC17 and 3/9 non-CC17, respectively). The rel_{Inc18} was only detected in one *vanA* plasmid from a ST16 *E. faecium* isolate

recovered in 1995 in the USA, which also contained rep_{pRE25} and ω-ε-ζ.

Van transposons were located in Inc18 derivatives ranging from 30 to 200 kb which contained rep_{pRE25} and/or rep_{Inc18} (n=22), and were frequently transferable (70%). In two cases, Inc18::Tn*1546* plasmids recovered from transconjugants and carrying both Inc18 rep genes showed larger size than those detected in wild type strains, illustrating reorganizations during the conjugation (Table 5).

VSE also contained Inc18-like plasmids (45-280 kb) with rep_{pRE25} (15%, 12/51 CC17), rep_{Inc18} (21%, 3/51 CC17 and 14/29 non-CC17), both rep_{pRE25}+rep_{Inc18} (5%, 1/51 CC17 and 3/29 non-CC17), rel_{Inc18} (1%, 1/29 non-CC17), ε-ζ (2%, 2/51 CC17); and ω (41%, 31/51 CC17 and 3/29 non-CC17).

Most rep_{Inc18} sequences were 100% identical to pIP816 (GenBank acc. no. AM932524) while the remaining showed variable identity (97%-98%) to different Inc18-like plasmids. The rep_{pRE25} showed 100% homology to that of pEF1 (GenBank acc. no. DQ198088) and the relaxase of Inc18 plasmids was 97%-99% homologous to the nickase of pI501 and pRE25 plasmids.

v) pHTβ-like (50-90kb)

Plasmids containing rep sequences identical to that of pHTβ and pMG1 were identified among VRE (35%; 22/63 CC17, 3/9 non-CC17) and VSE (5%; 3/53 CC17, 1/29 non-CC17) isolates from Europe, America and Saudi Arabia (1988-2008). They corresponded to a diversity of CC17 and non-CC17 clones. Most of the plasmids contain the three genes analyzed, although different combinations were observed in nine of them: $rep_{pHTβ}$+$rel_{pHTβ}$ (n=4); $rep_{pHTβ}$ (n=3); $rep_{pHTβ}$+*tra*A (n=1); and $rel_{pHTβ}$ (n=1). Different combinations with replicases (rep_{Inc18} or rep_{pRE25}) or relaxases (rel_{pEF1}) from other plasmids were observed among these plasmids (Table 5). Two strains from UK (ST18; 1992) and The Netherlands (ST16; 2002) harboured pHTβ-like plasmids encoding VanA type resistance, of ca. 50 kb and 85 kb respectively, only the second one being transferable.

All rep, rel and *tra*A sequences were 100% identical to that of pHTβ and pMG1.

(vi) Large plasmids (>150 kb)

Plasmids larger than 150 kb were identified in most VRE isolates (n=69/72; 96%) with the exception of two strains which corresponded to the first VRE isolates described in France in 1986 (ST25-CC9) and the UK in 1992 (ST18-CC17).

Most large plasmids were not associated with rep genes, although a number of them hybridized with probes specific for rep_{pLG1} and/or relaxases of pEF1 (sequences 99-100% identical at nucleotide level) or pAD1 (2 plasmids of ca. 150-160 kb, sequence identical at

nucleotide level). The exceptions corresponded to two isolates carrying VanA plasmids of ca. 175 kb (NLD) and 200 kb (USA) hybridizing to rel_{pEF1}/rep_{Inc18} and rel_{pEF1}/rep_{pRE25}, respectively (Table 5).

A number of isolates carried large plasmids which hybridized with *hyl* (30%) or *van* (13%) probes. Cotransfer of *hyl*-positive plasmids was achieved in 4 of 47 (8%) VRE isolates able to transfer vancomycin resistance. VanA large plasmids (145-300 kb) transferred by conjugation always contained rel_{pEF1}. The gene encoding the enterocin P was detected within large plasmids of ca. 150-200 kb which were co-transferred along with vancomycin resistance in 3/5 isolates.

Megaplasmids >150 kb were also abundand among VSE isolates (n=77/82; 94%).

vii) Untypeable plasmids

Plasmids of different sizes could not be identified with the schemes used. They included plasmids of **8-15 kb (**8 $rep^{(-)}/rel^{(-)}$ and 6 $rep^{(-)}/rel_{pCIZ2}^{(+)}$), **20-25 kb** (8 $rep^{(-)}/rel^{(-)}$), **30-100 kb** (25 $rep^{(-)}/rel^{(-)}$, 8 $rep^{(-)}/rel_{pEF1}$, 1 $rep^{(-)}/rel_{pHT\beta}$), and **>100 kb** (70 untypeable $rep^{(-)}/rel^{(-)}$ and 24 $rep^{(-)}/rel^{(+)}$ (22 rel_{pEF1} and 2 rel_{pAD1}) plasmids corresponding to 44 *vanA* and 19 *vanB* isolates. *vanA* was identified on $rep^{(-)}/rel_{pEF1}^{(+)}$ (n=8) or $rep^{(-)}/rel^{(-)}$ (n=2) plasmids of large size (145-300 kb) in 10/44 isolates.

Two major Inc18 and non-Inc18 clusters were observed among VRE plasmids. Comparison of *Cla*I restriction patterns of representative *vanA* plasmids from different countries, including prototypes pIP186 (Inc18) and pMG1 (pHTβ) allowed us to group plasmids within two major clusters (Figure 5).

Cluster I mostly includes plasmids containing rep_{pRUM} and Axe-Txe (1992-2005). They were mainly associated with the Tn*1546* variant “F” and showed variable size (30-90 kb) and rep content (derivatives of pRUM and/or pRE25 plasmids). All *vanA* plasmids from the USA clustered within this group.

Cluster II (n=8) comprised plasmids carrying rep_{Inc18} sequences (1986-2003), all but one also containing rep_{pRE25}, which were divided in two subclusters sharing >40% homology. Subcluster IIa (n=5) included four plasmids (30-40 kb) recovered from the first VRE detected in France and two of the first outbreak VRE strains from Brazil and Argentina (1986-1998) which exhibited >80% homology. Only the first VRE isolated in France in 1986, which corresponds to pIP816 (GenBank acc. no. NC_011140), lacked rep_{pRUM} and Axe-Txe, being an Inc18-type plasmid. The remaining ones are mosaics derivatives of pRUM and Inc18.Subcluster IIb (n=4) mostly includes mosaic plasmids carrying rep genes homologous to pHTβ and Inc18 plasmids (40-50 kb) as well as the

prototype pMG1 highly homologous to pHTβ. All plasmids contained IS*1216*-Tn*1546* variants.

The four plasmids containing *vanB* (50-60 kb) did not show significant similarity after digestion with restriction enzymes (data not shown).

***In silico* analysis of enterococcal plasmids.** An *in silico* comparative analysis of fully sequenced enterococcal plasmids available at GenBank database, either carrying Tn*1546* or not, shows the sharing of common genetic islands often flanked by specific insertion sequences among different plasmids (Figure 6). Plasmids lacking conjugation machineries might rely on a conduction phenomenon which is facilitated by co-integration with other transmissible plasmids (54). The notorious enrichment in insertion sequences can further promote IS-mediated arrangements between different plasmids.

CONCLUDING REMARKS

The results of this study reflect the penetration of vancomycin resistant transposons *vanA*-Tn*1546* and *vanB*-Tn*5382* in the contemporary human adapted *E. faecium* population (CC5, CC9, CC22, CC94 and CC17) and their further global expansion in hospitals driven by the CC17 polyclonal subcluster which comprises most of the isolates causing human infections and colonizing inpatients in hospitals around the world (20, 44, 79, 89). Within CC17, predominant clones ST16, ST17, ST18, or ST78 (which are considered the ancestral CC17 population) and others derived of them as ST192 or ST203 (derived from ST78), ST64 and ST132 (derived from ST18), seem to have driven the global expansion of glycopeptides resistance among enterococci (79). We observed particular and less disseminated STs only after the early 2000's, demonstrating that genetic diversification and recombination of CC17 strains might have occurred in recent years.

The similarity of most plasmid types (*plasmidome*) found among VRE and VSE strains reflect a common genetic background. Most VRE clinical isolates recovered from different continents in the last two decades belong to CC17 which generally harbour a plasmid pool of similar relaxases and rep initiator proteins to that of other human non-CC17 lineages. Differences in the plasmid content among isolates of different CCs are mostly due to the presence of broad host plasmids (e.g. Inc18) and the of traits carried by narrow host

plasmids (megaplasmids and small plasmids). Such differences reflect the wide possibilities of adaptation of these microorganisms to different environmental conditions.

Detailed analysis of representative VREfm isolates reflects a few acquisition events of Tn*1546* on a diversity of plasmids of narrow host range as RepA_N (pRUM and pLG1) and broad host range as Inc18, which are widely distributed among *Enterococcus faecium* population. The comprehensive analysis of *E. faecium* plasmids involved in vancomycin resistance showed a common conjugation system ($MOBP_7$ - pEF1), which is absent in *E. faecalis* species (22). With the exception of the Inc18 plasmid family, the plasmid types identified in the *E. faecium* isolates of this study have not been detected among *E. faecalis* clinical strains (see also results related to *E. faecalis* plasmidome in this thesis), neither were previously described in different genera or species, which strongly indicates their narrow-host range within *E. faecium* species. This apparent specificity might explain why this resistance is not widely disseminated in the two main enterococcal species.

The mosaicisms and the rearrangements observed in plasmids containing *vanA*-Tn*1546* or *vanB*-Tn*5382*, which were uncommonly observed among VSE strains, confirm the plasticity of *E. faecium* mobilome to adapt to different environments. Plasmid types carrying genes enhancing colonization (e.g. *hyl*-megaplasmids) might also have contributed to the success of CC17-VRE. The comparative *in silico* analysis of plasmid sequences available at Genbank databases with our results confirms the impact of LGT on the combinatorial evolution of MGE and lineages of enterococci.

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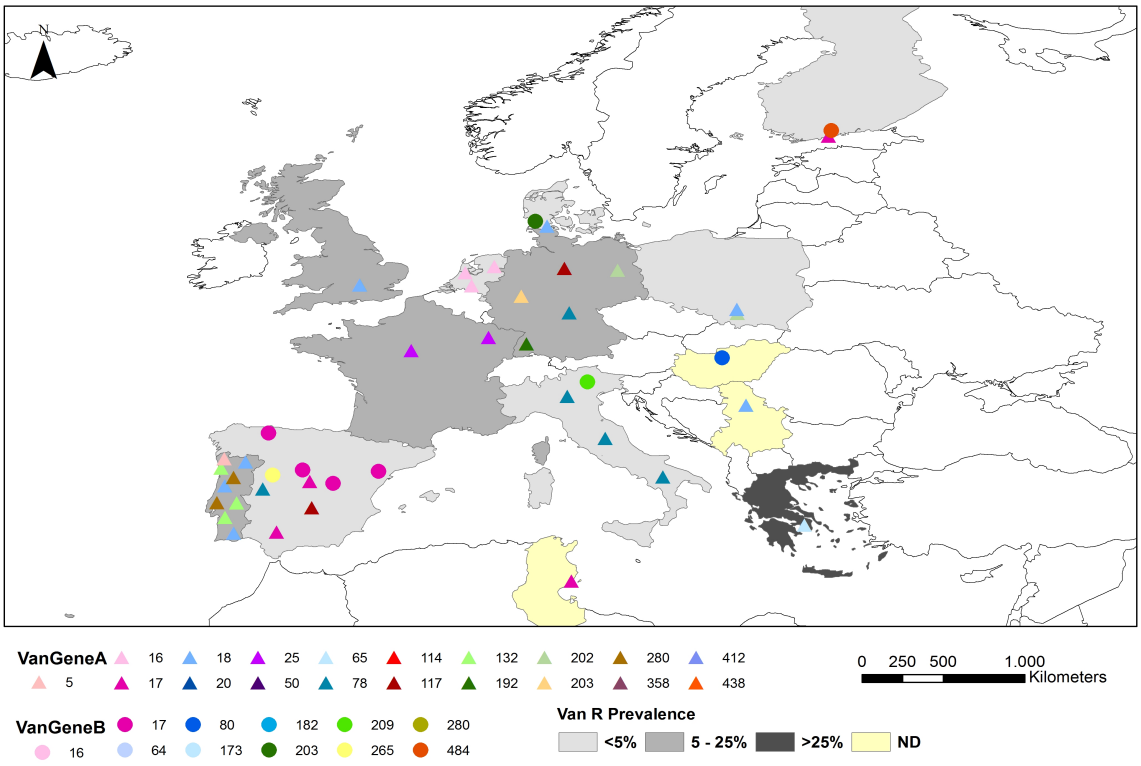


Figure 1. Geographic distribution of European *vanA* and *vanB Enterococcus faecium* isolates from this study (different STs are represented with different colours). Actual VRE rates are also shown accordingly to recent data from EARS-Net 2009 (www.rivm.nl.earss/).

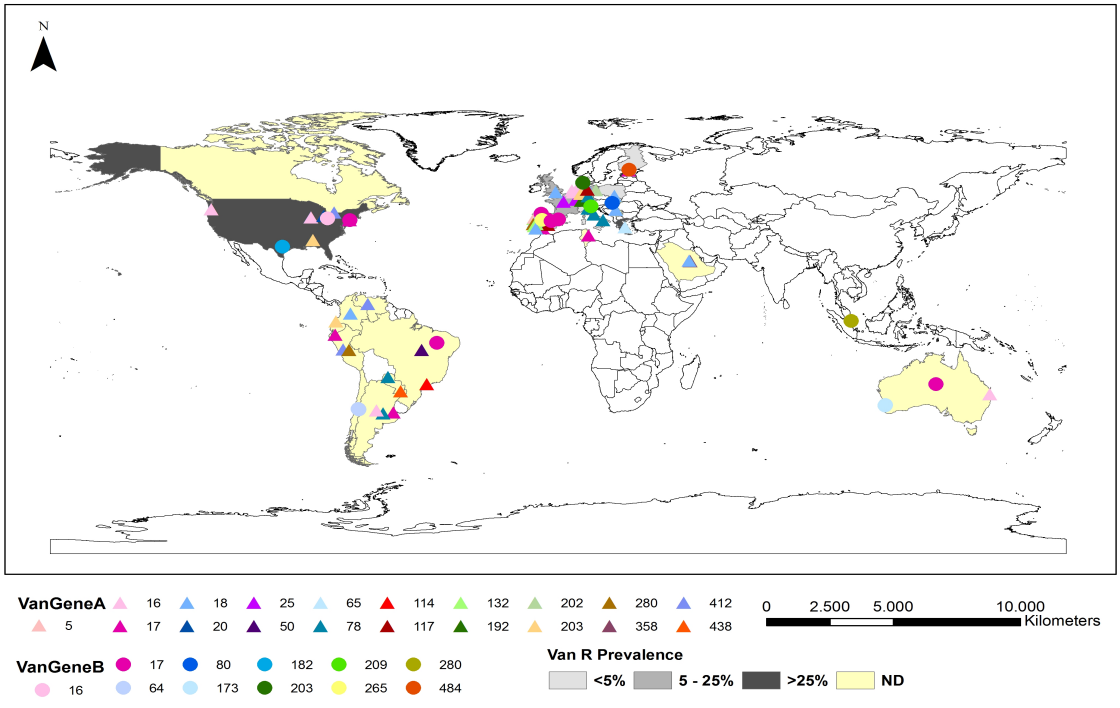


Figure 2. Geographic distribution of worldwide *vanA* and *vanB Enterococcus faecium* isolates included in this study (n=72; different STs are represented with different colours).

Table 1. Epidemiological background of VRE/VSE isolates included in this study.

MLST[a]			PFGE	N.º of	*van*	Country	City / Region	Date	Virulence / Bacteriocin profile								
CC	ST	Allelic profile	type	isolates	gene				*esp*	*acm*	*hyl*	*entA*	*entB*	*entP*	*entQ*	*bac32*	*bac43*
CC17	17	1-1-1-1-1-1-1	32	1	A	USA	New York	1991-92									
CC17	17	1-1-1-1-1-1-1	32	1	A	ARG	Buenos Aires	1997-98									
CC17	17	1-1-1-1-1-1-1	58	1	A	ECU	NI	NI									
CC17	17	1-1-1-1-1-1-1	55	1	A	FIN	Helsinki	1996-98									
CC17	17	1-1-1-1-1-1-1	18	1	A	ESP	Madrid	2003									
CC17	17	1-1-1-1-1-1-1	56*	1	A	ESP	Granada	2009									
CC17	17	1-1-1-1-1-1-1	52	1	A	SAR	Riyadh	2000-03									
CC17	17	1-1-1-1-1-1-1	53	1	A	TUN	Sfax	2003									
CC17	17	1-1-1-1-1-1-1	31	1	B	USA	New York	1991-92									
CC17	17	1-1-1-1-1-1-1	46	1	B	AUS	NI	1998									
CC17	17	1-1-1-1-1-1-1	19	1	B	ESP	Madrid	2004									
CC17	17	1-1-1-1-1-1-1	19	1	B	ESP	Madrid	2005									
CC17	17	1-1-1-1-1-1-1	45	1	B	ESP	Burgos	2006									
CC17	17	1-1-1-1-1-1-1	45	1	B	ESP	Soria	2004-2006									
CC17	17	1-1-1-1-1-1-1	34	1	B	BRA	NI	NI									
CC17	17	1-1-1-1-1-1-1	6	1	VSE	NOR	Bergen	1999									
CC17	17	1-1-1-1-1-1-1	21	1	VSE	DNK	Aarhus	1999-2000									
CC17	17	1-1-1-1-1-1-1	AREF-P	1	VSE	ESP	Madrid	1995									
CC17	17	1-1-1-1-1-1-1	AREF-B	1	VSE	ESP	Madrid	1996									
CC17	17	1-1-1-1-1-1-1	AREF-8	1	VSE	ESP	Madrid	2008									
CC17	16	1-2-1-1-1-1-1	26	1	A	USA	Chicago	1995									
CC17	16	1-2-1-1-1-1-1	41	1	A	AUS	Brisbane	1997-1999									
CC17	16	1-2-1-1-1-1-1	8	1	A	NLD	Amsterdam	1999									
CC17	16	1-2-1-1-1-1-1	9	1	A	NLD	Utrecht	2000									
CC17	16	1-2-1-1-1-1-1	9	1	A	USA	Oregon	2001									
CC17	16	1-2-1-1-1-1-1	9**	1	A	NLD	Amersfoort	2002									
CC17	16	1-2-1-1-1-1-1	AREF-T**	4	VSE	ESP	Madrid	2001-04		n=3							
CC17	16	1-2-1-1-1-1-1	38	1	A	ARG		2000									
CC17	16	1-2-1-1-1-1-1	27	1	B	USA	Cleveland	1996									
CC17	63	1-1-1-21-1-1-1	AREF-W.2	1	VSE	ESP	Madrid	2000									
CC17	103	1-14-1-21-1-1-1	AREF-W	1	VSE	ESP	Madrid	2001									
CC17	103	1-14-1-21-1-1-1	AREF-W.3	1	VSE	ESP	Madrid	2001									
CC17	182	1-1-1-1-1-19-1	29	1	B	USA	Texas	1992									
CC17	202	1-1-1-1-1-7-1	36	1	A	POL	Krakow	2005									
CC17	202	1-1-1-1-1-7-1	24	1	A	DEU	Berlin	2006									
CC17	209	37-1-1-1-1-1-1	15	1	B	ITA	Northern Italy	2002									
CC17	280	1-3-1-1-1-1-1	17	1	A	PRT	Porto, Viseu	2002-2003									
CC17	280	1-3-1-1-1-1-1	17	2	B	SIN	Singapore	2004		n=1							
CC17	280	1-3-1-1-1-1-1	55	1	A	PER	NI	NI									
CC17	64	7-1-1-1-1-1-1	39	1	B	CHI	Santiago	2003									
CC17	64	7-1-1-1-1-1-1	40	1	B	CHI	Santiago	2004									
CC17	117	9-1-1-1-1-1-1	5	1	A	DEU	Berlin	1998									
CC17	117	9-1-1-1-1-1-1	28	1	A	ESP	Madrid	2008									
CC17	78	15-1-1-1-1-1-1	37	1	A	ARG	NI	1998									
CC17	78	15-1-1-1-1-1-1	11	1	A	ITA	Several cities	2001-03									
CC17	78	15-1-1-1-1-1-1	11	1	A	DEU	Freiburg	2002									
CC17	78	15-1-1-1-1-1-1	11	1	A	ESP	Barcelona	2006									
CC17	78	15-1-1-1-1-1-1	51	1	A	PRG	Asunción	2005									
CC17	438	15-1-1-1-1-56-1	51	1	A	PRG	Asunción	2006									
CC17	192	15-1-1-1-1-7-1	16	1	A	DEU	Freiburg	2004									
CC17	203	15-1-1-1-1-20-1	30	1	A	USA	Alabama	2004-05									
CC17	203	15-1-1-1-1-20-1	23	1	A	DEU	Tübingen	2005									
CC17	203	15-1-1-1-1-20-1	57	1	A	ECU	NI	NI									
CC17	203	15-1-1-1-1-20-1	48	1	B	DNK	NI	2005-08									
CC17	203	15-1-1-1-1-20-1	AREF-9	1	VSE	ESP	Madrid	2006									
CC17	203	15-1-1-1-1-20-1	AREF-9.3	1	VSE	ESP	Madrid	2006									
CC17	203	15-1-1-1-1-20-1	AREF-9.1	1	VSE	ESP	Madrid	2007									
CC17	412	15-1-1-44-1-20-1	50	1	A	CAN	Ontario	2005									
CC17	412	15-1-1-44-1-20-1	50	1	A	VEN	NI	NI									
CC17	412	15-1-1-44-1-20-1	56	1	A	PER	NI	NI									
CC17	18	7-1-1-1-5-1-1	3	1	A	GBR	Oxford	1992									
CC17	18	7-1-1-1-5-1-1	ND	1	A	SAR	Riyadh	2000-2003									
CC17	18	7-1-1-1-5-1-1	22	1	A	SER	Belgrade	2005									
CC17	18	7-1-1-1-5-1-1	13	1	A	PRT	Porto, Coimbra	2002-2003									
CC17	18	7-1-1-1-5-1-1	14	1	A	PRT	Coimbra	NI									
CC17	18	7-1-1-1-5-1-1	49	1	A	DNK	NI	2005-08									
CC17	18	7-1-1-1-5-1-1	43	1	A	POL	Krakow	2005									
CC17	18	7-1-1-1-5-1-1	C	2	A	COL	NI	NI			n=1						
CC17	18	7-1-1-1-5-1-1	AREF-A	1	VSE	ESP	Madrid	1995									
CC17	18	7-1-1-1-5-1-1	AREF-C	1	VSE	ESP	Madrid	1996									
CC17	18	7-1-1-1-5-1-1	AREF-D	13	VSE	ESP	Madrid	1997-2005	n=8	n=11	n=1		7			1	

Table 1. *Continued.*

MLST[a]			PFGE	N.º of	*van*	Country	City / Region	Date	Virulence / Bacteriocin profile								
CC	ST	Allelic profile	type	isolates	gene				*esp*	*acm*	*hyl*	*entA*	*entB*	*entP*	*entQ*	*bac32*	*bac43*
CC17	18	7-1-1-1-5-1-1	AREF-D.1	1	VSE	ESP	Madrid	2002									
CC17	18	7-1-1-1-5-1-1	AREF-E	1	VSE	ESP	Madrid	1997									
CC17	18	7-1-1-1-5-1-1	AREF-G	1	VSE	ESP	Madrid	1998									
CC17	18	7-1-1-1-5-1-1	AREF-Z	1	VSE	ESP	Madrid	1998									
CC17	18	7-1-1-1-5-1-1	AREF-J	1	VSE	ESP	Madrid	1999									
CC17	18	7-1-1-1-5-1-1	AREF-N	2	VSE	ESP	Madrid	2000	n=1								
CC17	18	7-1-1-1-5-1-1	AREF-M	1	VSE	ESP	Madrid	2000									
CC17	18	7-1-1-1-5-1-1	AREF-Y	1	VSE	ESP	Madrid	2001									
CC17	18	7-1-1-1-5-1-1	AREF-U	1	VSE	ESP	Madrid	2002									
CC17	18	7-1-1-1-5-1-1	AREF-1	1	VSE	ESP	Madrid	2003									
CC17	18	7-1-1-1-5-1-1	AREF-2	2	VSE	ESP	Madrid	2004	n=1		n=1						
CC17	18	7-1-1-1-5-1-1	AREF-3	1	VSE	ESP	Madrid	2004									
CC17	18	7-1-1-1-5-1-1	AREF-4	1	VSE	ESP	Madrid	2006									
CC17	18	7-1-1-1-5-1-1	AREF-5	1	VSE	ESP	Madrid	2006									
CC17	18	7-1-1-1-5-1-1	AREF-6	1	VSE	ESP	Madrid	2007									
CC17	18	7-1-1-1-5-1-1	AREF-7	1	VSE	ESP	Madrid	2008									
CC17	18	7-1-1-1-5-1-1	ASEF-9	1	VSE	ESP	Madrid	1996									
CC17	18	7-1-1-1-5-1-1	ASEF-12	1	VSE	ESP	Madrid	1997									
CC17	18	7-1-1-1-5-1-1	ASEF-24	1	VSE	ESP	Madrid	2000									
CC17	125	7-3-1-1-5-1-1	AREF-Q	1	VSE	ESP	Madrid	2000									
CC17	132	7-1-1-1-12-1-1	10	1	A	PRT	Porto, Coimbra	1999-2001									
CC17	132	7-1-1-1-12-1-1	54	1	A	PRT	Porto	2002									
CC17	80	9-1-1-1-12-1-1	20	1	B	HUN	Budapest	2004									
CC17	173	7-1-1-123-12-1-1	42	1	B	AUS	Perth	2000									
CC17	173	7-1-1-123-12-1-1	42	1	B	AUS	Perth	2001									
CC17	484	55-2-1-1-1-1-1	47	1	B	FIN	Helsinki	1996-98									
CC1	4	8-4-1-3-1-1-5	ASEF-31	1	VSE	ESP	Madrid	2001									
CC9	25	9-3-1-6-1-1-1	1	1	A	FRA	NI	1986									
CC9	25	9-3-1-6-1-1-1	2	1	A	FRA	Nancy	1987									
CC9	50	15-1-6-6-1-1-1	35	1	A	BRA	NI	NI									
CC9	266	15-1-6-6-1-7-1	AREF-10	1	VSE	ESP	Madrid	2004									
CC9	358	9-2-1-32-1-1-1	44	1	A	SAR	Riyadh	2000-03									
CC9	29	4-5-1-3-1-1-1	ASEF-34	1	VSE	ESP	Madrid	2007									
CC5	5	5-2-1-9-1-1-5	12	1	A	PRT	Porto	2002									
CC94	40	10-8-8-8-6-10-6	ASEF-32	1	VSE	ESP	Madrid	2002									
CC94	69	13-8-8-8-6-27-6	ASEF-30	1	VSE	ESP	Madrid	2001									
CC94	69	13-8-8-8-6-27-6	ASEF-35	1	VSE	ESP	Madrid	2003									
CC94	69	13-8-8-8-6-27-6	ASEF-44	1	VSE	ESP	Madrid	2005									
CC94	85	13-8-15-8-11-10-6	ASEF-17	1	VSE	ESP	Madrid	1997									
CC94	178	9-2-1-11-1-14-6	ASEF-18	1	VSE	ESP	Madrid	1997									
CC94	178	9-2-1-11-1-14-6	ASEF-19	1	VSE	ESP	Madrid	1999									
CC22	22	2-3-1-2-1-1-1	ASEF-2	1	VSE	ESP	Madrid	1995									
CC22	22	2-3-1-2-1-1-1	ASEF-13	1	VSE	ESP	Madrid	1997									
CC22	22	2-3-1-2-1-1-1	ASEF-22	1	VSE	ESP	Madrid	1999									
CC22	214	2-3-2-2-1-1-1	ASEF-36	1	VSE	ESP	Madrid	2006									
CC22	214	2-3-2-2-1-1-1	ASEF-37	1	VSE	ESP	Madrid	2006									
CC22	32	3-3-1-2-1-1-1	ASEF-5	1	VSE	ESP	Madrid	1995									
CC22	71	15-3-1-2-1-1-1	ASEF-3	1	VSE	ESP	Madrid	1995									
CC22	420	5-3-38-2-1-1-1	ASEF-38	1	VSE	ESP	Madrid	2007									
ST	102	2-4-1-9-1-1-10	ASEF-20	1	VSE	ESP	Madrid	1999									
ST	102	2-4-1-9-1-1-10	ASEF-25	1	VSE	ESP	Madrid	2000									
ST	102	2-4-1-9-1-1-10	ASEF-29	1	VSE	ESP	Madrid	2001									
ST	102	2-4-1-9-1-1-10	AREF-S	1	VSE	ESP	Madrid	2001									
ST	20	5-1-1-1-8-6-1	25	1	A	USA	Detroit	1992-94									
CS	114	5-3-21-18-6-32-3	33	1	A	BRA	S. Paulo	1998									
CS	65	1-2-1-20-1-1-9	7	1	A	GRC	Athens	1999									
CS	265	9-4-1-6-21-20-5	4	1	B	ESP	Madrid	1998									
ST	419	47-1-32-1-14-1-18	AREF-11	1	VSE	ESP	Madrid	2005									

Abbreviations: CC, clonal complex; ST, sequence type; CS, singleton; VSE, vancomcyin-susceptible *Enterococcus faecium*; ARG, Argentina; AUS, Australia; BRA, Brazil; CHI, Chile; COL, Colombia; DEU, Germany; DNK, Denmark; ECU, Ecuador; ESP, Spain; FRA, France; GBR, Great Britain; GRC, Greece; HUN, Hungary; ITA, Italy; NLD, The Netherlands; NOR, Norway; PER, Peru; POL, Poland; PRT, Portugal; SER, Serbia; SIN, Singapore; TUN, Tunisia; USA, United States of America; VEN, Venezuela. [a]MLST designation was established following the standard guidelines (www.mlst.net).

Table 2*. Tn*1546* diversity of *vanA E. faecium* isolates.



IS	Tn*1546* type	Tn*1546* PCR mapping[a] p1p2 (22-1330)	p3p4 (1222-2353)	p5p6 (2227 3525)	p7p8 (2769-4042)	p9p10 (3569-4793)	p11p12 (4675-6353)	p13p14 (6229-8021)	p15p16 (6979 8920)	p17p18 (8889-10473)	p19p1 (10403-10830)	N.º of isolates	MLST	Country (ies)	Date	Location	*vanA* plasmid size in kb
	A[a]	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	6	CC9 (ST25, ST50), CC17 (ST78)	FRA, DEU, ESP, IT, BRA	1986-2006	P	25 (n=2); >200 (n=4)
	D[a]	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	5	CC5 (ST5), CC17 (ST16, ST192, ST203)	DEU, NLD, PT, AUS	1999-2005	P	30-60 (n=4), >150 (n=2)
	M[a]	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	1	CC17 (ST17)	FIN	1996-1998	P	300
	F[b]	(-)	(+)	(+)	(+)	(+)	(++)[h]	(+)	(+)	(+)	(+)	10	CC17 (ST16, ST17), ST20, ST65, ST114	ARG, GRC, BRA, ECU, USA	1992-2005	P	35-55 (n=9); 170-200 (n=3)
	F1	(-)	(-)	(+)	(+)	(+)	(++)[h]	(+)	(+)	(+)	(+)	2	CC17 (ST18, ST202)	POL	2005	P	60
	F2	(-)	(+)	(-)	(-)	(+)	(++)[h]	(+)	(+)	(+)	(+)	1	CC17 (ST78)	ARG	1998	P	50
IS*1251*	F3	(-)	(-)	(-)	(-)	(+)	(++)[h]	(+)	(+)	(+)	(+)	2	CC17 (ST18)	COL, SER	2005	P	30-40
	F4	(-)	(+)	(+)	(+)	(+)	(-)[h]	(+)	(+)	(+)	(+)	4	CC17 (ST17, ST78, ST412, ST438)	CAN, BRA, PAR	2005-2006	P	50-55 (n=3); >200 (n=1)
	F5	(-)	(-)	(-)	(-)	(-)	(-)[h]	(+)	(+)	(+)	(+)	1	CC17 (ST18)	DEN	2005-2008	P	60
	F6	(+)	(+)	(-)	(+)	(+)	(++)[h]	(+)	(+)	(+)	(+)	1	CC17 (ST280)	PER	2006-2008	P	30
	NEW	(+)	(+)	(+)	(+)	(+)	(++)[h]	(+)	(++)[j]	(+)	(+)	1	CC17 (ST412)	VEN	2006-2008	P	30
	NEW	(-)	(+)	(+)	(+)	(+)	(++)[h]	(+)	(++)[j]	(+)	(+)	1	CC17 (ST412)	PER	2006-2008	P	30
	C[c]	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(++)[i]	(+)	1	CC17 (ST117)	DEU	1998	C	Not applicable
IS*Ef1*	PP-4[d]	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(++)[r]	(+)	2	CC17 (ST18, ST117)	ESP, PRT	2002-2008	P	90
	PP-5[d]	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(++)[i]	(-)	2	CC17 (ST18, ST280)	PRT	2003	P	85 (n=2)
	PP-13[d]	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(-)[j]	(-)	(+)	1	CC17 (ST132)	PRT	2001	P	50
	PP-23[d]	(-)	(-)	(+)	(-)	(-)	(+)	(+)	(-)[j]	(-)	(+)	1	CC17 (ST132)	PRT	2002	P	50
IS*1216*	G[a]	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(-)[j]	(+)	(+)	1	CC17 (ST18)	GRB	1992	P	50
	X[a]	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(-)[j]	(-)	(+)	2	CC17 (ST16)	NLD	2000-2002	P	30 (n=1); 85 (n=1)
	B[f]	(+)	(+)	(+)	(+)	(+)	(-)[j]	(+)	(-)[j]	(+)	(+)	1	CC17 (ST17)	SAR	2000-03	P	55
	J*[f]	(-)	(+)	(+)	(+)	(+)	(-)[j]	(+)	(+)	(+)	(+)	2	CC17 (ST18)	COL, SAR	2000-03	P	45
	PP-16[d]	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)[j]	(+)	(+)	2	CC17 (ST17, ST202)	ESP, DEU	2006-2009	P	30 (n=2)
	T[a]	(-)	(-)	(-)	(-)	(++)[j]	(+)	(+)	(++)[j]	(+)	(+)	1	CC17 (ST17)	TUN	2003	P	145
	EF-*1546*-5[e]	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(++)[j]	(+)	(+)	1	CC17 (ST17)	ESP	2003	P	40
IS*1485*	H*[f]	(++)[k]	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	1	CC9 (ST358)	SAR	2000-03	P	50

Table 2 Footnote.

*IS, insertion sequence; MLST, multilocus sequence typing; CC, clonal complex; ST, sequence type; ARG, Argentina; AUS, Australia; BRA, Brazil; COL, Colombia; DEU, Germany; ECU, Ecuador; ESP, Spain; FIN, Finland; FRA, France; GBR, Great Britain; GRC, Greece; ITA, Italy; NLD, The Netherlands; PER, Peru; POL, Poland; PRT, Portugal; SER, Serbia; TUN, Tunisia; USA, United States of America; VEN, Venezuela; P, plasmid; C, chromosome. (+) amplification; (-) no amplification; (++) amplification of sequences larger than those of the expected sizes.

[a]Tn*1546* types A, D, G, M, T and X were designated as previously described by Woodford *et al.* (90).

[b]Tn*1546* variants similar to "type F" were previously described as F1/F2 types (87). In this study we identified six subvariants of "type F" designated as F1, F2, F3, F4, F5 and F6.

[c]Tn*1546* type C was previously described by Werner *et al.* (84).

[d]Tn*1546* types "PP-4", "PP-5", "PP-13" and "PP-16" were classified as described (55), being types "PP-4" and "PP-5" widely disseminated in Portuguese hospitals. PP-16 has been also designated as "type A3" (84).

[e]For those that did not have a specific previously described type, we used our own designation (EF-1546-X).

[f]Tn*1546* types B, H* and J* were recently described by Khan *et al*. (41).

[g]Tn*1546* PCR mapping is based on the results obtained by a PCR overlapping assay described by Woodford *et al.* (90).

[h]Sequencing of the amplicon or positive amplification using IS*1251*-F and p11 in the case of the absence of amplicon showed IS*1251*in *vanS-vanH* region as described (87).

[i]Positive amplification using IS*Ef1*-F and p18 as described (55).

[i*]In adittion to IS*Ef1*, the Spanish isolate also contained a IS*1216* within *vanY-vanZ* region.

[j]Sequencing of the amplicon or positive amplifciation using IS*1216*-R and p15 in the case of absence of amplicon showed IS*1216* in *vanX-vanY* region.

[k]Presence of IS*1485* as previously described in Khan *et al.* (41).

Table 3*. Diversity of *vanB* transposons among *E. faecium* isolates.

		Transposon PCR mapping[a]									
Transposon	***vanB* allele**	***van*RSYWHBX RFLP**	***vanS*$_B$-*vanY*$_B$**	**Tn*5382*-like**	***pbp5*-Tn*5382***	**ORF28-Tn*1549***	**N.º of isolates**	**MLST**	**Country**	**Date**	**Location**
Tn*1547*	*vanB1*	RFLP-1	*vanB1*	-	-	-	2	CC17 (ST17, ST182)	USA	1992	C
Tn*1549*/Tn*5382*	*vanB2*	RFLP-2	*vanB2*	-	-	+	1	ST265	ESP	1998	P
	vanB2	RFLP-2	*vanB2*	+	-	+	3	CC17 (ST17, ST280)	ESP, SIN	2004-05	P
	vanB2	RFLP-2	*vanB2*	+	-	+	6	CC17 (ST17, ST80, ST203, ST280)	ESP, HUN, DEN, AUS, SIN	1998-2008	C
	vanB2	RFLP-2	*vanB2*	++[b]	-	+	3	CC17 (ST64)	CHI	2003-04	C
	vanB2	RFLP-2*	*vanB2**[c]	+	+	+	4	CC17 (ST16, ST173, ST484)	FIN, USA, AUS	1996-2001	C
Tn*1549*/Tn*5382*	*vanB1*	RFLP-3	*vanB1*[d]	+	-	+	1	CC17 (ST209)	ITA	2002	C

*MLST, multilocus sequence typing; CC, clonal complex; ST, sequence type; AUS, Australia; CHI, Chile; ESP, Spain; FIN, Finland; HUN, Hungary; ITA, Italy; SIN, Singapore; USA, United States of America; P, plasmid; C, chromosome.

[a]+, amplification; -, no amplification; ++, amplification of sequences larger than those of the expected size.

[b]Sequencing of the fragment revealed the presence of IS*Enfa110*.

[c]Sequencing of the fragment revealed the presence of IS*Enfa200*.

[d]Sequencing of *vanS*$_B$-*vanY*$_B$ revealed a new *vanB1* type.

Table 4. Diversity of plasmid modules among VRE (n=52 VanA and 20 VanB) and VSE isolates (n=82).

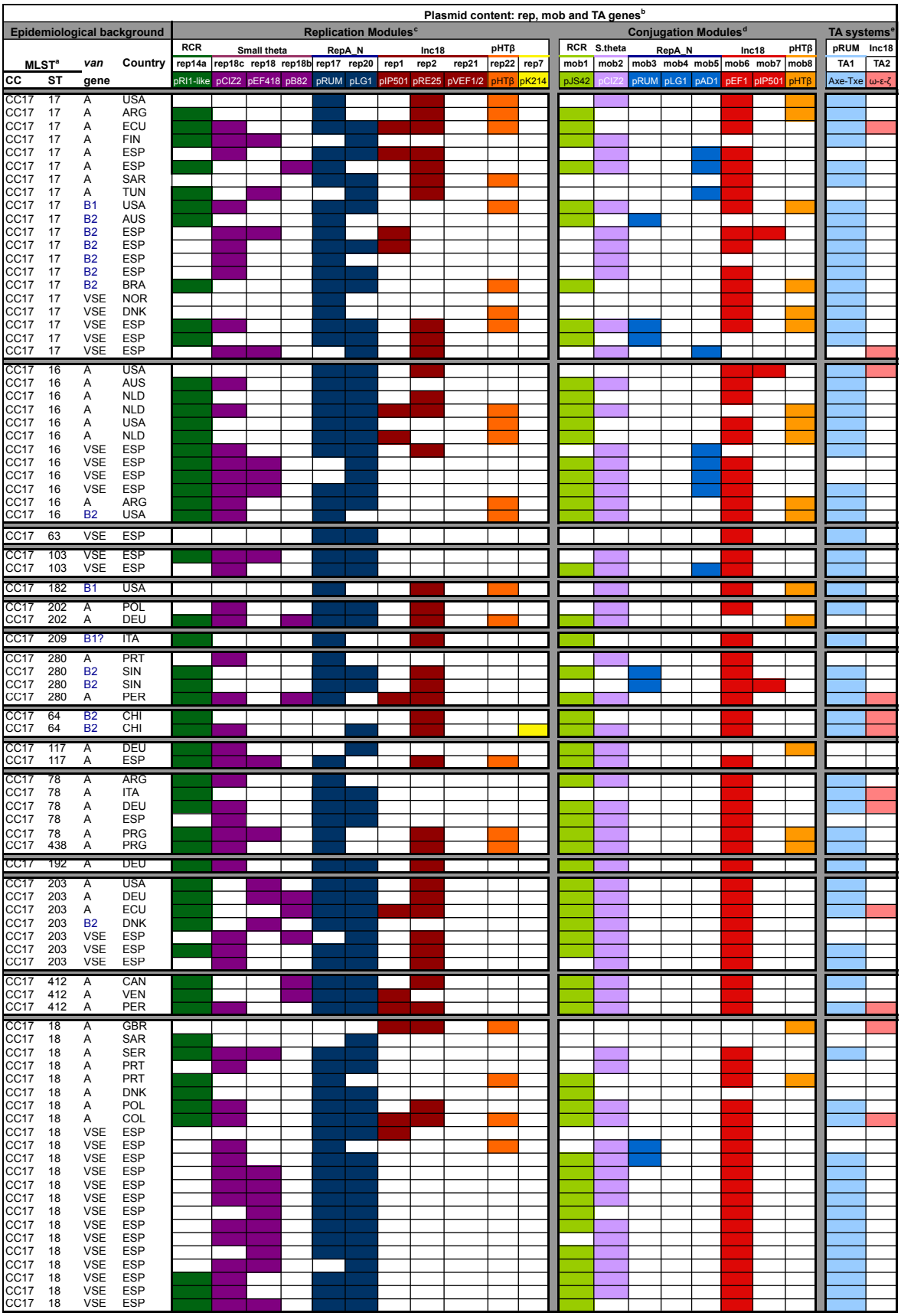
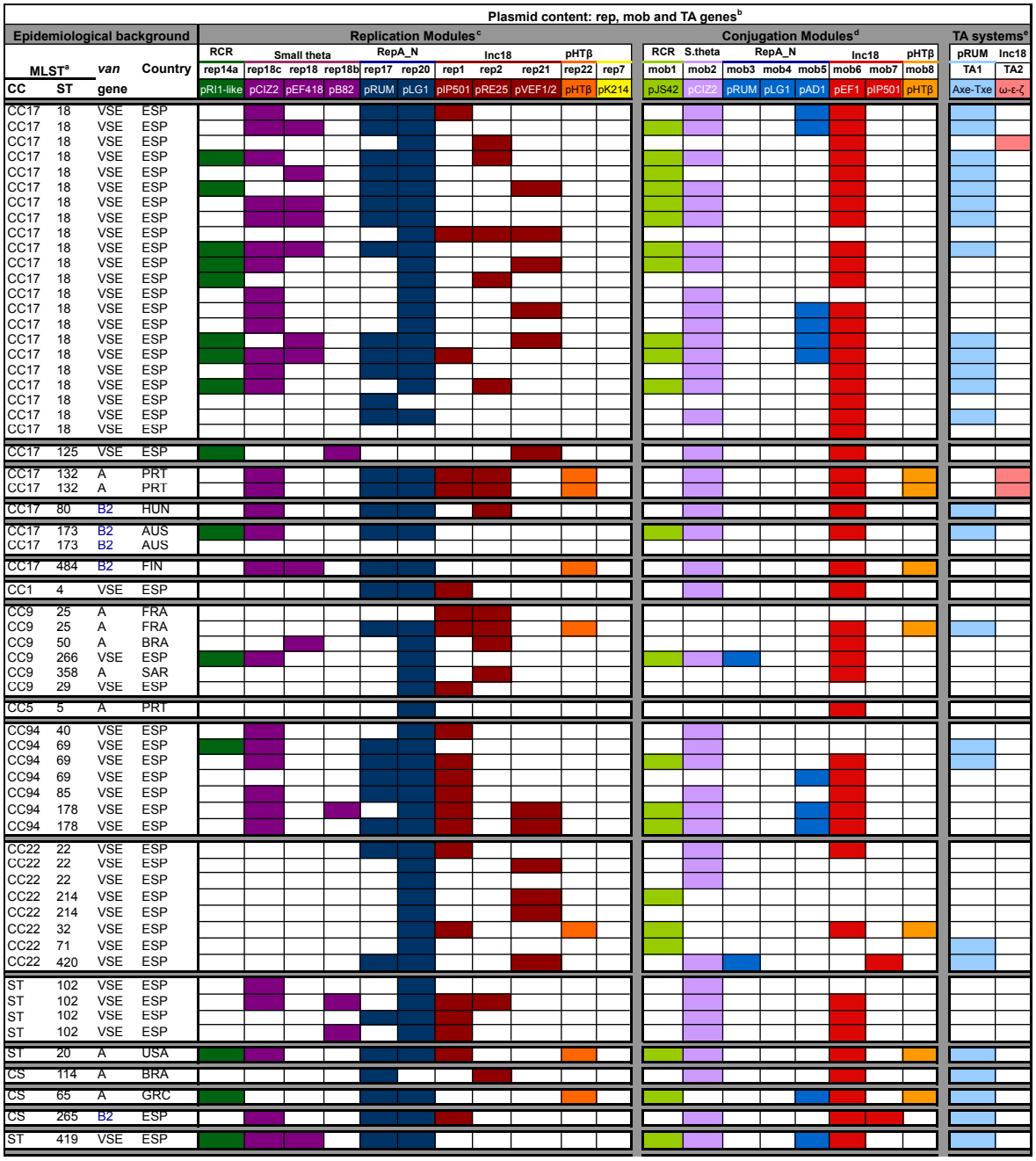


Table 4. Diversity of plasmids among VRE and VSE isolates (*continued*).



Abbreviations: VRE, vancomycin-resistant *Enterococcus faecium*; VSE, vancomycin-susceptible *Enterococcus faecium*; rep (replicases); mob (mobilization/relaxases); TA (toxin-antitoxin). [a]MLST results according to www.mlst.net.

[b]Plasmid genes (*rep, mob, TA*) previously associated with a given plasmid family are represented with a colour; rep, mob and TA genes belonging to the same plasmid family are represented with the same range of colours: *rep* genes are in darker shades, *mob* genes are in weak shade, and TA genes are in the lighter shades as the following: dark and weak green (RCR, Rolling-circle replicating plasmids), dark and weak violet (S.theta, Small theta replicating plasmids); dark, weak and light blue (RepA_N including pRUM, pLG1 and pheromone-responsive (pAD1) plasmids); dark, weak and light red (Inc18), dark and weak orange (pHTβ/pMG1 plasmids) and yellow (pK214 from *Lactococcus lactis*).

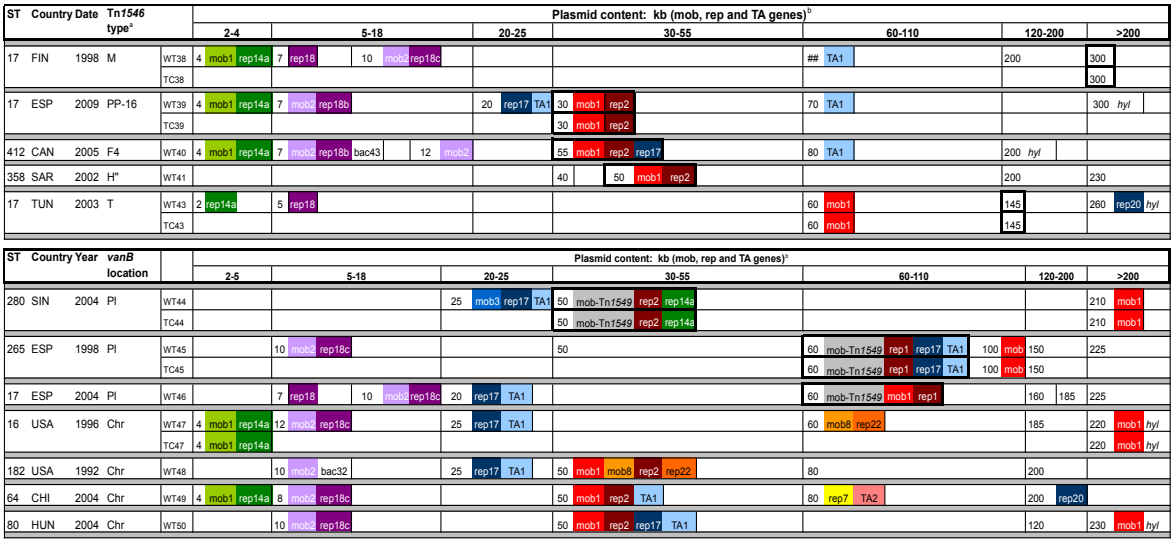
[c]Rep families are named RepX where X indicates different rep-families according to Jensen *et al*. (32) – within each family the sequencing analysis revealed homology with the following plasmids: rep1$_{pIP501}$, rep from Inc18 plasmids as pIP501, pIP816 and pRE25; rep2$_{pRE25}$, rep from pRE25 (pRE25 contains rep1 and rep2) and pEF1; rep7$_{pUSA02}$, rep from pK214; rep14a$_{pRI1}$, rep from RCR plasmids pEFNP1, pJS42 and/or pRI1; rep17$_{pRUM}$, rep from pRUM; rep18$_{pEF418}$, rep from pEF418; rep18b, rep from pB82, rep18c, rep from pCIZ2; rep20, rep from pLG1; rep21, rep from pVEF1, pVEF2, pVEF3 and pVEF4 plasmids; and rep22, rep from pHTβ and pMG1 plasmids. Rep14a, rep 18b, rep18c, rep21 and rep22 are not included in Jensen's scheme (or the primers are different) and the numbers were arbitrarily attributed following that numeration.

[d]Mob genes were arbitrarily designated from mob1 to mob8 with correspondence with different plasmid types: mob1, pJS42; mob2, mob from p200B, pCIZ2 and/or pB82 plasmids; mob3, pRUM; mob4, pLG1; mob5, mob from pAD1 and the pathogenicity island of V583; mob6, pEF1; mob7, mob from pIP501 and pRE25; mob8, mob from pHTβ and pMG1 plasmids. [e]Toxin-antitoxin system from pRUM (Axe-Txe) and Inc18 (ω-ε-ζ) plasmids were arbitrarily designated as TA1 and TA2, respectively.

Table 5. Diversity of plasmids among representative *vanA* and *vanB E. faecium* isolates.

ST	Country	Date	Tn*1546* type[a]		Plasmid content: kb (mob, rep and TA genes)[b]						
					2-4	5-18	20-25	30-55	60-110	120-200	>200
25	FRA	1988	A	WT1				40 mob1 rep1 rep2 rep17 TA1	60 mob1 mob8 rep22	135	
				TC1				40 mob1 rep1 rep2 rep17 TA1	60 mob1		
114	BRA	1998	F	WT2		15 mobZ bac32		40 rep1 rep17 TA1	80 mob1 rep2		230
				TC2		15 mobZ bac32		40 mob1 rep1 rep2 rep17 TA1	80 mob1		230
17	ARG	1998	F	WT3	4 mob1 rep14a	15		40 rep1 rep17 TA1	90 mob8 rep2 rep22	200 mob1	
				TC3		15		40 mob1 rep1 rep2 rep17 TA1 50 mob1			
17	ESP	2003	EF-1546-5	WT4		10 mobZ rep18c		40 mob1 rep1 rep2	60 rep17	160 mob5	250 280
				TC4				40 mob1 rep1 rep2		160 mob5	280
16	USA	1995	F	WT5			20	40 mob1 mob7 rep2 rep17 TA1 TA2		150 200	
				TC5				40 mob1 rep2 rep17 TA1		200	
132	PRT	2001	PP-13	WT6		12 mobZ rep18c		30 rep1 rep2 TA2 50 mob1 rep2 rep17 TA2	60 mob8 rep22	170	300
				TC6					110 mob8 rep1 rep2 rep22 TA2	170	
132	PRT	2002	PP-23	WT7		10 mobZ rep18c		50 mob1 rep1 rep2 rep17 TA2	60 mob8 rep22	200 rep20	
				TC7				50 mob1 rep1 rep2 rep17 TA2			
202	POL	2005	F1	WT8		12 mobZ rep18c bac32	25 TA1	50 mob1	60 mob1 rep2 rep17 TA1 80	120 180	230 250
				TC8		12 mobZ rep18c bac32		50 mob1	60 mob1 rep2 TA1	120 180	230 250
18	POL	2005	F1	WT9	4 mob1 rep14a	12 mobZ rep18c bac32	25 rep17 TA1	50 mob1	60 rep2 rep17 TA1 80	150 180	220 260 *hyl*
				TC9	4 mob1 rep14a			50 mob1	60 rep2 rep17 TA1		220
280	PRT	2003	PP-5	WT10		12 mobZ rep18c		50	85 mob1 rep17		300 *hyl*
				TC10					85 mob1 rep17		
18	PRT	2002	PP-4	WT11		12 mobZ rep18c		40	90 mob1 rep17	140	230
				TC11					90 mob1 rep17		
18	PRT	NI	PP-5	WT12	4 mob1 rep14a			50 mob1 rep17	60 mob8 rep22 80 mob1 rep17		245
				TC12	4 mob1 rep14a				90 mob1 rep17		
17	USA	1992	F	WT13		15 mobZ bac32		45 mob1 rep17 TA1	80 mob8 rep2 rep22	200	240
				TC13				45 mob1 TA1		200	240
17	SAR	2000	F4	WT14	4 mob1 rep14a	15 mobZ bac32	20 TA1	45 mob1 rep2 rep17 TA1	60 rep22		240 *hyl*
				TC14	4 mob1 rep14a			45 mob1 rep2 rep17 TA1			
78	PRG	2005	F4	WT15	4 mob1 rep14a	12 mobZ rep18c 15 rep18	20 rep2	50 mob1 rep17 TA1	70 mob8 rep22		240
				TC15	4 mob1 rep14a	12 mobZ rep18c 15 rep18		50 mob1 rep17 TA1			
202	DEU	2006	PP-16	WT16	4 mob1 rep14a	7 mobZ rep18b bac43 10 mobZ rep18c	20	30 rep2 rep17	60 mob8 rep22	120 190	220
5	PRT	2002	D	WT17						150 mob1 rep20	
				TC17						150 mob1 rep20	
16	ARG	2000	F	WT18	4 mob1 rep14a	15 mobZ rep18c	25 rep17 TA1		60 mob8 rep22	170 mob1	250
16	NLD	1999	D	WT19	2 mob1 rep14a		25 rep17 TA1			175 mob1 rep2	
				TC19						175 mob1 rep2	
20	USA	1992	F	WT20	4 mob1 rep14a	7 10 mobZ rep18c	22 rep17 TA1		60 mob8 rep22	200 mob1 rep1 rep20	
				TC20	4 mob1 rep14a	10 mobZ rep18c				200 mob1 rep1 rep20	
78	DEU	2002	A	WT21	2 mob1 rep14a	12 mobZ rep18c	25 rep17 TA1	50 TA2		170	240 mob1 rep20
				TC21							240 mob1 rep20
78	ITA	2002	A	WT22	2 rep14a			50 mob1 rep17 TA1 TA2			240 mob1 rep20
				TC22				50 mob1 rep17 TA1 TA2			240 mob1 rep20
17	BRA	NI	F4	WT23	4 mob1 rep14a	15 bac32		40 rep17 TA1	85 mob8 rep22		230 mob1 rep20
				TC23	4 mob1 rep14a				85 rep22		230 mob1 rep20
50	BRA	NI	A	WT24		5 rep18	25 rep2				200 mob1
				TC24		5 rep18	25 rep2				200 mob1
16	NLD	2002	X	WT25	4 mob1 rep14a		20 rep17 TA1	45	60 mob1 85 mob8 rep1 rep22	135 170 *hyl*	
				TC25					85 mob8 rep1 rep22		
18	GRB	1992	G	WT26			25	50 mob8 rep1 rep2 rep22 TA2			
				TC26			25	50 rep1 rep2 TA2			
16	USA	2001	F	WT27	4 mob1 rep14a		25	40 rep17 TA1	60 mob8 rep22		220 mob1 *hyl*
				TC27	4 mob1 rep14a			40 rep17 TA1			
18	SER	2005	F3	WT28	4 rep14a	7 rep18 18 mobZ rep18c bac32	25	40 rep17 TA1	60 mob1 rep17 TA1 92	150 200	280 *hyl*
				TC28	4 rep14a	18 mobZ bac32	25	40 rep17 TA1		150 200	
203	USA	2005	F	WT29	4 mob1 rep14a	6 rep18 12 mobZ	25 rep2	40 rep17 TA1	60 mob1 rep17	180	
				TC29	4 mob1	6 rep18 12 mobZ		40 rep17 TA1	60 mob1 rep17		
65	GRC	1999	F	WT30	4 mob1 rep14a			30 rep17 TA1	60 mob8 rep22	150 mob5	230 mob1 *hyl*
				TC30				30 rep17 TA1		150 mob5	
78	ARG	1998	F	WT31	4 mob1 rep14a	10 mobZ rep18c		55 rep17 TA1	75 mob1		250
				TC31		10 mobZ rep18c		55 rep17 TA1	75 mob1		
16	AUS	1999	D	WT32	4 mob1 rep14a	10 mobZ rep18c			60 rep17 TA1		220 mob1 *hyl*
				TC32	4 mob1 rep14a	10 mobZ rep18c			60 rep17 TA1		220 mob1 *hyl*
192	DEU	2004	D	WT33	4 mob1 rep14a	10 mobZ rep18c		50 rep2	60 mob1 rep17 TA1		200 mob1 *hyl*
				TC33	4 mob1 rep14a	10 mobZ rep18c		50 rep2	60 mob1 rep17 TA1		200 mob1 *hyl*
16	NLD	2000	X	WT34	4 mob1 rep14a	7 10 mobZ rep18c	25 rep17 TA1	30 rep1 rep2	60 mob8 rep22	150 mob1 entP	250
				TC34	4 mob1 rep14a			50 rep1 rep2	60 mob8 rep22		
25	FRA	1986	A	WT35				30 rep1 rep2			
203	DEU	2005	D	WT36	4 mob1 rep14a	7 mobZ rep18b bac43 15 rep18		40 rep2	60 mob1 rep17 TA1	185	225
117	DEU	1998	C	WT37	4 mob1 rep14a	8 mobZ rep18c		30	60 mob8	200	

Table 5. *Continued.*



Abbreviations: ST (sequence type); ARG, Argentina; AUS, Australia; BRA, Brazil; CHI, Chile; COL, Colombia; DEU, Germany; DNK, Denmark; ECU, Ecuador; ESP, Spain; FIN, Finland; FRA, France; GBR, Great Britain; GRC, Greece; HUN, Hungary; ITA, Italy; NLD, The Netherlands; NOR, Norway; PER, Peru; POL, Poland; PRT, Portugal; SAR, South Arabia; SER, Serbia; SIN, Singapore; TUN, Tunisia; USA, United States of America; VEN, Venezuela; rep (replicases); mob (mobilization/relaxases); TA (toxin-antitoxin).

[a]Tn*1546* designation is based on the results obtained by a PCR overlapping assay described by Woodford *et al.* (90).

[b]Total plasmid content per each donor/transconjugant pair is represented in different categories/ranges according to the approximately size in Kb. Plasmid genes (*rep, mob, TA*) and sizes previously associated with a given plasmid family or published plasmid are represented with a colour; rep, mob and TA genes belonging to the same plasmid family are represented with the same range of colours following the same criteria explained in Table 4; for each plasmid mob (1-5), rep (1-22) and TA (1-2) genes searched are indicated with numbers as in Table 4; genes hybridizing in the same band as *vanA* or *vanB* plasmids appear in bold rectangules.

Table 6*. Diversity of plasmids among representative VSE isolates from Spain.

CC	ST	Date	Type	Plasmid content: kb (mob, rep and TA genes)*						
				2-4	5-18	20-25	30-55	60-110	120-200	>200
CC17	18	1995	ARE							230 mob1 rep1 rep20
CC17	18	1996	ARE				50 rep22 *ermB*			
CC17	18	1997	ARE				50 mob3 rep17 *ermB*	75 mob1 rep17		
CC17	18	1997	ARE		10 mobZ rep18c	25 rep17 TA1		75 mob1		
CC17	18	1998	ARE				30 rep17 50 mob1	75 mob1	170 rep20	
CC17	18	2000	ARE		10 mobZ rep18c	20 rep17 TA1			170 mob1	
CC17	18	2002	ARE		10 mobZ rep18c 15 rep17					280 mob1 mob5 rep20 *hyl*
CC17	18	1997	ARE				40 rep2			280 mob1 *hyl*
CC17	18	1998	ARE	4 rep14a	10 mobZ rep18c	20 rep17 TA1	45 rep2	75 mob1 rep14a		
CC17	18	2000	ARE				45 rep1 rep2		190 rep20	
CC17	18	2001	ARE		10 mobZ rep18c	20 rep17		75 mob1		285 mob1 rep20 *hyl*
CC17	18	2004	ARE							
CC17	18	2004	ARE		10 mobZ rep18c					310 *hyl*
CC17	18	2004	ARE		12 mobZ rep18c					300 mob1 mob5 *hyl*
CC17	18	2006	ARE		10 mobZ 15 rep17					280 mob5 rep20 *hyl*
CC17	18	2007	ARE			25 mobZ rep18c rep17 TA1		75 mob1		270 mob1 *hyl*
CC17	18	1996	ASE						190 mob1 rep20	
CC17	18	1997	ASE				50 rep17 TA1		190 mob1 rep20 rep17	
CC17	17	1995	ARE					60 mob3 rep17 rep2 *ermB*	190 mob1 rep20	
CC17	63	2000	ARE		18 rep17 TA1					
CC17	103	2001	ARE		10 mobZ rep18c			75 mob1		
CC17	103	2001	ARE		10 mobZ rep18c		30 rep17	75 mob1		280 mob1 mob5 rep20 *hyl*
CC17	16	2001	ARE					80 rep2		275 mob5 *hyl*
CC17	16	2004	ARE				55 mob1 rep17	70 mob1		295 mob1 rep20
CC17	203	2006	ARE		10 mobZ rep18c			70 mob1		375 *hyl*
CC17	203	2007	ARE						160 rep20	
CC17	442	2005	ASE				50 rep17	60 rep2 100 rep20		
CC1	4	2001	ASE							250 mob1 rep17 *ermB*
CC9	29	2007	ASE						170 rep20	
CC94	69	2001	ASE							
CC94	69	2003	ASE		10 mobZ rep18c		30 rep17 55 rep17 TA1			300 rep17 *ermB*
CC94	69	2005	ASE						200 mob1 rep1 rep17	
CC94	85	1997	ASE		7 mobZ rep18c 15 mobZ rep18c				150 rep20	260 mob1 rep1 rep17
CC94	178	1997	ASE						200 mob1 rep1 rep20	
CC94	178	1999	ASE		7 mobZ rep18c					280 mob1 rep1 rep20
CC22	22	1995	ASE							
CC22	22	1997	ASE							
CC22	71	1995	ASE							
CC22	214	2006	ASE							270 mob1 rep1
CC22	214	2006	ASE					100 TA1		
S	102	1999	ASE		7 mobZ rep18c 15 mobZ rep18c		30 mob1 rep2			280 mob1 rep1 rep20
S	102	2000	ASE							
S	102	2001	ASE							
S	474	2004	ASE							250 mob1 rep1 rep17 rep20
	New	2000	ASE				50 rep2 rep17 rep14a		200 rep20	
	New	2007	ASE				60 rep2 rep17	100 rep20		
	New	1997	ASE							240 mob1 rep17
	New	2001	ASE							240 mob4 rep1 rep17 rep20

*CC, clonal complex; ST (sequence type); ARE, ampicillin-resistant *E. faecium*; ASE, ampicillin-susceptible *E. faecium*.
[a]Total plasmid content per each isolate is represented in different categories/ranges according to the approximately size in Kb. Plasmid genes (*rep, mob, TA*) and sizes previously associated with a given plasmid family or published plasmid are represented with a colour; rep, mob and TA genes belonging to the same plasmid family are represented with the same range of colours following the same criteria explained in Table 4; for each plasmid mob (1-5), rep (1-22) and TA (1) genes searched are indicated with numbers as in Table 4.

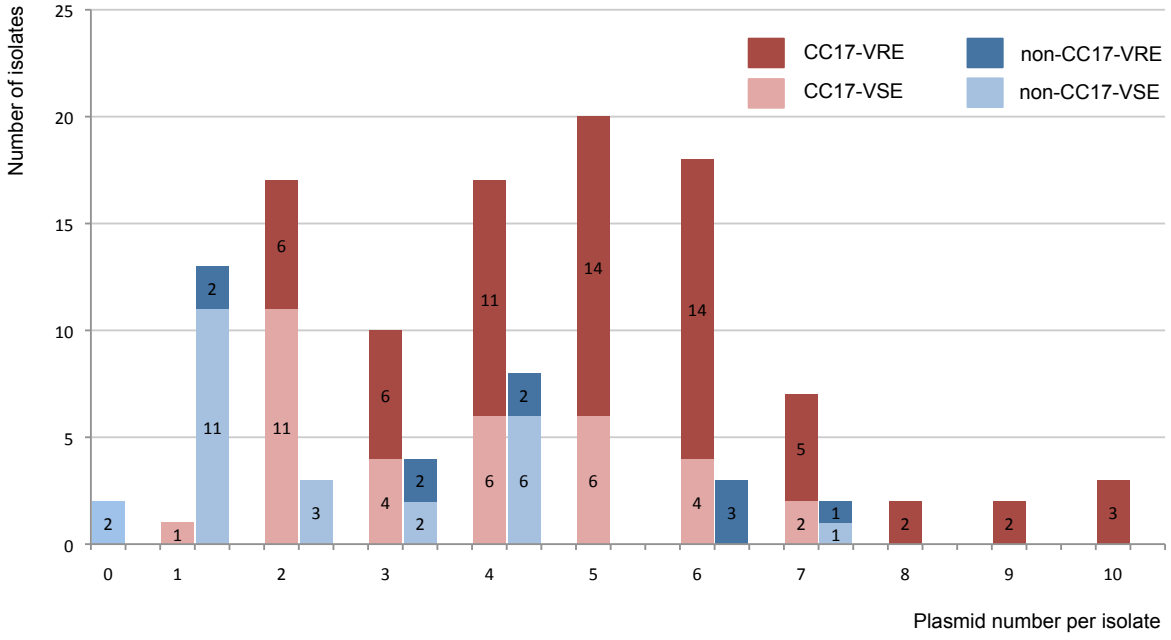


Figure 3. Plasmid number (1-10/cell) of CC17 and non-CC17 isolates (VRE and VSE) typed by S1-PFGE.

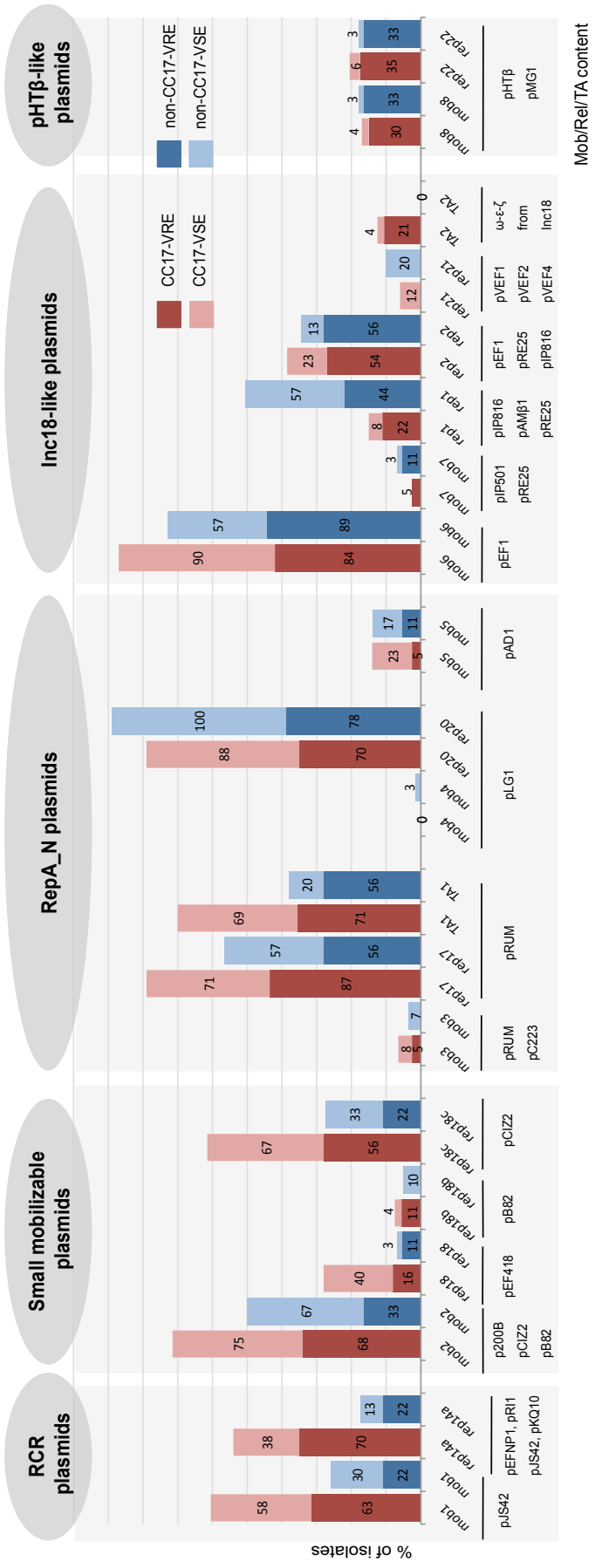


Figure 4. Plasmid content (mob/rep/TA) of CC17 and non-CC17 *E. faecium* isolates.
Numbers inside bars correspond to the individual % of CC17-VRE/non-VRE (red) and non-CC17-VRE/VSE (blue) containing each mob (mobilization), rep (replication) and TA (toxin-antitoxin) gene. Total of isolates, n=154; 72 VRE, vancomycin-resistant *E. faecium* (n=63 CC17 and n=9 non-CC17) and 82 VSE, vancomycin-susceptible *E. faecium* (n=52 CC17 and n=30 non-CC17). Each plasmid mob (1-7), rep (1-22) and TA (1-2) genes analysed by PCR are indicated with numbers as in Table 4, and the highest aminoacid sequence homology of the different genes is indicated above by one or more plasmids.

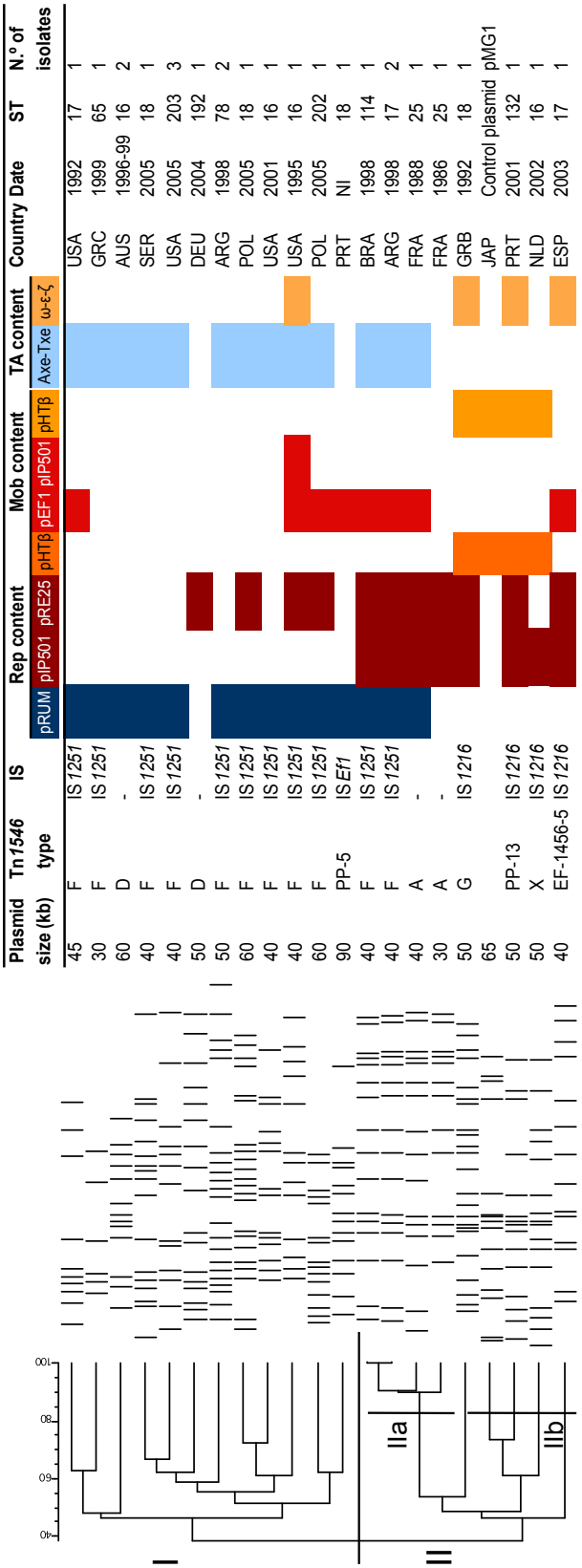


Figure 5. Computer analysis of *Cla*I-digested plasmid DNA of representative vancomycin-resistant VanA-type *E. faecium*.
Abbreviations: IS, insertion sequence; Rep, replicases; mob, mobilization; TA, toxin-antitoxin; ST, sequence type; ARG, Argentina; AUS, Australia; BRA, Brazil; DEU, Germany; ESP, Spain; FRA, France; GBR, Great Britain; JAP, Japan; NLD, The Netherlands; POL, Poland; PRT, Portugal; SER, Serbia; USA, United States of America. Plasmid genes (*rep*, *mob*, *TA*) previously associated with a given plasmid type are represented with different colours following the same criteria explained in Tables 4 and 5.

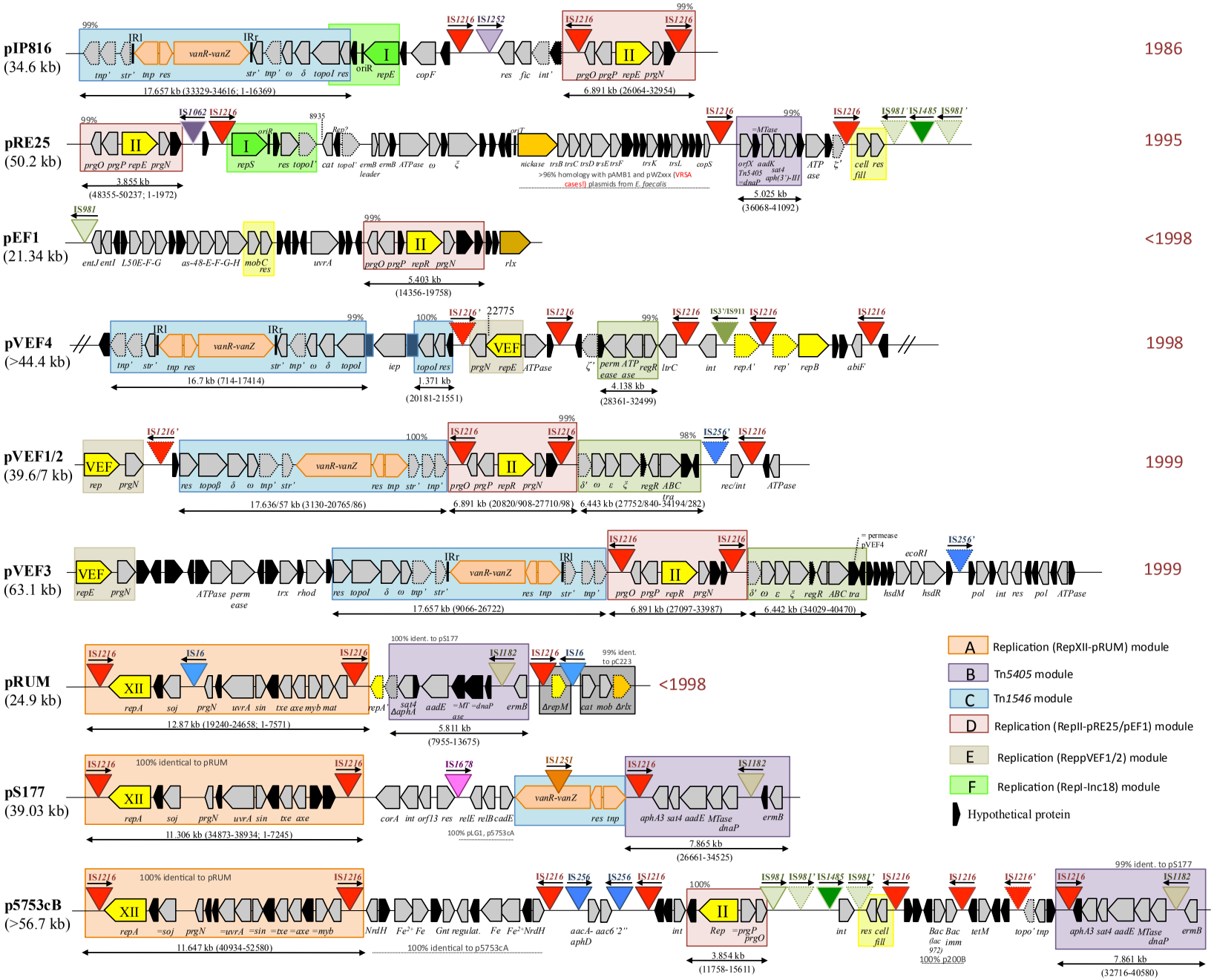


Figure 6. *In silico* analysis of enterococcal sequenced plasmids.

Table S1. Main plasmid types representative of the plasmid families found among *Enterococcus*.

Plasmid family	Size	Examples	Host	Rep1	Rep2	Rep3	Rel	TA	Bac	Hyl
Rolling-circle replicating plasmids	2-4 kb	pSJ42	*Enterococcus faecium*	rep14a	-	-	MobV4	-	-	-
		pRI1	*Enterococcus faecium*	rep14a	-	-	MobV4	-	-	-
		pEFNP1	*Enterococcus faecium*	rep14a	-	-	-	-	-	-
		pAMα1	*Enterococcus faecalis*	rep6	-	-	MobV1	-	-	-
Small theta-replicating plasmids	5-18 kb	p200B	*Enterococcus faecium*	rep18	-	-	MobP7	-	-	-
		pEF418	*Enterococcus faecium*	rep18	-	-	-	-	-	-
		pB82	*Enterococcus faecium*	rep18b	-	-	MobP7	-	Bac43	-
		pCIZ2	*Enterococcus faecium*	rep18c	-	-	MobP7	-	Bac32	-
Inc18	21-60 kb	pIP816	*Enterococcus faecium*	rep1	rep2	-	-	-	-	-
		pRE25	*Enterococcus faecalis*	rep1	rep2	-	MobQ3	ω–ε–ζ	-	-
		pEF1	*Enterococcus faecium*	-	rep2	-	MobP7	-	-	-
		pVEF1	*Enterococcus faecium*	-	rep2	rep21	-	ω–ε–ζ	-	-
		pVEF2	*Enterococcus faecium*	-	rep2	rep21	-	ω–ε–ζ	-	-
		pVEF3	*Enterococcus faecium*	-	rep2	rep21	-	ω–ε–ζ	-	-
RepA_N	25 kb	pRUM	*Enterococcus faecium*	rep17	-	-	MobP7	Axe-Txe	-	-
	281 kb	pLG1	*Enterococcus faecium*	rep20	-	-	MobP7	-	-	Hyl
* Pheromone-responsive	35-70 kb	pAD1	*Enterococcus faecalis*	rep9	-	-	MobC2	*par*	-	-
		pCF10	*Enterococcus faecalis*	rep9	-	-	MobP7	*par*	-	-
		pAM373	*Enterococcus faecalis*	rep8	-	-	MobC2	-	-	-
pHTβ-like	60-90 kb	pMG1	*Enterococcus faecium*	rep22	-	-	MobPu	-	-	-
		pHTβ	*Enterococcus faecium*	rep22	-	-	MobPu	-	-	-
New	>150 kb	New	*Enterococcus faecium*	(rep1)	rep20	-	MobP7	-	EntP	Hyl
		New	*Enterococcus faecium*	-	-	-	MobC2	-	-	-
	30-55 kb	New	*Enterococcus faecium*	(rep2)	rep17	-	MobP7	Axe-Txe	-	-

Rep (replicases); mob (mobilization/relaxases); TA (toxin-antitoxin).
Plasmid genes (*rep, mob, TA*) previously associated with a given plasmid family are represented with a colour; rep, mob and TA genes belonging to the same plasmid family are represented with the same range of colours as in the former Table 4 of this manuscript.
Rep families are named RepX where X indicates different rep-families according to Jensen *et al*. (32) and our own primers (Table 3 of section 6.1.7. of appendix of this thesis - Rep14a, rep 18b, rep18c, rep21 and rep22 are not included in Jensen's scheme and the numbers were arbitrarily attributed following that numeration). Mob genes are designated accordingly to the families they belong (22).

Influence of pAD1 and Inc18-plasmids in the worldwide spread of vancomycin-resistant *Enterococcus faecalis* outbreak strains (1987-2004)

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ABSTRACT

Vancomycin resistant (VR) enterococci are more commonly associated with *E. faecium* than *E. faecalis* (Efs). However, the spread of VREfs is of concern since all vancomycin-resistant *Staphylococcus aureus* (VRSA) have mostly been associated with patients co-colonized with Efs containing Inc18-like VanA plasmids. Plasmid diversity among VREfs was analyzed in order to better understand the scarce dissemination of vancomycin resistance within this species and the potential transferability to other hosts. We analysed 33 VREfs (27 *van*A, 4 *van*B and one *van*G) isolates from 13 countries recovered from 1987 to 2004. They are representative isolates (16 PFGE types; 16 sequence types) of well characterized clonal outbreaks, most of them belonging to HiRCC (CC2, CC9 and CC87). Plasmid analysis included determination of the size and the whole plasmid content (S1-PFGE) and characterization of Van plasmids by comparison of their *Eco*RI/*Cla*I-RFLP profiles, identification of relaxases (rel), replication initiator proteins (rep), and toxin-antitoxin systems (TA) by PCR. Results were confirmed by S1/I-*Ceu*-I-hybridization and sequencing. VREfs contained a variable number of plasmids (1-4/cell) ranging from 20 to 200 kb. A diversity of sequences compatible with plasmids from different families (small theta-replicating plasmids, RepA_N including pheromone-responsive plasmids, Inc18, and pHTβ-like) was detected. While *vanB2*-Tn*5382* and *vanG* were chromosomally located, Tn*1546* (7 variants, with predominance of the complete Tn*1546*) were located on plasmids ranging from 30 to 200 kb, with some common types identified in different countries over years. Most of *vanA*-plasmids hybridized with probes for rel_{pAD1}, rep_{pAD1} and par_{pAD1} genes in different combinations, although they often contained rep_{Inc18}. Those recovered from VREfs isolated in Michigan besides *vanA*-*S. aureus* also contained rel_{Inc18} (sequences identical to those of pRE25). Differences in VanA plasmid size and rep/rel/TA content between wild types and their corresponding transconjugants were frequently observed (50% of cases). *vanA*-plasmids from Efs are mainly derivatives of the pheromone-responsive pAD1 which frequently contain rep_{Inc18}. Their narrow host and the frequent rearrangements observed during the conjugation might explain the maintenance of pAD1-Tn*1546* within this species. The transferability of VREfs plasmids to *S. aureus* seems to be associated to the presence of the broad host conjugative module of Inc18 plasmids.

INTRODUCTION

Enterococcus is one of the leading causes of nosocomial infections, *Enterococcus faecalis* (Efs) being the species more frequently identified followed by *Enterococcus faecium*. Nevertheless, vancomycin resistant enterococci (VRE) are mainly associated with *E. faecium* which has facilitated the selection and expansion of these microorganisms as one of the main multidrug-resistant pathogen in hospitals around the world (57, 65). The overrepresentation of genetic determinants coding for glycopeptide resistance among *E. faecium* remains to be explained.

Methicillin-resistant *Staphylococcus aureus* (MRSA) and VRE are the most common multidrug-resistant pathogens recovered from nosocomial infections and their concomitant isolation might facilitate the emergence and further expansion of vancomycin-resistant *S. aureus* (VRSA) (29, 46, 47). Of special concern is the selection pressure exercised by glycopeptides which have been increasingly used as the first-line therapeutic options in the treatment of human infections caused by MRSA, multidrug-resistant enterococci, and have also been increasingly used for the treatment of pseudomembranous colitis due to *Clostridium difficile*, and coagulase-negative staphylococcal infections (30, 37). Eleven cases of VRSA have been described to date, of which nine were reported in the USA, one in Iran and other in India (1, 17, 50). Of note is the fact that seven of the nine U.S. cases described to date occurred in the same area (southeastern Michigan) (58, 68), although the underlying factors for this preferential geographic location are unknown.

Some plasmids from *E. faecalis* have been extensively studied at molecular level as pheromone-responsive plasmids represented by pAD1 or pCF10 or Inc18-plasmids represented by pRE25 (19, 51). In most of the VRE from Michigan, VanA plasmids from VRE co-isolated with VRSA were identified as members of the Inc18 family (69). However, little is known about the plasmids associated with vancomycin resistance among *E. faecalis* from hospitalized patients worldwide.

Our purpose was to analyze the plasmid diversity among VREfs causing outbreaks in different countries since first description in 1987 in order to understand the scarce dissemination of glycopeptide resistance among *E. faecalis* and the potential transferability of these plasmids to other more pathogenic microorganisms.

MATERIALS AND METHODS

Bacterial strains and epidemiological background. Thirty-two vancomycin-resistant *E. faecalis* (27 *vanA,* 4 *vanB* and 1 *vanG*) recovered from hospitalized patients in 13 countries between 1987 and 2004 were studied. They are representative isolates from Europe (n=17; Cyprus, Hungary, Italy, Poland, Portugal, Serbia, Spain, UK), North America (n=10; USA) and South America (n=4; Argentina, Brazil, Chile), and Australia (n=1), and most of them caused clonal outbreaks clinically documented (Table 1). The collection include epidemiologically relevant *E. faecalis* strains as V583, the first known *vanB*-VREfs isolated in the USA (45), the Australian isolate BM4518 carrying *vanG* (16), representative VREfs isolates recovered in Houston/Texas in 1994 and Bogotá/Colombia during 2001-2006 (2), WBH13 which transferred *vanA* to one of the U.S. (Michigan) methicillin-resistant *S. aureus* (MRSA) (18), and representatives of the epidemic *E. faecalis* clone associated with nosocomial outbreaks in Portugal, Spain and Italy (20).

Susceptibility to 10 antibiotics was determined by the disk diffusion method following CLSI guidelines (6). Relationship among isolates, which was established by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), has been mostly previously described by our group (48, 49).

Characterization of vancomycin-resistant transposons. PCR was performed for detection of genes coding for resistance to glycopeptides. The backbone structure of *vanA*-Tn*1546* was determined by the PCR mapping assay as described by Woodford *et al.* (66). The diversity of *vanB*-tranposons was established after comparing the RFLP patterns corresponding to *Bsp*HI/*Dra*I (New England Biolabs Inc., UK) digested *van*RSYWHBX amplicons obtained by long PCR (5,959-bp) (12). Chromosomal and/or plasmid location of *van* genes was assessed by hybridization of I-*Ceu*I-digested genomic DNA with *vanA*, *vanB* and 23S rRNA probes. DNA southern transfer and hybridization were accomplished by standard procedures (23). Hybridization experiments were carried out by using the Gene Images AlkPhos Direct labeling and detection system (Amersham GB, GE Healthcare Life Sciences UK Limited) following the manufacturer's instructions. Conjugation transfer of *van* transposons was performed by the filter mating method at 1:1 donor:recipient ratio using *E. faecalis* strain JH2-2 as recipient strain (fusidic acid and rifampin resistant, plasmid free). Transconjugants were selected on Brain Heart Infusion agar plates supplemented with vancomycin (6 µg/ml) and fusidic acid (20 µg/ml), with or without rifampin (30 µg/ml).

Plasmid analysis. Plasmid content and size of both *E. faecalis* wild type strains and transconjugants were determined by using the method described by Barton *et al.* (3, 23). Plasmid location of *van* genes was assessed by hybridization of S1-digested genomic DNA with *vanA* or *vanB* probes. The classification of *E. faecalis* plasmids was based on the presence of specific modules for basic plasmid functions as replication (Rep, rep initiator protein), mobilization (Rel, relaxases) and stability (TA, toxin-antitoxin systems) using recently developed PCR typing schemes that allow the identification of different plasmid families identified in enterococci and other Gram-positive genera (22, 26, 32). The rep sequences were designated according to the plasmid type in which they were originally identified as well as the numeric nomenclature used by Jensen *et al*. (32) and our own (see Table 3 in the section 6.1.7.3. of material and methods of this thesis).

PCR products were sequenced in order to confirm the specificity of the method and to analyze similarities with available sequences. Plasmid and/or chromosomal location of rel/rep/TA sequences were determined by hybridization of I-*Ceu*I/S1-digested genomic DNA with rel/rep/TA probes obtained by PCR using DNA from reference plasmids. Correlation of the genes amplified with vancomycin resistant plasmids was established when probes for *van* genes and genes encoding replication, relaxases or TA systems hybridized in the same plasmid band. Relatedness among plasmids of similar size (<150 kb) and similar replicon-relaxase gene content was established by comparison of their *Eco*RI and *Cla*I-digested DNA patterns (21). Plasmid DNA was obtained by using a protocol based on the alkaline lysis method described by Handwerger *et al.* (22, 28; see protocol 2 in the section 6.3. of the appendix of this thesis).

RESULTS AND DISCUSSION

Dissemination of vancomycin resistance is caused by clonal spread of *E. faecalis*. The 32 VREfs were classified as 16 PFGE types that corresponded to 16 different sequence types (STs) and mostly belonged to major hospital-derived clonal complexes (CC) CC2, CC9 and CC87 (n=20 isolates). Specific singletons as ST103 and ST158 were also identified in more than one isolate in the USA (n= 2 each), and the remaining isolates were identified as ST4, ST16, ST30, ST49, ST88, ST159, ST192, and ST201 (one each). Several PFGE-patterns corresponding to strains of the main CCs were detected in different countries for extended periods of time. They belonged to CC2 (ST6, isolates from

Spain, Portugal and Italy recovered since 1993 to date), CC9 (ST9, isolates recovered in Spain and Argentina from 2001 to 2003), and CC87 (ST28, isolates identified in Cyprus, Serbia and the UK from 1989 to 2004) (49). CC2, CC9 and CC87 have been described as causing infections in hospitalized patients worldwide, being considered of high-risk within the clinical setting (63). CC2 is one of the most widespread clonal complexes, while specific CCs (CC40 which is absent in this study, and CC87) might be endemic in particular geographical regions (33, 35, 48). ST4, ST16, ST30, ST88, ST103, ST159 have also been associated with human strains from the Netherlands, Poland, Portugal, Spain, Thailand, and the USA since the 1950's, but their relevance to the whole *E. faecalis* population structure remains to be proved (21, 33, 40, 48).

The susceptibility to antimicrobial agents did not vary greatly between clonal complexes or countries, with all isolates expressing resistance to erythromycin (100%) besides vancomycin. Most of them were also resistant to ciprofloxacin (91%), teicoplanin (78%), tetracycline (76%), high-levels of gentamicin (88%) and streptomycin (61%), and to a lesser extent to chloramphenicol (39%) (Table 1).

A few transposon platforms carry vancomycin resistance among *E. faecalis* strains from different countries. The PCR overlapping assay showed the presence of 7 Tn*1546* variants being "type A", which represents the complete transposon, the most frequently found (n=20 isolates from 8 countries including Argentina, Brazil, Italy, Hungary, Serbia, Spain, UK, and the USA). The Tn*1546*-types PP-4 (n=2) and PP-15 (n=1) containing IS*Ef1* and IS*1216* within *vanX-vanY* region, respectively, were only identified in isolates from Portugal (43). The Tn*1546* type F, which contains IS*1251* within *vanS-vanH* region, and type X (43, 63) were identified in VREfs from the USA and Cyprus (one each), respectively. The remaining two Tn*1546* types corresponded to two new variants, either showing alterations within *vanX-vanY* region (Poland) or within ORF2-*vanR* (Italy). The *vanA* gene was located on plasmids in all strains studied and successfully transferred by conjugation in 89% of the cases (n=24/27).

The diversification and evolutionary history of Tn*1546* have been carefully analyzed in several studies over the past 15 years, by different methods and applying different nomenclatures for designating the variants generated by insertions, deletions and point mutations (15, 31, 43, 54, 62, 64, 66). Most of transposons analyzed in these studies were recovered from *E. faecium* isolates of different ecological niches and countries due to predominance of vancomycin resistance among isolates of this enterococcal species. In contrast to the diversification of Tn*1546* by different recombinatorial processes, vancomycin resistant *E. faecalis* strains harbour the entire Tn*1546* (type A) possibly

contributing, at least in part, to the poor dissemination of *vanA* type resistance between *E. faecalis* isolates.

VanB isolates harbouring *vanB1*-Tn*1547* (n=1, the known *E. faecalis* V583 from the USA) or *vanB2*-Tn*5382*/Tn*1549* (n=3, Poland, Chile and USA), the last being the most widespread VanB-type transposon described in enterococci, were identified (11, 12, 41). The *vanB* gene was transferred by conjugation in 50% of strains (n=2/4) and hybridized on chromosomal bands in all of them. *vanG* was chromosomally located and its low frequency conjugative transfer to JH2-2 was previously described as being associated with the movement, from chromosome to chromosome, of genetic elements of ca. 240 kb (16).

Vancomycin resistant *E. faecalis* isolates harbour a diversity of plasmids belonging to different families. VREfs isolates carried a variable number of plasmids (1-4/cell; 20-200 kb) of different families inferred by the content of sequences linked to different replication, conjugation and maintenance systems. The diversity of rep, rel and TA genes for each isolate is shown in Figure 1.

Sequences of rep-initiator proteins of *small theta-replicating plasmids* ($rep6_{pAMα1}$, 27%); *pheromone-responsive plasmids* ($rep9_{pCF10}$, 94%; $rep8_{pAM373}$, 9%); *plasmids from the Inc18 family* ($rep1_{pIP501}$, 58%; $rep2_{pRE25}$, 52%); or *pHTβ-like family* ($rep22_{pHTβ}$, 6%) were detected. Similarly, relaxases associated with the conjugation modules of the same plasmid families, small theta-replicating ($MobV_1$-pAMα1, 36%), pheromone ($MobC_2$-pAD1, 92%; $MobP_7$-pCF10, 24%), Inc18 ($MobQ_3$ of the Inc18 plasmids pTEF1, pRE25 or pIP816, 18%), or pHTβ (MobP-pHTβ, 6%) were detected. VanB and VanG *E. faecalis* contained relaxases associated with Tn*1549* or the *vanG* transposable element, respectively (data not shown; 16). The TA system *par* was identified in 76% of *vanA* strains, all of them positive for rel_{pAD1}, whereas the TA systems ω–ε–ζ from Inc18 plasmids (12%) and Axe-Txe from pRUM (6%), frequently found among *E. faecium* strains, were scarcely present among *E. faecalis*. Toxin-antitoxin systems described in gram negatives (*maz*EF and *rel*BE) were absent.

We were able to identify a number of replication and conjugation genes associated with different plasmid types among *E. faecalis* strains from different countries although they shared an overall similar plasmid background with clear overrepresentation of pheromone-responsive plasmids. *E. faecalis* isolates contained theta-replicating plasmids from the different RepA_N, Inc18 and pHTβ-like plasmid families, and lacked rolling-circle replicating plasmids which are frequent among *E. faecium* isolates from different countries (22, 24, 32). Both pheromone-responsive (RepA_N family) and Inc18 plasmids have been

previously associated with *E. faecalis* strains, but this is the first study describing the presence of pHTβ-like genes in *E. faecalis* species. Plasmids containing rep and rel genes from pHTβ/pMG1 were detected in isolates from Argentina and the USA, and the presence of a replication gene from these plasmids suggests their ability to replicate among *E. faecalis* species. pHTβ-like and pMG1 plasmids have been previously isolated in *E. faecium* from hospitalized patients in different countries (22, 59), though their prevalence in *E. faecalis* seems to be low (this study). The presence of these plasmids in *E. faecalis*, as well as of Axe-Txe from pRUM, which has also been commonly associated with *E. faecium* species, and the reverse occurrence of pheromone-responsive plasmids in *E. faecium*, indicates these species share a common mobilome with some elements of narw host being confined to particular enterococcal species (22, 27, 44, 61).

***vanA* is located on conjugative mosaic plasmids derivatives of pheromone-responsive and Inc18 plasmid families.** Hybridizations experiments to confirm the associations of VRE plasmids with sequences obtained by PCR were accomplished in a selected number of strains and transconjugants. Most *vanA* plasmids analyzed hybridized with probes for $rep_{pAD1,}$ rel_{pAD1}, and par_{pAD1} genes in different combinations, and often also with $rep1_{pIP501}$ and/or $rep2_{pRE25}$ linked to Inc18 plasmids. Only some isolates from Michigan-USA (6/8 VREfs corresponding to 1 ST2, 1 ST4, 1 ST28, 1 ST103, 2 ST158, all recovered from 2002) contained a relaxase linked to the conjugation module of Inc18 (rel_{Inc18}) which showed >97% homology to the relaxase/nickase of pRE25 and pIP501 plasmids (GenBank accession numbers X92945 and L39769, respectively). The size of plasmids containing Tn*1546* (30-200 kb) varied during the conjugation process for half of the strains tested, recombinant plasmids recovered from transconjugants showing larger (n=7/12) or lower (n=5/12) size than those of the wild type strains. Analysis of the *van*-plasmid patterns after digestion with restriction enzymes revealed a high diversity of RFLP profiles although some of such patterns from isolates recovered in different countries over the years showed common bands (data not shown), probably reflecting the rearrangements observed during the conjugation process. This was also reinforced by the differences observed in the rep/rel/TA content of wild type strains and their vancomycin-resistant transconjugants (representative examples are shown in Table 2).

To date, most epidemiological studies analyzing VREfs do not provide information about *vanA* plasmids (18, 36, 44, 45, 53, 66, 67). The few analysis available include *vanA* *E. faecalis* recovered in Michigan, USA due to the alarm caused by the transmission to the *Staphylococcus aureus* strains from the same patients (7 of 11). Five of these plasmids were identified as members of the Inc18 family as they contain sequences linked

to the replication and conjugation systems of these plasmids (68, 69). Our results confirm the wide spread of Inc18 genes among *E. faecalis* strains from different countries. However, it is worthwhile to highlight that the presence of the Inc18 conjugation system was limited to VREfs from Michigan. *vanA* plasmids of our collection could not be classified strictly as Inc18 as most of them corresponded to mosaic genetic structures harbouring sequences from both pheromone-responsive and Inc18-like genes, some of them carrying only genes related with pheromone plasmids.

The acquisition of enterococcal plasmids by *S. aureus* is impaired by the inefficient replication of enterococcal plasmids in this host and also by a type III-like restriction endonuclease system that acts as a barrier for the transference of DNA from other bacterial genus (8-10, 60). However, enterococcal plasmids can be transferred and maintained after cointegration with other plasmids able to replicate in *S. aureus* (53, 60). The presence of plasmids with the conjugation machinery of Inc18 plasmids in VREfs strains may amplify the potential of Tn*1546* transfer to *S. aureus*, although it seems to be rarely found in *Enterococcus faecium* and *Enterococcus faecalis* from different origins and locations (data not shown; 22, 69).

Changes in the plasmid size and gene content between *E. faecalis* donors and transconjugants mostly involved genes of pheromone-responsive plasmids. The pathogenicity island (PAI) of *E. faecalis* contains a region with high homology to the pheromone-responsive conjugative plasmids pAM373 and pAD1 (52). Coburn *et al*. demonstrated the formation of cointegrates of excised circular intermediate DNA fragments of the PAI and pheromone plasmids (e.g. pTEF1-like) by homologous recombination, with transfer of a large PAI region using the plasmid conjugation system (7). Similar processes of lateral genetic transfer between homologous sequences could explain the loss of relaxase in some of our transconjugants and the changes in size observed in several of them. Recently, Laverde Gomez *et al*. have shown the precise transfer of a ca. 200 kb *E. faecalis* PAI with concomitant transfer of a mosaic 66 kb plasmid pLG2 containing modules of Inc18 and pheromone-responsive plasmids (34). Although a relaxase gene was not identified, the authors postulated that this plasmid could support PAI transfer with conjugative modules necessary to cell-to-cell transfer. Other recent study by Manson *et al*. also demonstrated that transfer of *E. faecalis* PAI and flanking chromosome sequences (e.g., antibiotic resistance determinants) only occurs in the presence of a co-resident pheromone-responsive conjugative plasmid, confirming the role of these plasmid types in the genome plasticity and evolution of *E. faecalis* species (39). Although the *esp* gene of PAI E. faecalis could not be amplified in transconjugants we can not discard the presence of a truncated island or other genetic island or ICEs with conjugation modules homologous to that of the vancomycin resistant plasmids. Further

ongoing studies in our lab are focused to elucidate the mechanism responsible to such arrangements.

CONCLUDING REMARKS

Vancomycin resistance among *Enterococcus faecalis* is caused by both clonal spread and dissemination of conjugative plasmids. VREfs clinical isolates recovered from different continents in the last 20 years mainly belong to major human-adapted clonal complexes (CC2, CC9 and CC87) and contain plasmids mostly associated with the pheromone-responsive (narrow host) and Inc18-type (broad host) plasmids. Both the narrow host range and the frequent rearrangements observed during the conjugation are consistent with the maintenance of pAD1-like::Tn*1546* within the *E. faecalis* species. The transferability of *vanA-E. faecalis* plasmids to *S. aureus* might be associated with the presence of the broad host conjugative module of Inc18 plasmids.

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Table 1. Epidemiological and genetic backgrounds of vancomycin-resistant *E. faecalis* causing outbreaks in different countries during 1987-2004.

CC	ST	PFGE type	Date	Origin	*E. faecalis* group	Resistance profile[a]	Virulence profile[b]	*Van-Tn* type[c]	Transfer frequency	WT plasmid content (kb)[d]	TC plasmid content (kb)[d]	Reference
CC2	6	B	1987	USA (St. Louise)	VanB	VAN, ERY, CIP, GEN, STR, CHL	asa, gel	*vanB1*-RFLP-1	$<10^{-6}$	20, 50, 70	20	45
	6	B	1993	Italy (Ancona)	VanA	VAN, TEC, ERY, CIP, TET, GEN, CHL	*asa, gel*	A	-	20, 90, **100**	-	20
	6	B	1999	Spain (Valencia)	VanA	VAN, TEC, ERY, CIP, TET, GEN, CHL	asa, gel	A	$1x10^{-4}$	75, **100**	**160**	20
	6	B	2000	Italy	VanA	VAN, TEC, ERY, CIP, TET, GEN, STR, CHL	asa, gel	A	$5x10^{-3}$	60, **120**	**120**	20
	6	B	2001	Portugal (Porto)	VanA	VAN, TEC, ERY, CIP, TET, GEN	*asa, gel, cyl, esp*	PP-4	ND	20, **75**, 140	**75**	20, 21
	6	B	2001	Portugal (Porto)	VanA	VAN, TEC, ERY, CIP, TET, GEN, STR, CHL	asa, gel, cyl	PP-15	ND	**30**, 40, 75	**110**	20, 21
	6	B	2002	USA (Detroit, Michigan)	VanA	VAN, ERY, CIP, GEN, STR	asa, gel, cyl	A	$6x10^{-6}$	40, 65, 85, **200**	60, **100**	49
	2	A	1994	USA (Houston, Texas)	VanB	VAN, ERY, CIP, GEN	*gel*	*vanB2*-RFLP-2	-	50, 65, 85	-	2
	2	A	2002	USA (Detroit, Michigan)	VanA	VAN, TEC, ERY, CIP, GEN	asa, gel	A	$2x10^{-3}$	**40**, 55, 100	**35**	49
	2	A	2003	USA (Detroit, Michigan)	VanA	VAN, ERY, CIP, GEN, STR	asa, gel, cyl	A	$3x10^{-4}$	**50**, 70, 100	**50**, 95	49
CC9	9	C	2001	Spain (La Coruña)	VanA	VAN, TEC, ERY, CIP, GEN, STR	asa, gel, esp	A	$4x10^{-3}$	**100**, 115	80, **90**	49
	9	C	2003	Spain (Palma de Mallorca)	VanA	VAN, TEC, ERY, CIP, GEN, STR, CHL	asa, gel, esp	A	$2x10^{-5}$	90, **100**	**40**, 100	49
	9	C	NK	Argentina (Buenos Aires)	VanA	VAN, TEC, ERY, CIP, TET, GEN, STR	asa, gel, esp	A	$2x10^{-4}$	75, **120**	**40**, 65	49
	318	D	NK	Brazil	VanA	VAN, TEC, ERY, CIP, TET, GEN, CHL	*asa, gel, esp*	A	-	20, 35, **50**, 75	-	49
	318	ND	NK	Brazil	VanA	VAN, TEC, ERY, CIP, TET, GEN, CHL	asa, gel, esp	A	ND	**40**, 80	**40**	49
CC87	28	E	1989	UK (London)	VanA	VAN, TEC, ERY, TET, GEN, STR, CHL	*asa, gel, cyl, esp*	A	$8x10^{-3}$	70, **100**	**100**	42
	28	E	2002	Serbia (Belgrade)	VanA	VAN, TEC, ERY, CIP, TET, GEN, STR	gel	A	$1x10^{-6}$	**70**, 100	70, **100**	56
	28	E	2004	Cyprus (Nicosia)	VanA	VAN, TEC, ERY, CIP, TET, GEN, STR	*asa, gel*	X	-	**50**, 75	-	13
	87	F	2004	Poland	VanA	VAN, TEC, ERY, CIP, TET, GEN, STR, CHL	asa, gel, cyl, esp	New	ND	30, 50, 105	40, **65**	33
	87	F	2000	Poland	VanB	VAN, ERY, CIP, TET, GEN, STR, CHL	asa, gel, cyl	*vanB2*-RFLP-2	ND	80, 100	80	33
CC30	30	G	1997	Australia	VanG	VAN, ERY, CIP, TET, GEN	*gel*	-	-	ND	-	16
CC88	88	H	2002	Italy	VanA	VAN, TEC, ERY, CIP, TET, GEN	gel	New	ND	60, **80**	110, **140**	49
ST	4	I	2002	USA (Detroit, Michigan)	VanA	VAN, TEC, ERY, CIP, GEN, STR	*asa, gel, cyl, esp*	A	$1.2x10^{-3}$	30, **50**, 100, 135	30, 80, **110**	18
CS	103	J	2002	USA (Detroit, Michigan)	VanA	VAN, TEC, ERY, CIP, TET, GEN, STR	asa, gel	F	ND	**125**	**200**	49
	103	J	2002	USA (Detroit, Michigan)	VanA	VAN, TEC, ERY, CIP, TET, GEN	*asa, gel*	A	$5x10^{-5}$	**50**	**50**	49
CS	158	K	2002	USA (Detroit, Michigan)	VanA	VAN, TEC, ERY, CIP, TET, STR, CHL	asa, gel, cyl, esp	A	$4x10^{-5}$	**50**, 60, 80, 100	**50**, 60, 80, 100	49
	158	K	2002	USA (Detroit, Michigan)	VanA	VAN, TEC, ERY, CIP, TET, GEN, STR, CHL	*asa, gel, cyl, esp*	A	ND	**30**, 60, 80	**40**	49
CS	159	L	2002	Portugal (Viseu)	VanA	VAN, TEC, ERY, CIP, TET	*asa, gel, cyl, esp*	PP-4	ND	**85**	**85**	21
CS	201	M	2004	Chile (Santiago)	VanB	VAN, ERY, CIP, TET, GEN, STR, CHL	*asa, gel, cyl, esp*	*vanB2*-RFLP-2	-	35, 50, 95	-	38
CS	192	N	NK	Hungary (Budapest)	VanA	VAN, TEC, ERY, CIP, TET, GEN, STR	asa, gel	A	ND	35, **90**	**90**	49
ST	16	O	NK	Hungary (Budapest)	VanA	VAN, TEC, ERY, TET, GEN, STR	asa, *cyl, esp*	A	ND	90, **100**	**100**	49
ST	49	P	NK	Spain (Cataluña)	VanA	VAN, TEC, ERY, CIP, TET	asa, gel	A	ND	**100**	**100**	14

Abbreviations: ND, not done; NK, not known; CC, clonal complex; CS, singleton; ST, sequence type; WT, wild-type; TC, transconjugant; -, negative or not applicable.

[a]VAN, vancomycin; TEC, teicoplanin; ERY, erythromycin; CIP, ciprofloxacin; TET, tetracycline; GEN, high-level resistance to gentamicin; STR, high-level resistance to streptomycin; CHL, chloramphenicol.

[b]Underlined putative virulence genes are transferred by conjugation.

[c]The Tn*1546* designation is based on PCR mapping as described previously (43, 66). The two new variants showed alterations within *vanX-vanY* region (Poland) or within ORF2-*vanR* (Italy).

[d]The number of plasmids is separated by commas and numbers in bold indicate plasmids hybridizing with a *vanA* gene probe.

Table 2. Plasmid characterization of representative *vanA E. faecalis* isolated from European and American hospitals.

Isolate	PFGE	ST	CC[a]	Country	Year	Tn*1546* type[b]	nt: Rep/Mob/TA (kb)[c] 20-30 Kb	50-80 Kb	85-110 Kb	>110 Kb
2724	B	6	CC2	Italy	1993	A	20-kb rep9 *par*		100-kb rep9 mob5 rep2	
Negative conjugation (failed with JH2-2 recipient strain)										
229710	B	6	CC2	Portugal	1996	PP-4		75-kb rep9 mob5 *par*	85-kb mob5 rep1 rep2	
TC-229710									85-kb mob5 rep1	
WBH18	A	2	CC2	USA	2003	A		50-kb rep9 mob5 *par* rep1 mob7	100-kb rep9 mob5	
TC-WBH18								50-kb rep9 rep1 mob7	95-kb rep9 mob5	
1940	C	9	CC9	Spain	2001	A			100-kb rep9 mob5 *par* rep1	115-kb mob5
TC-1940								80-kb mob5	90-kb rep9 mob5 *par* rep1	
EN455	D	9	CC9	Argentina	NI	A		75-kb rep9 mob5 *par*	110-kb rep9 *par*	
TC-EN455								50-kb rep9 *par*		
12201	E	28	CC87	London	1989	A		70-kb rep9 mob5 *par*	100-kb mob5 rep1 rep2	
TC-12201									100-kb mob5 rep1 rep2	
WBH13	G	4	CS	USA	2002	A	30-kb	50-kb rep9 mob5 rep1 mob7	100-kb	135-kb
TC-WBH13							30-kb	80-kb mob5	110-kb mob5 mob7	

Abbreviations: rep (rep initiator protein); Mob (relaxases); TA (toxin-antitoxin systems); TC (transconjugant); CS (singleton).

[a]MLST designation was established following the standard guidelines (www.mlst.net).

[b]Tn*1546* designation is based on the results obtained by a PCR overlapping assay previously described (43, 66).

[c]The total plasmid content is represented for both wild-type and transconjugants (TC) in different categories according to the approximately size in Kb. Sequences corresponding to different plasmid modules are shown in different colours (rep, mob and TA genes belonging to the same plasmid family are represented with the same range of colours following the same criteria explained in Table 4 of section 3.2.2.1. (former paper) in Results of this thesis). Rep sequences are designated according to the numeric nomenclature used by Jensen *et al*. (32) and our own (available in Table 3 of section 6.1.7.3. in material and methods of this thesis); genes hybridizing in the same band as *vanA* plasmids appear in bold rectangules. The correspondence of plasmid sequences in the schemes used with those of plasmids previously published are as follows: rep9 (rep from pAD1, pTEF1, pTEF2, pBEE99, pMG2200), rep1 (rep from Inc18 plasmids as pTEF1, pRE25, pIP816, paMβ1), rep2 (second rep of pRE25), mob5 from $MobC_2$ family (relaxases from the *E. faecalis* pathogenicity island, pAD1, and pTEF1), and mob7 from $MobQ_3$ family (relaxase from the Inc18 plasmids pRE25 and pIP501).

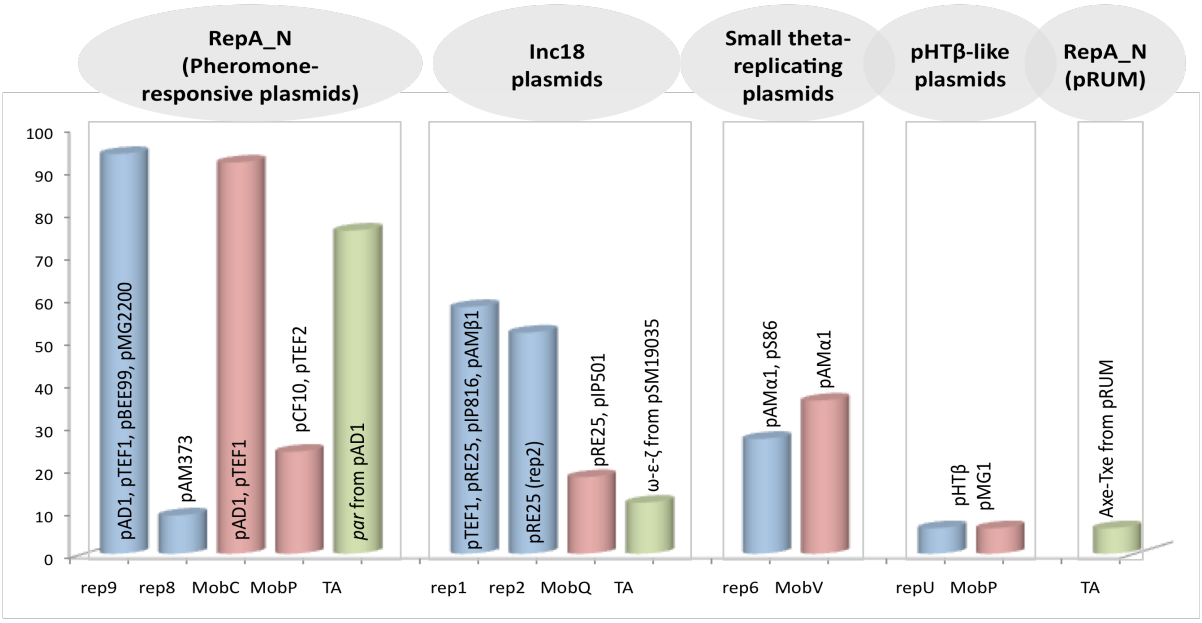


Figure 1. Percentage of Rep/Mob/TA content of vancomycin resistant *E. faecalis* strains (*vanA, van*B and *van*G) from different countries during 1987-2005.
Abbreviations: Rep, replication (represented in blue); Mob, relaxases (represented in red); TA, Toxin-Antitoxin systems (represented in green); U, unique.
Rep genes are referred according to the numeric nomenclature used by Jensen *et al*. (32), and relaxases are named by the Mob subfamilies which are designated by capital letters according to Garcillán-Barcia *et al*. (25). The names of the published plasmids showing the highest homologies to Rep/Mob/TA sequences identified in this study are showed inside bars.
Members of a given family of plasmids were grouped as follows: RepA_N which comprises, both pheromone-responsive (pAD1, pCF10, pTEF1, pTEF2, pBEE99, pMG2200, pAM373) and pRUM plasmids; Inc18-like plasmids (pRE25, pIP816, pAMβ1 and the ω-ε-ζ system); small theta-replicating plasmids (pS86, pAMα1); and pHTβ/pMG1-like plasmids. Particular plasmids are mentioned twice within each family (pTEF1 within pheromone-responsive and Inc18 plasmids or pRE25 within Inc18 plasmids), which corresponds to the amplification of different rep genes within the same plasmid type.

Spread and persistence of Inc18::Tn*1546* plasmids among enterococci from poultry food products in Portugal

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ABSTRACT

The population structure (clones, transposons and plasmids) of 50 vancomycin-resistant *Enterococcus* strains from chicken retail samples and healthy humans in Portugal (1999-2004) was analysed. These isolates belonged mainly to *Enterococcus faecium* CC9 and CC17 lineages in poultry and humans, respectively. Glycopeptide resistance was associated with the presence of *vanA*-Tn*1546* and eight variants of this transposon were identified on Inc18-derivatives plasmids of different sizes (50-165 kb). A diversity of plasmid families, both rolling-circle and theta-replicating (Inc18, RepA_N, pHTβ) plasmids, was observed among isolates studied. Our results indicate that specific clones and different plasmid types have facilitated the spread and maintenance of vancomycin resistance among enterococci recovered from the community.

INTRODUCTION

Vancomycin resistant enterococci (VRE) were initially detected in isolates from hospitalized patients in France and England in 1986 and 1987 respectively (23, 42). The increasing recovery from food borne animals and healthy humans in Europe during the following years was associated with the wide use of the glycopeptide's analogue avoparcin as a growth promoter (1, 20). The risk of transmission of VRE of animal origin to humans motivated the adoption of different intervention measures as the ban of this animal growth-promoter agent use in the EU by 1997 (32). Although a noteworthy reduction in the occurrence of animals and humans carrying VRE was noted after the EU withdrawal in some countries (12, 21, 32), other geographical areas still report high rates of VRE (4, 7, 14, 16, 26-30).

Recent studies have documented the global spread of vancomycin resistant *Enterococcus faecium* clones belonging to the clonal complexes (CC) 5 and CC17 and their recovery from both swine and humans (8 and references herein). Also, it has been observed a predominant location of *vanA* on untypeable megaplasmids of >150 kb in *E. faecium* from swine (8) or association of *vanA* with Inc18 plasmids in *E. faecium* from poultry animals and farmers (10, 33, 36-38). However, the number of studies assessing the risk to transfer clones or genetic elements from foods of animal origin to resident human flora remains scarce (8, 10, 14, 19, 24, 25, 37).

We have previously described the occurrence of VRE in poultry retail products and among healthy individuals in Portugal, one of the EU countries with high rates of VRE (29, 30; www.earss.rivm.nl). In this work we analyze the genetic context of 50 representative VRE strains from these hosts in order to explain the maintenance of VRE in the community and the risk of VRE transmission from animals to humans.

MATERIAL AND METHODS

Epidemiological background. Forty-three VanA isolates (28 *E. faecium*, 9 *E. durans*, 5 *E. hirae*, 1 *E. faecalis*) recovered from the inner carcass of retail poultry corresponding to 9 different brands widely commercialised throughout Portugal (1999-2001) and 7 *E. faecium* obtained from faecal samples of healthy humans (2001-2004), were analyzed. VRE isolates from poultry represent clonally unrelated strains isolated in different surveillance studies including a number of widespread strains which were persistently recovered among food samples of different commercial Portuguese brands (29). Isolates from humans comprise all VRE isolates identified from healthy humans in a surveillance recent study (30). These isolates were identified by using a multiplex PCR assay able to differentiate *E. faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*, *E. hirae* and *E. durans* by specific amplification of intragenic fragments of $ddl_{E.\ faecium}$, $ddl_{E.\ faecalis}$, *vanC-1*, *vanC-2,* *murG* and $mur\text{-}2_{ed}$ genes, respectively (2, 6). Susceptibility to 14 antibiotics was determined by standard procedures following CLSI guidelines.

Clonal relatedness. Clonal relatedness among isolates of the same species was established by PFGE of *Sma*I-digested genomic DNA. Representative PFGE types of *E. faecium* and *E. faecalis* were also analyzed by multilocus sequence typing (MLST) (17, 35; http://www.mlst.net).

Characterization and transferability of Tn1546. The backbone structure of Tn*1546* was determined by a PCR overlapping assay described by Woodford *et al.* (31, 45), and its location was assessed by hybridization of I-*Ceu*I-digested genomic DNA using *van*A and 23S rRNA probes. Conjugation experiments were performed by the filter mating method at a 1:1 donor-recipient ratio using *E. faecium* strains GE-1, BM4105RF and/or 64/3 and *E. faecalis* JH2-2 as recipients strains (29).

Plasmid analysis. Plasmid content and size were determined on representative transconjugants isolates using the method described by Barton *et al.* (3). The classification of plasmids was based on the presence of specific modules for basic plasmid functions as replication (Rep-initiator proteins), mobilization (relaxases, Rel) and stability (toxin-antitoxin systems, TA) of enterococcal plasmids using different PCR typing methods recently developed (9, 13, 18). The different enterococcal plasmid families identified to date are described in detail in section 1.3.3. of the Introduction this thesis. Relatedness among representative plasmids was established by comparison of their *Eco*RI-digested DNA patterns by standard methods, where plasmid DNA was obtained by using an alkaline lysis based method modified as described (9).

RESULTS AND DISCUSSION

Different enterococcal species contribute to the spread of vancomycin resistance among poultry with predominance of CC9 *E. faecium.* The 28 *E. faecium* isolates from poultry were grouped in 14 PFGE types, four types representing more than one isolate and being persistently recovered from poultry samples of different commercial brands over years (1999-2001). A diversity of sequence types (ST) belonging to the clonal complex (CC) 9, a host specific lineage of poultry (17), was associated with all but one *E. faecium* isolates (10 PFGE types) tested. CC9 isolates comprised isolates of sequence types ST9, ST196, ST394, ST395, ST405, ST450 (one clone each) and ST236 (n=2). The exception was the singleton ST406. ST236 and its single locus variant (SLV) ST395 corresponded to the most widespread and persistent clones identified among *E. faecium* poultry isolates. The only *E. faecalis* isolate recovered from poultry was identified as ST16, which is widely distributed among *E. faecalis* from animals and humans of different locations (35). The 7 *E. faecium* isolates from humans grouped in 5 PFGE types which belong to CC17/ST18 (n=3 clones/4 isolates), CC9 (n=1/2) and the singleton ST89 (n=1/1). Other enterococcal species were classified into 10 PFGE types (8/9 *E. durans*, 2/5 *E. hirae*).

Most isolates were resistant to tetracycline (88%), erythromycin (88%), quinupristin/dalfopristin (71%), teicoplanin (64%), streptomycin (60%), nitrofurantoin (55%), ciprofloxacin (52%), and to a lesser extent to kanamycin (12%), gentamicin and chloramphenicol (5% each).

A diversity of Tn*1546* are spread among poultry and healthy humans. Eight known Tn*1546* variants previously described were identified among the 50 *vanA* isolates (31; Figure 1). Poultry VRE isolates contained the entire Tn*1546* (designed as type A, 38%) and Tn*1546* variants lacking insertion sequences (PP-3, 3%), or carrying IS*19* (type S, 18%) and IS*1216* (types PP-1, 14%, and PP-11, 3%). The Tn*1546* type D harbouring IS*1216* within ORF1 (*tnpA*) was identified in both human and poultry isolates (14%). Types PP-5 (7%) and PP-6 (3%) carrying IS*Ef1* within *vanX-vanY* were confined to human isolates.

Tn*1546* was always located on plasmids which were successfully transferred by conjugation in 48% of the cases (24/50) at transfer frequencies ranging from 10^{-6} to 10^{-8}, which is much lower that the usual rates observed for plasmids of VRE isolates from hospitalized patients. Co-transfer of resistance to erythromycin and/or streptomycin was observed in 46% and 21% of the isolates, respectively.

Diversity of plasmid families among different enterococcal species from poultry and healthy humans. Plasmids were classified in different families according to the replication machinery (see Table 6 of section 1.3.3.1 in the Introduction of this thesis) (Figure 2). Combinatorial diversity of replicases, relaxases and toxin-antitoxin systems of different plasmid families was observed for clonal enterococcal lineages of different species and hosts (poultry *E. faecium* CC9, poultry *E. durans/E. hirae*, and healthy humans *E. faecium* CC17). They can be summarize as follows:

- Plasmids showing homology to the *rolling-circle replicating (RCR) plasmids* pEFNP1, pRI1, pJS42 and pKQ10 (14%) were identified in all enterococcal species with the exception of *E. durans* and *E. faecalis* from poultry. These cryptic plasmids have been recently identified in human and animal strains from different countries (18).
- Plasmids carrying rep sequences highly homologous to the *small theta-replicating plasmids* pCIZ2 (62%) were confined to healthy humans, while pEF418/p200B and pB82 (52% each) were only observed among poultry isolates. These small theta-replicating plasmids are associated with the production of bacteriocins (5, 40).
- Plasmids of the *RepA_N family* included pLG1 (48%), pRUM (12%), and the pheromone-responsive plasmid pAD1 (2%) (22, 43). Derivatives of pLG1, a megaplasmid widespread from clinical and swine isolates in different countries (8, 22), was identified in *E. faecium* and *E. durans* isolates but not in *E. hirae* and *E. faecalis*. pRUM was exclusively detected in CC17 *E. faecium* human isolates. The replication protein from the pheromone-responsive plasmid pAD1 was only detected in the *E. faecalis* isolate. *E. faecalis* vancomycin-resistant plasmids from poultry were also identified as being of the pheromone-responsive type in Korea (24).
- Plasmids assigned to the *Inc18 family* contained rep proteins from several Inc18-like plasmids as pIP501 and pRE25 (rep1), a second rep present in pRE25 (rep2) and $rep_{pVEF1/2}$, and were distributed among human and animal isolates of different species. $Rep1_{pIP501}$, the prototype of the Inc18 family, was rare (4%) and confined to *E. faecium* species. Rep2, linked to pRE25 (40%), and $rep_{pVEF1/2}$ (22%) were observed in all isolates but *E. hirae* and *E. faecalis* from poultry. pRE25 and pVEF1/2 plasmids and its derivatives have been identified in animal VRE from different locations (10, 18, 33, 37).
- Plasmids containing rep sequences with identity to that of *pHTβ/pMG1-like plasmids* (8%; 41) were mostly associated with *E. faecium* from humans.

The conjugation systems of the plasmids included in this study were inferred by the presence of relaxases of the families $MOBC_2$, MOBP, $MOBQ_3$ and $MOBV_4$ (11).

Sequences assigned to subfamily $MOBC_2$ (34%) showed variable identity with relaxases of different pheromone-responsive plasmids and were distributed among all enterococcal species from humans and poultry. Sequences assigned of the $MOBP_7$ subfamily were highly related to relaxases of plasmids belonging to pEF1 (58%), and families of RepA_N (pLG1, 46%, pRUM, 2%), small theta plasmids (pB82/p200B, 36%) and pHTβ (30%). They were detected in most of the isolates with the exception of rel_{pRUM} which was confined to the *E. faecium* CC17 isolate from human origin. The $MOBQ_3$, represented by relaxases of the Inc18 plasmids pIP501 and pRE25, was only detected in the *E. faecalis* isolate from poultry (2%). The $MOBV_4$ was linked to RCR plasmids which were spread in all species (22%). Sixteen isolates (32%), including isolates from the four prevalent clones, did not contained any of the relaxase genes tested.

The ω-ε-ζ toxin-antitoxin system of the Inc18 family was detected in 26% of the isolates, all identified as *E. faecium*. Of note, this plasmid addiction system was previously associated with the persistence of vancomycin-resistance in animal and human strains from Norwegian poultry farms (39). The *par* system from pAD1 was identified in the *E. faecalis* isolate, associated with pheromone responsive plasmids.

Inc18::Tn*1546* plasmids are disseminated among enterococcal species from poultry. VanA transconjugants contained 1-4 plasmids (25-300 kb) and a specific *vanA* probe hybridized with DNA of one or two large plasmids ranging from 50 to 165 kb in each transconjugant or wild type strain. Plasmid types carrying Tn*1546*-*vanA* (60 kb, 80 kb and 150 kb) were predominant and recovered from isolates of different clones from the same or different species.

Representative vancomycin-resistant plasmids were selected for hybridization and RFLP analysis. Most VanA plasmids contained Rep proteins homologous to that of Inc18 derivatives (pRE25, pEF1, pIP816, pVEF1/2) and a relaxase homologous to that of pEF1 (GenBank accession number DQ198088; 34). Nevertheless, some of the plasmids could not be correlated with the rep or rel genes tested, thus highlighting the existence of yet unknown plasmid types.

Three *vanA* plasmids from poultry VRE arbitrarily designated as plasmids A, B and C on the basis of their enzyme digested patterns were identified. Plasmid “A” (60-80 kb) was isolated from 4 *E. faecium* and 2 *E. hirae* (one PFGE-type each specie) recovered from three different commercial brands (1999-2000); ii) plasmid “B” (50 kb) was identified in two *E. faecium* strains of different brands (2001); and iii) plasmid “C” (75 kb) was linked to one *E. faecium* and one *E. durans* (1999).

CONCLUDING REMARKS

The results of this study indicate that the spread of VRE among retail poultry in Portugal is mostly caused by both clonal spread of CC9 *E. faecium* and dissemination of Inc18 plasmids, among different enterococcal species. The Inc18-like plasmids is a family of broad host range widely distributed among streptococci and enterococci from community-based hosts, and also from hospitalized patients (9, 46). The frequent presence of *vanA* Inc18 plasmids of enterococci from both poultry (this and 37, 38) and hospitalized humans suggests possible acquisition of Tn*1546* from an animal origin and/or transmission through food chain (9, 15).

Comparative analysis of the plasmid content among different *E. faeicum* lineages CC9, CC17 revealed a common pool of plasmids of different families highlighting the adaptation of particular mobile genetic elements to ecological conditions shared by organisms of different species and genera belonging to the same genetic exchange communities (Coque *et al*. Mobile genetic elements and lateral genetic transfer in enterococci, *in press*).

VRE clones and plasmids containing Tn*1546* may persist in the absence of selective pressure as demonstrated the recovery years after the banning of antibiotics as growth animal promoters (7, 24, 27, 28). Further studies are necessary to evaluate the similarity among plasmids of particular families recovered in different geographical areas and the factors responsible for the maintenance of a community VRE reservoir in absence of direct selective pressure.

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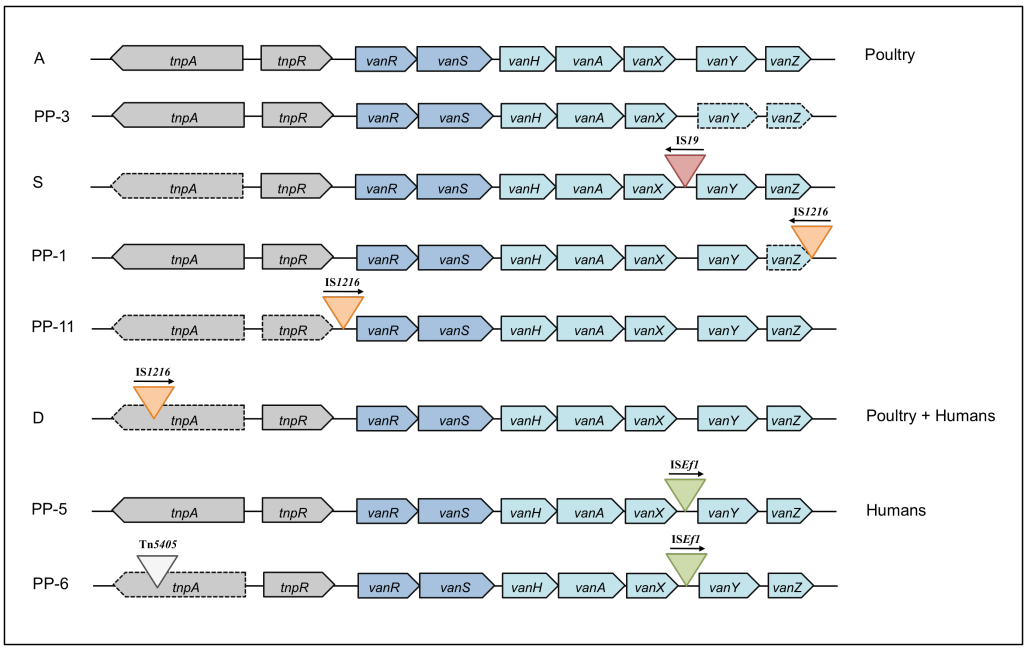


Figure 1. Genetic map of Tn*1546* variants found in this study.
The entire Tn*1546* transposon (designed here and other papers as type A) and different Tn*1546* types lacking insertion sequences (PP-3) or carrying IS*19* or IS*Efm1* (S), IS*Ef1* (PP-5 and PP-6) and IS*1216V* (D, PP-1 and PP-11)] are represented. Tn*1546* designation is based on the results obtained by a PCR mapping assay described by Woodford *et al*. (31, 45). The position of genes and open reading frames and the direction of transcription are depicted with open arrows. IS elements and a Tn*5405* fragment are represented by triangles. Sequences not fully characterized are indicated by dots.

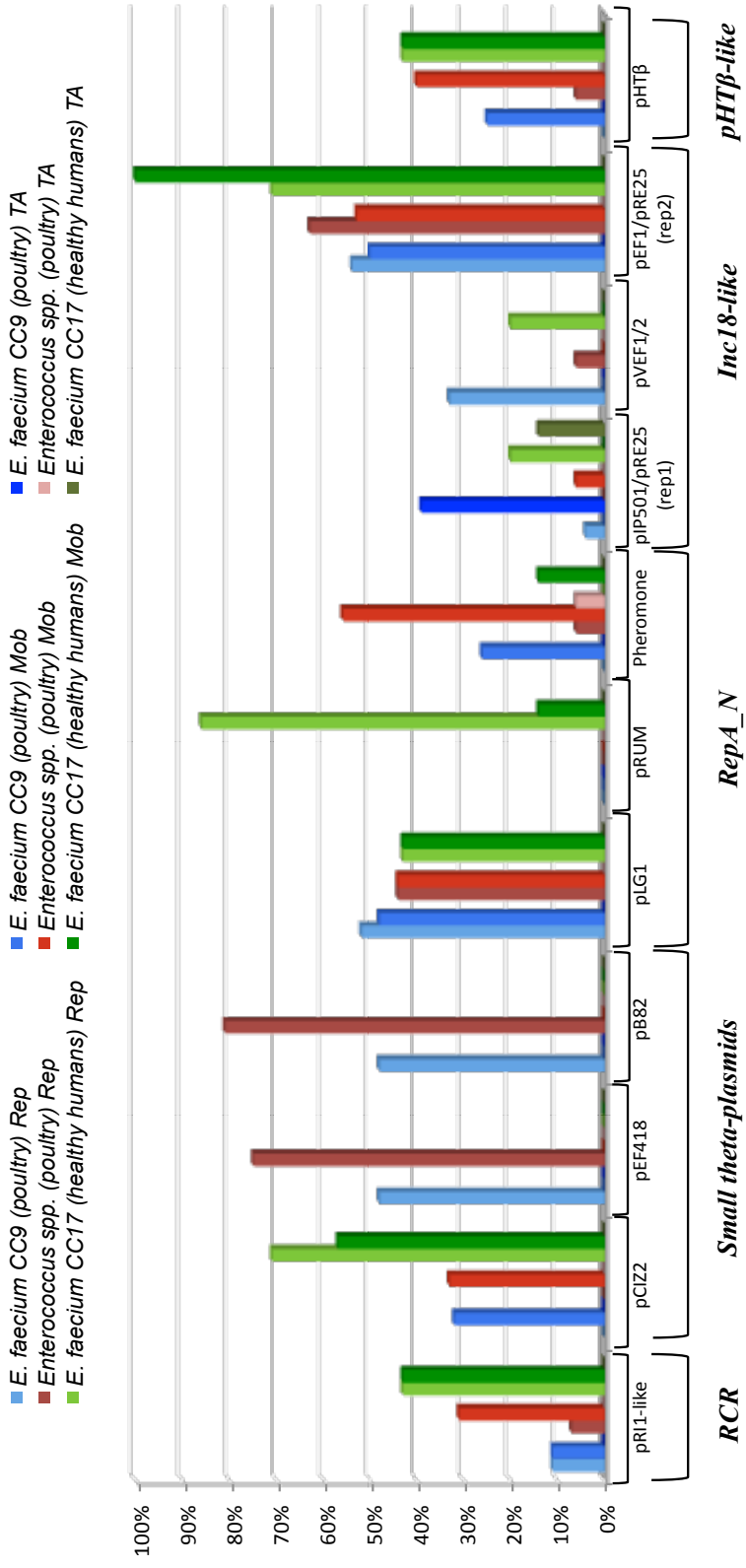


Figure 2. Replication (Rep), mobilization (Mob) and toxin-antitoxin (TA) gene content of *vanA* strains (n=50) from samples of retail poultry (n=43, 27 *E. faecium* and 16 *Enterococcus* spp.) and healthy humans (n=7 *E. faecium*) recovered in Portugal during 1999-2004.
E. faecium isolates from poultry (CC9) are represented in different ranges of blue, *Enterococcus* spp. are represented in different ranges of red and *E. faecium* from healthy humans (mostly CC17) are represented in different ranges of green.

Spread and diversification of pRUM and Inc18-like plasmids carrying Tn*1546* among CC17 *Enterococcus faecium* from hospitals in Portugal (1996-2008)

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ABSTRACT

The plasmid diversity among vancomycin-resistant *Enterococcus faecium* (VREfm) from Portuguese hospitals and aquatic surroundings was analyzed in order to better understand the plasmid ecology and the dramatic recent spread of VRE in our country. We analysed 75 VREfm-*vanA* mostly belonging to CC17 (43 PFGE types containing 18 Tn*1546* types) from hospitalized patients in 6 hospitals of different cities (n=62) and from hospital waste waters and river samples (n=13) (1996-2008). Plasmid characterization included determination of size and content, comparison of *Eco*RI/*Cla*I-RFLP patterns, and identification of 12 relaxases (rel), 24 rep initiator proteins (rep) and 5 toxin-antitoxin systems (TA) by PCR, hybridization and sequencing. A high diversity of plasmid content was observed (1-8 /cell, 4-350 kb). All Tn*1546* types, mostly containing IS*Ef1* or IS*1216*, were located on plasmids of variable size (30-250 kb; 95% conjugative). We identified 28 different RFLP plasmid patterns with overrepresentation of 2 profiles corresponding to plasmids types of 60 kb and 90 kb, the most disseminated among hospitals and environment and persistently recovered over years. CC17-VRE isolates harboured plasmids with similar content based on their replication machinery, corresponding to different families of rolling-circle (pEFNP1/pRI1) and theta-replicating (small mobilizable pCIZ2/pB82-like, Inc18, RepA_N, pHTβ-like) plasmids, and megaplasmids. Some isolates contained either plasmids with more than one replication or conjugation modules or plasmids which could not be classified with the schemes used. The toxin-antitoxin Axe-Txe and omega-epsilon-zeta systems from pRUM and Inc18, respectively, were infrequent. VanA-plasmids predominantly contained relaxases from pEF1, mostly associated with rep_{pRUM} and eventually with rep_{Inc18}. *E. faecium* isolates recovered from Portuguese hospitals contained plasmids with similar gene content suggesting a common evolutionary trend in intra and interhospital dissemination of VREfm. The conjugative plasmids driving the spread of vancomycin resistance in our area are mainly derivatives of pEF1 and pRUM.

INTRODUCTION

Vancomycin-resistant enterococci (VRE) have increasingly been reported worldwide since first description in the late 80's, with remarkable differences in the rates of VRE of distinct geographical areas (2, 29, 45). While these microorganisms are established in most hospitals from North America since years (2, 5, 27, 29, 40), their overall occurrence in Europe has remained low, with sporadic cases of nosocomial outbreaks being reported (22, 23). Nevertheless, the epidemiology of VRE in Europe seems to be changing in the last years, as they are increasingly being isolated in European hospitals, with nosocomial outbreaks being described more often [Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net), 2009] (45). Portugal is one of the European countries with a high VRE prevalence over the last 5 years (>20%) (www.earss.rivm.nl).

As in other areas, most VRE isolates from Portugal are identified as *Enterococcus faecium* clonal complex (CC) 17 carrying a diversity of Tn*1546* encoding VanA (7, 33, 45, 48), with some variants confined to particular institutions and others widely disseminated among hospitals throughout the country (32). Tn*1546* is frequently transferable by conjugation, although little is known about the molecular epidemiology of the plasmids in which they are located. To date, the few plasmids carrying Tn*1546* correspond to the plasmid families RepA_N (pheromone-responsiveness plasmids and derivatives of pRUM and pLG1 megaplasmid), Inc18, and pHTβ (4, 18, 20, 34, 37, 41, 42, 44) highlighting the promiscuity of this transposon. Some VRE plasmids causing nosocomial outbreaks in specific countries have been recently characterized but information about those recovered in Europe remains scarce (19, 34).

In order to the better understanding of the recent rapid spread of VRE in Portugal, we have further analyzed a collection of vancomycin-resistant *E. faecium* isolates from clinical and environmental sources and we have characterized the plasmids carrying the most common Tn*1546* variants.

MATERIAL AND METHODS

Bacterial strains and epidemiological background. Seventy-five VanA *Enterococcus faecium* isolates carrying Tn*1546* from different regions of Portugal were analyzed in this study. They included 62 clinical isolates obtained from 1996 to 2008 in 6 hospitals of different cities (Coimbra, Covilhã, Matosinhos, Porto, Vila Real and Viseu) located in Northern and Central Portugal, 10 isolates from waste waters of two hospitals and 3 isolates from the estuary of the river Douro recovered in Porto area during 2001-2003. VREfm isolates causing outbreaks or strains widely disseminated among hospitals and/or particular hosts are represented in this collection. Most of the isolates were previously included in surveillance studies from our lab (7, 31, 33).

For recent isolates (2007-2008) not previously analyzed, phenotypic and genotypic assays were performed following standard procedures. Susceptibility against 15 antibiotics was determined by the agar dilution method following CLSI standard procedures (only high-levels of resistance, HLR, to aminoglycosides were considered). Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested genomic DNA following the criteria described by Tenover *et al.* (33, 39) and Multilocus Sequence Typing (MLST) (7; http://efaecium.mlst.net). The presence of genes coding for virulence or epidemicity factors, collagen-binding adhesin (*acm*), enterococcal surface protein (*esp*), hyaluronidase (hyl_{Efm}), cytolysin/hemolysin (*cyl*), gelatinase (*gelE*) and aggregation substance (*agg*) was investigated by PCR (30, 43).

Characterization and genomic location of Tn*1546*. The backbone of Tn*1546* was determined by a PCR overlapping assay as reported (32, 50). Conjugation experiments were performed by filter mating and transconjugants analysed by comparison of their *Sma*I-PFGE electrophoresis with those of recipient and donor strains (33). The plasmid location of *vanA*-Tn*1546* was confirmed by hybridization of S1-digested genomic DNA with a *vanA* probe following standard procedures.

Plasmid analysis. The content and size of plasmids from both the *E. faecium* wild type strains and their transconjugants were determined by using either the technique described by Barton *et al.* (plasmids >10 kb) and the alkaline lysis extraction method of Kado & Liu (plasmids <10 kb) (see protocols 1 and 4 described in section 6.3. of the appendix of this thesis) (1, 8, 21).

Classification of *E. faecium* plasmids was based on the presence of specific modules for basic plasmid functions as replication (rep-initiator proteins), mobilization (relaxases) and stability (toxin-antitoxin systems and bacteriocins). *Relaxases* (rel) were sought by a

multiplex-PCR-based relaxase typing method which differentiates 12 relaxases of the $MOBQ_3$, MOBP, $MOBC_2$ and MOBV families related to 27 known plasmids (14, 15). Rep *rep-initiator proteins* (rep) were investigated by amplification of 24 replicons, which allows discriminating among 111 DNA sequences from 102 published plasmids (9, 19). The designation of rep sequences is based on the plasmid type in which they were initially identified, as well by as the numeric nomenclature used by Jensen *et al*. (19). *Toxin-antitoxin systems* (TA) previously identified among streptococci and enterococci (Axe-Txe, ω–ε–ζ, *par*) or gram negative bacteria (*maz*EF, *rel*BE) were detected by different PCR reactions (9). Finally, some *bacteriocins* (bac) previously found on enterococcal plasmids (*entA, entB, entP, entQ, entL50A/B, entJ/I, bac32 and bac43*) were searched by PCR (9). Primers and PCR conditions are described in Table 3 (material and methods section) of this thesis. PCR products of representative genes were sequenced in order to confirm the specificity of the method and to analyze similarities with other well-characterized plasmids. Plasmid and/or chromosomal location of rel/rep/TA/bac sequences in representative strains were determined by hybridization of rel/rep/TA/bac specific probes obtained by PCR using DNA from reference plasmids with plasmid DNA in Kado gels and I-*Ceu*I/S1-digested genomic DNA. Typing of vancomycin-resistant plasmids was performed by correlation of probes for *vanA* and genes encoding replication, relaxases or TA systems hybridizing in the same plasmid band.

Characterization of vancomycin-resistant plasmids. Extraction of plasmid DNA was obtained by using a modified protocol of the alkaline lysis method described by Handwerger *et al.* (9, 17). Relationships among plasmids of similar size were established by comparison of RFLP patterns obtained after digestion with different restriction enzymes (*Eco*RI, *Hind*III and *Cla*I). These methods are detailed described in section 6.3. of the Appendix of this thesis (protocols 2 and 5). The plasmids were designated with capital letters following the nomenclature of previous studies (6).

RESULTS AND DISCUSSION

VRE isolates of nosocomial origin belong to the CC17 lineage. Forty-three PFGE types were established among the 75 *vanA* isolates studied (35 PFGE types among 62 clinical and 1 hospital sewage isolates; 8 PFGE types/12 environmental isolates). Thirteen PFGE types were represented by more than one isolate and correspond to strains recovered in single hospitals (n=6), different hospitals (n=5) or strains recovered from environmental samples for extended periods of time (n=2). The majority of isolates (69/75; 92%) belong to the CC17. Within the CC17 lineage, most widespread and persistent clones correspond to the sequence type (ST) 18 and its single locus variant (SLV) ST132, as well as ST280, which were identified since 2001 (7; this study). Isolates recovered before 2000 were mainly associated with ST16 and ST80 from CC17, and non-CC17 sequence types. The six non-CC17 isolates encompassed some of the first VRE isolates described in Portugal which were collected in three different hospitals (ST190, ST366, ST367, ST391) and a pandemic ST5/CC5 clone globally disseminated among swine of different continents (6).

The presence of Acm, Esp, Hyl and Agg was inferred in 76%, 35%, 9% and 4% of the isolates studied, respectively, with isolates of the same clonal type often showing a variable content of these traits. The pediocin-like enterocin A was detected in the chromosome of 65% of the isolates. Previous studies have suggested that enterocin A could be considered a species-specific *E. faecium* putative virulence determinant (24, 46), which might confer an advantage to isolates lacking other epidemicity markers as *esp* and hyl_{Efm} genes, frequently recovered in our collection (>50% isolates). Among the remaining bacteriocins tested, only *bac32* (15%, n=11; ST18 and ST132) and *entP* (1%, 1 ST18) were detected.

All but one isolate were resistant to vancomycin (MIC 32 to 256 µg/ml), and 73 out of 75 (97%) were resistant to teicoplanin. Most isolates also expressed resistance to erythromycin (97%), ciprofloxacin (92%), kanamycin (86%), and ampicillin (66%); and to a lesser extent to tetracycline (53%), gentamicin (45%), streptomycin (41%) and chloramphenicol (37%).

This study confirms the spread of vancomycin resistance among the contemporary CC17 *E. faecium* polyclonal subcluster responsible for most of the isolates causing human infections and colonizing hospitalized persons worldwide (10, 26, 49). Selection of one of the main CC17 genotypes, the ST18 lineage, seems to have occurred since at least 2001 in hospitals from the mid-northern part of our country. A wider expansion may have occurred as ST18 isolates have been recently described in hospitals located in the Southern region of Portugal (28).

A diversity of related Tn*1546* variants are located on conjugative plasmids. Eighteen Tn*1546* types were identified among the 75 *van*A isolates. They corresponded to the entire sequence of Tn*1546* (the "type A"; n=3), Tn*1546* variants with deletions upstream *vanR* (2 types; n=2), with undetermined insertions within the *vanX-vanY* region (1 type; n=1); and containing IS*Ef1* (5 types; n=40) or IS*1216* (8 types; n=29) at different locations of the backbone. The isolate (ST78, 2007) susceptible to both glycopeptides (MIC of 4 mg/L) contained alterations within the *vanS-vanH* intergenic region (including a IS*1216*), a region involved in the expression and regulation of the resistance to vancomycin, and it constitutes the first description of a *vanA* isolate phenotypically susceptible to vancomycin in our clinical collection. The isolate susceptible to teicoplanin carried a Tn*1546* variant containing an insertion in the *vanY* gene that would affect the transcription of *vanZ* and it might explain the lack of susceptibility to this glicopeptide as previously reported (32, 47).

Tn*1546* was always located on plasmids ranging from 30 to 150 kb, and successfully transferred by conjugation in 95% of the cases (n=71/75) with a frequency ranging from $10^{-1}$to 10^{-8}.

VRE isolates contain plasmid of different families. A variety of plasmids was observed among the 75 *vanA* isolates (1-8/cell, 4-350 kb, Figure 1). The diversity and the occurrence of Rep/Mob/TA genes among isolates are shown in Table 1 and Figure 2, respectively.

Plasmids of different families were identified based on the presence of the replication sequences detected by hybridization and further sequencing. They were classified as: i) small rolling-circle replicating (RCR) plasmids (4-7 kb) since they contained $rep14a_{pRI1}$ sequences linked to putative RCR plasmids pEFNP1, pJS42, pRI1 and pKQ10; ii) small theta-replicating plasmids (6-18 kb) containing sequences highly related to pCIZ2 ($rep18c_{pCIZ2}$), pB82 ($rep18b_{pB82}$), and pEF418 ($rep18_{pEF418}$); some of them carrying *bac32;* iii) plasmids of the family RepA_N (20-150 kb) corresponding to derivatives of pRUM ($rep17_{pRUM}$) and pheromone-responsive plasmids pAD1 and pCF10 ($rep9_{pCF10}$); iv) Inc18 plasmids (30-200 kb) with sequences homologous to pRE25, pIP501, pIP816 and pEF1 ($rep1_{pIP501}$ and/or $rep2_{pRE25}$); and v) pHTβ/pMG1 derivatives (60-110 kb) with sequences identical to $rep22_{pHT\beta}$. Megaplasmids (>150 kb) were nontypeable as they did not hybridized with any of the rep genes tested, indicating the occurrence of unknown plasmid types.

Plasmids were associated with relaxases of MOB subfamilies $MOBP_7$, associated with theta replicating plasmids of families RepA_N (pRUM), pHTβ, pEF1 and small pCIZ2 derivatives; $MOBV_4$, confined to RCR plasmids; and $MOBC_2$ linked to pheromone-responsive plasmids. Megaplasmids (175-220 kb) often contained sequences from other

plasmid types highlighting the rearrangements between enterococcal plasmids. Replication and mobilization modules of the pheromone-responsive plasmids were identified in four and five *E. faecium* isolates, respectively. Although these plasmids exhibit a narrow host range and have been confined to *E. faecalis,* our data suggest their ability to replicate among other enterococcal species.

Toxin-antitoxin systems linked to plasmid pRUM (Axe-Txe) or Inc18 (ω-ε-ζ) were identified in 16% and 14% of the isolates, respectively.

Characterization of VanA plasmids. *vanA* plasmids from 57 VRE isolates were selected for detailed characterization. Twenty-six RFLP patterns were clustered in three major groups associated with particular Tn*1546* types. Some RFLP types were overrepresented (Table 2).

Group I grouped plasmids of variable size (40-150 kb) showing 5 RFLP patterns designed as A, B, C, D, and E harbouring Tn*1546* without any ISs (n=5 isolates; 1996-2002). These plasmids contain rep_{pRUM} sequences (n=3), a mosaic plasmid with rep_{pRUM} and rep_{pRE25} (n=1), and one plasmid with no rep genes detected. Four plasmids contained rel_{pEF1}.

Group II comprised plasmids from 40 to 110 kb showing 9 RFLP patterns (types F, G, H, I, J, K, L, M, N) and carrying 5 different IS*Ef1*-Tn1*546* variants (n=33 isolates; 1997-2008). One plasmid of this group designated as “type G” (75-90 kb) was the most commonly recovered and widely distributed (20 isolates, 11 PFGE types; 4 Tn*1546* variants) as it was recovered from all hospitals analyzed, river and urban sewage from 2001 to 2007 (Figure 3). Plasmid “type H” (85 kb) was also recovered among vancomycin-resistant *E. faecalis* isolates from Portuguese hospitals and aquatic sources for several years (data not shown). Plasmid types “I” (85 kb) and “J” (90-100 kb) identified in three different *E. faecium* strains each, were persistent over years in one hospital. Group II mostly includes plasmids carrying $rep_{pRUM,}$ in different combinations; rep_{pRUM} (n=6), rep_{pRUM} + rep_{pRE25} (n=3) or rep_{pRUM} + rep_{pIP501} (n=1). Others were Inc18 derivatives containing rep_{pRE25} (n=1) or rep_{pRE25} + rep_{pIP501} (n=1), and one was non-typeable. These plasmids contained rel_{pEF1} (n=6), rel_{pAD1} (n=1) or were not correlated with any of the relaxases tested (n=2); and contained ω-ε-ζ (n=1).

Group III comprises plasmids from 30 to 120 kb corresponding to 12 RFLP patterns (types O, P, Q, R, S, T, U, V, W, X, Y, Z) and carrying 8 IS*1216*-Tn1*546* variants (n=19 isolates; 2001-2003). Plasmid “type P” (60-65 kb) was identified in 5 isolates (3 PFGE types; 5 Tn*1546* variants) recovered from one hospital and close sewage plant from 2001 to 2003. This type has been also identified in a vancomycin-resistant *E. faecium* isolate recovered from swine in 2007 (plasmid “type P1”), highlighting the stability and transmissibility of particular VanA plasmids in different hosts (6). Plasmids clustered in the

group III were mostly classified as members of the Inc18-family based on the presence of the sequences (rep_{pIP501} and rep_{pRE25}, n=9). Some plasmids also carried rep_{pRUM} (n=6); and two did not harbor any rep gene. They contained rel_{pEF1} (n=9), rel_{pCIZ2} (n=1) or none relaxase (n=3). The TA systems ω-ε-ζ (n=3) and Axe-Txe (n=2) were mostly confined to IS*1216*-Tn*1546*-containing plasmids of this group.

Vancomycin-resistant plasmids linked to pRUM and its toxin-antitoxin system Axe-Txe have been recently described preferentially among CC17 *E. faecium* strains from different European countries (16, 34). Although the frequency of Axe-Txe was low, our data corroborate those studies. On the contrary, plasmids containing rep and rel genes homologous to that of pHTβ and pMG1 plasmids, which have been involved in the spread of VRE in Japan and USA, were not associated with Tn*1546* in our collection (41).

Rearrangements observed in *vanA* plasmids during conjugation inferred by similarity of RFLP patterns and variability of plasmid sizes were frequent among plasmids containing Tn1*546*::IS*1216*. Those harbouring Tn1*546*::IS*Ef1* correspond to plasmid type G which showed an identical RFLP over years.

Plasmid sequences identified in this study. Sequencing analysis revealed that rep_{pRUM} was 95%-96% identical to RepA_pRUM (GenBank accession number AF507977); most $rep1_{pIP501}$ sequences were 98%-100% identical to RepE of pIP816, a member of the Inc18 family (GenBank accession number AM932524); and $rep2_{pRE25}$ showed 100% homology to that of pEF1 (GenBank accession number DQ198088). Sequences identified as rel_{pEF1} showed 98%-100% homology to orf34_pEF1. The relaxase of the pheromone-responsive plasmids “type H” displayed <70% homology with EP0019_pAM373 (GenBank acc. no. NC_002630) and EFA0025_pTEF1 (GenBank acc. no. AE016830), indicating the presence of a new mobilization protein.

CONCLUDING REMARKS

Despite the number of studies about the diversity of Tn*1546* variants among VRE from different ecological origins and countries, only a few studies have analyzed the diversity of plasmids in which they are located (12, 13, 32, 37, 47, 50). This is the first study characterizing vancomycin-resistant plasmids from *E. faecium* isolates of hospitalized patients in Portugal. Our data suggest that intra- and interhospital widespread of VREfm in Portuguese hospitals is driven by both clonal spread of CC17 strains and dissemination of particular highly transmissible pRUM and Inc18 plasmids among clones of *E. faecium* and to a lesser extent, other enterococcal species. The Tn*1546* diversity

detected among related plasmids from different strains recovered in distinct hospitals suggests evolution of specific plasmids by different genetic events. Plasmids of VREfm isolates recovered from different hospitals and aquatic environments were similar which reflect wide spread of highly epidemic elements in hospital and community settings.

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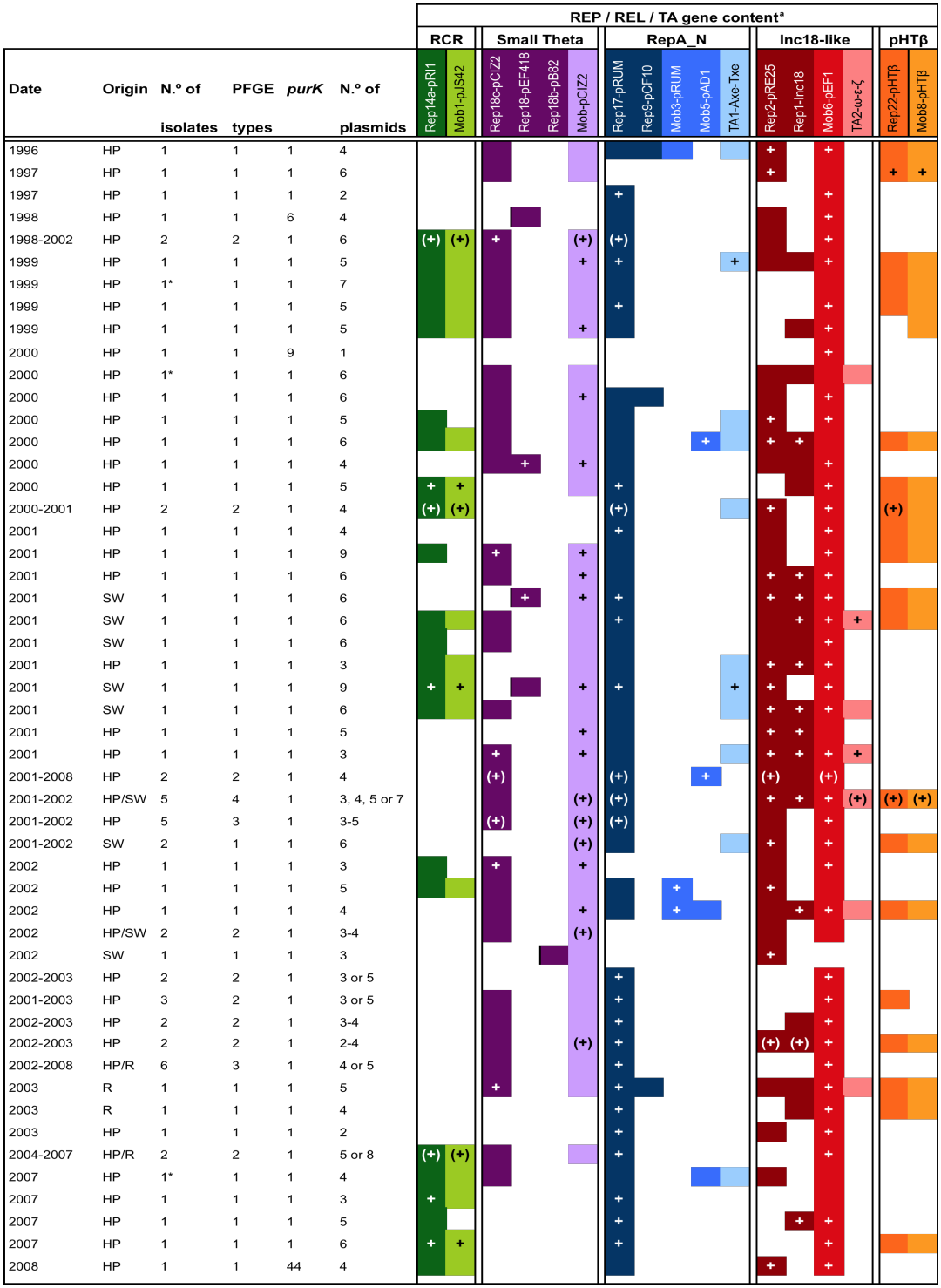
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Table 1. Plasmid content (replication, mobilization and toxin-antitoxin genes) of *vanA E. faecium* isolates from hospitals and aquatic environments of Portugal.



						REP / REL / TA gene content[a]															
						RCR		Small Theta				RepA_N				Inc18-like				pHTβ	
Date	Origin	N.º of isolates	PFGE types	*purK*	N.º of plasmids	Rep14a-pRI1	Mob1-pJS42	Rep18c-pCIZ2	Rep18-pEF418	Rep18b-pB82	Mob-pCIZ2	Rep17-pRUM	Rep9-pCF10	Mob3-pRUM	Mob5-pAD1	TA1-Axe-Txe	Rep2-pRE25	Rep1-Inc18	Mob6-pEF1	TA2-ω-ε-ζ	Rep22-pHTβ	Mob8-pHTβ
1996	HP	1	1	1	4												+		+		
1997	HP	1	1	1	6												+				+ +
1997	HP	1	1	1	2							+							+		
1998	HP	1	1	6	4														+		
1998-2002	HP	2	2	1	6	(+)	(+)	+			(+)	(+)							+		
1999	HP	1	1	1	5						+	+				+			+		
1999	HP	1*	1	1	7																
1999	HP	1	1	1	5							+							+		
1999	HP	1	1	1	5						+								+		
2000	HP	1	1	9	1														+		
2000	HP	1*	1	1	6																
2000	HP	1	1	1	6						+								+		
2000	HP	1	1	1	5												+		+		
2000	HP	1	1	1	6										+		+	+			
2000	HP	1	1	1	4				+		+								+		
2000	HP	1	1	1	5	+	+					+							+		
2000-2001	HP	2	2	1	4	(+)	(+)					(+)					+		+	(+)	
2001	HP	1	1	1	4							+							+		
2001	HP	1	1	1	9			+			+								+		
2001	HP	1	1	1	6						+						+	+	+		
2001	SW	1	1	1	6				+		+	+					+	+	+		
2001	SW	1	1	1	6							+						+	+	+	
2001	SW	1	1	1	6														+		
2001	HP	1	1	1	3												+	+	+		
2001	SW	1	1	1	9	+	+				+	+				+	+		+		
2001	SW	1	1	1	6												+	+	+		
2001	HP	1	1	1	5						+						+	+			
2001	HP	1	1	1	3			+			+						+	+	+	+	
2001-2008	HP	2	2	1	4			(+)				(+)			+		(+)		(+)		
2001-2002	HP/SW	5	4	1	3, 4, 5 or 7						(+)	(+)					+	+	+	(+)	(+) (+)
2001-2002	HP	5	3	1	3-5			(+)			(+)	(+)							+		
2001-2002	SW	2	1	1	6						(+)						+		+		
2002	HP	1	1	1	3			+			+								+		
2002	HP	1	1	1	5								+				+				
2002	HP	1	1	1	4						+		+					+	+		
2002	HP/SW	2	2	1	3-4						(+)										
2002	SW	1	1	1	3												+				
2002-2003	HP	2	2	1	3 or 5							+							+		
2001-2003	HP	3	2	1	3 or 5							+							+		
2002-2003	HP	2	2	1	3-4							+							+		
2002-2003	HP	2	2	1	2-4						(+)	+					(+)	(+)	+		
2002-2008	HP/R	6	3	1	4 or 5							+							+		
2003	R	1	1	1	5			+				+							+		
2003	R	1	1	1	4							+							+		
2003	HP	1	1	1	2							+							+		
2004-2007	HP/R	2	2	1	5 or 8	(+)	(+)					+							+		
2007	HP	1*	1	1	4																
2007	HP	1	1	1	3	+						+									
2007	HP	1	1	1	5							+						+	+		
2007	HP	1	1	1	6	+	+					+							+		
2008	HP	1	1	44	4												+		+		

Abbreviations: Rep (rep initiator protein); Rel/Mob (relaxase); TA (toxin-antitoxin systems); HP, hospitalized patient; SW, sewage; R, river. *Isolates that did not transferred *vanA* by conjugation.

[a]The content of mob, rep and TA genes identified for each isolate by PCR is indicated by different colours, each colour being associated with a given plasmid according with previously published studies and GenBank databases. The isolates are grouped by their content in plasmids. Each range of colour corresponds to a plasmid family (RCR, small theta plasmids, RepA_N which includes pRUM and pheromone-responsive plasmids, Inc18 and pHTβ). Rep genes are indicated according to the plasmid type in which they were identified, as well as by the numeric nomenclature used by Jensen *et al*. (19) and ours (Table 3 of section 6.1.7.3. of appendix of this thesis). Genes transferable by conjugation appear with + and variable transfer results among different isolates with the same plasmid content appear in brackets ().

Table 2. Plasmid types and features of representative *vanA* tranconjugants from hospitals and aquatic sources.

Plasmid group	Nº of isolates	Plasmid type[a]	Plasmid(s) size (kb)[b]	Plasmid content			N.º PFGE types	MLST	Source[c]	Date	Tn*1546* type(s)	IS
				Rep[c]	Relaxase(s)	TA						
I	1	A	60	$rep17_{pRUM}$	pEF1	-	1	non-CC17	HP_A	1998	A	none
	1	B	80	$rep17_{pRUM}$	pEF1	-	1	CC17	HP_A	2000	A	none
	1	C	150	-	pEF1	-	1	non-CC17	HP_C	2002	D	none
	1	D	40	$rep2_{pRE25}$, $rep17_{pRUM}$	pEF1	-	1	CC17	HP_A	1996	PP-10	none
	1	E	60	$rep17_{pRUM}$	-	-	1	CC17	HP_A	2002	PP-27	none
II	1	F	110	$rep2_{pRE25}$	-	-	1	CC17	HP_A	1997	PP-4	IS*Ef1*
	20	**G**	75, 85, 90	$rep17_{pRUM}$, ($rep1_{pIP501}$), ($rep2_{pRE25}$)	pEF1	-	11	CC17	$HP_{A, B, C, D}$, R, UW	2001-2007	PP-3, PP-4, PP-5, PP-24	IS*Ef1*
	1	H	85	$rep1_{Inc18}$, $rep2_{pRE25}$	pAD1	-	1	CC17	HP_A	2000	PP-9	IS*Ef1*
	3	I	85	($rep17_{pRUM}$)	pEF1	-	3	CC17	HP_A	1997-2001	PP-4, PP-9	IS*Ef1*
	3	J	90, 100	$rep17_{pRUM}$, ($rep2_{pRE25}$)	pEF1	-	3	CC17	HP_A	1998-2001	PP-4	IS*Ef1*
	2	K	85	$rep17_{pRUM}$, ($rep2_{pRE25}$)	pEF1	-	2	CC17	HP_A	2000	PP-5	IS*Ef1*
	1	L	60	$rep17_{pRUM}$	pEF1	ω-ε-ζ	1	CC17	HP_C	2002	PP-5	IS*Ef1*
	1	M	40	-	-	-	1	non-CC17	HP_E	2007	PP-5	IS*Ef1*
	1	N	50	$rep17_{pRUM}$	pEF1	-	1	CC17	HP_B	2008	PP-5	IS*Ef1*
III	1	O	120	-	pEF1	-	1	CC17	HP_A	2001	PP-2-b	IS*1216V*
	5	**P**	60	$rep1_{Inc18}$, $rep2_{pRE25}$, $rep17_{pRUM}$	pEF1	ω-ε-ζ	3	CC17	HP_C, HW_C, R	2001-2003	PP-13, PP-17, PP-20, PP-23, X	IS*1216V*
	3	Q	60	$rep17_{pRUM}$	(pEF1), (pCIZ2)	-	2	CC17	HP_A, HP_B	2002	PP-2-b	IS*1216V*
	1	R	60	-	pEF1	-	1	CC17	HP_B	NI	PP-2-b	IS*1216V*
	1	S	60	$rep1_{Inc18}$, $rep2_{pRE25}$	pEF1	ω-ε-ζ	1	CC17	HP_C	2001	PP-13	IS*1216V*
	1	T	110	$rep1_{Inc18}$, $rep2_{pRE25}$, $rep17_{pRUM}$	pEF1	ω-ε-ζ	1	CC17	HP_C	2001	PP-13	IS*1216V*
	1	U	65	$rep2_{pRE25}$, $rep17_{pRUM}$	pEF1	Axe-Txe	1	CC17	HP_C	2001	PP-15	IS*1216V*
	2	V	30	$rep1_{Inc18}$, $rep2_{pRE25}$	-	-	2	CC17	HW_F	2001	PP-16	IS*1216V*
	1	W	40	$rep2_{pRE25}$	-	-	1	CC17	HW_F	2001	PP-16	IS*1216V*
	1	X	55	$rep1_{Inc18}$, $rep2_{pRE25}$	pEF1	-	1	CC17	HW_C	2001	PP-17	IS*1216V*
	1	Y	50	$rep1_{Inc18}$, $rep17_{pRUM}$	pEF1	-	1	CC17	HP_C	2001	PP-13	IS*1216V*
	1	Z	30	$rep2_{pRE25}$, $rep17_{pRUM}$	-	Axe-Txe	1	CC17	HW_F	2002	PP-16	IS*1216V*

Abbreviations: IS, insertion sequence; CC, clonal complex; HP, hospital; HW, hospital wastewater; UW, urban sewage; R, river.
[a]Plasmid types were designated by capital letters from A to Z, accordingly to *Eco*RI, *Hind*III and *Cla*I restriction profiles. The most disseminated plasmid types G and P appear in bold.
[b]Approximate plasmid sizes (kb) found in transconjugants presenting similar plasmid patterns.
[c]Rep genes are indicated according to the plasmid type in which they were identified, as well as by the numeric nomenclature used by Jensen *et al*. (19).
[d]Representative VRE isolates were recovered from five hospitals in five cities: Coimbra (A), Viseu (B), Porto (C), Matosinhos (D), Covilhã (E), and from sewages close to two hospitals in Oporto (C, F).

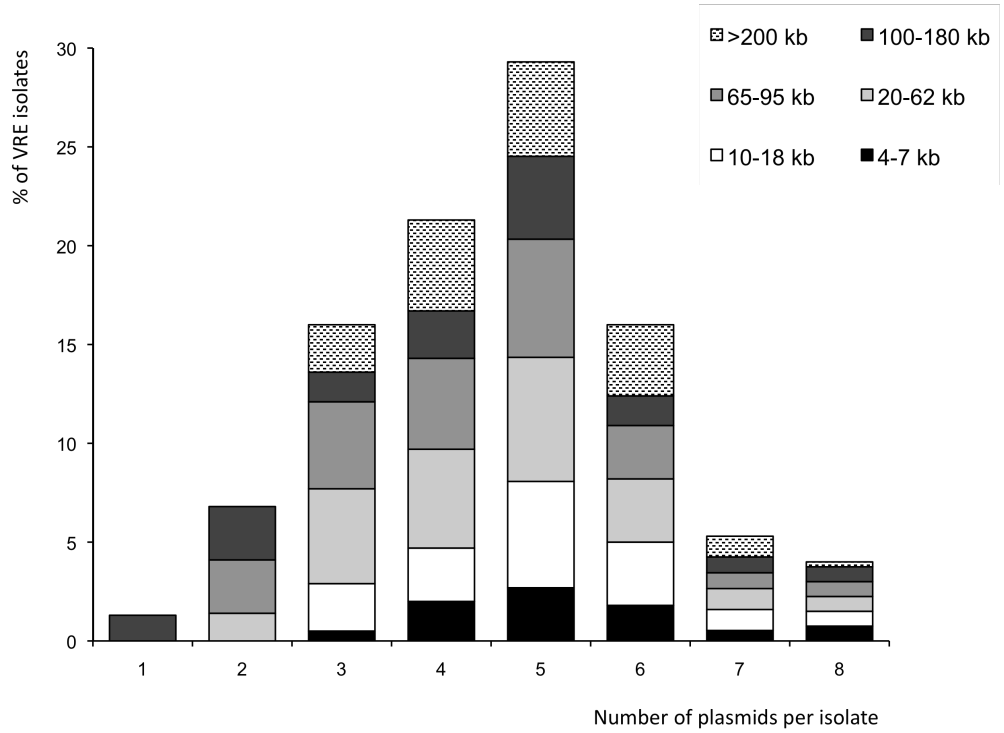


Figure 1. Number and size of plasmids among VRE isolates analyzed.

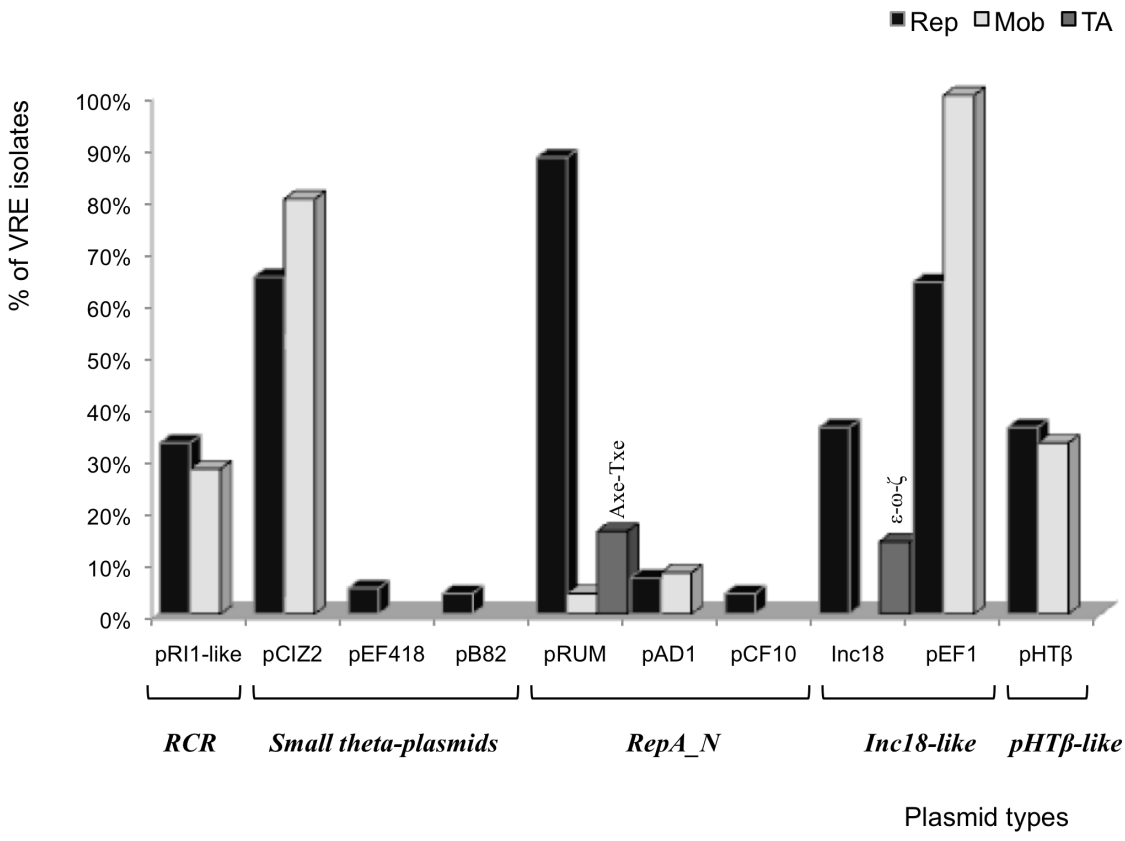


Figure 2. Plasmid content of 75 *vanA* isolates recovered from Portuguese hospitals and aquatic environments during 1996-2008. Rep, rep initiator proteins; Mob, relaxases; TA, toxin-antitoxin systems.

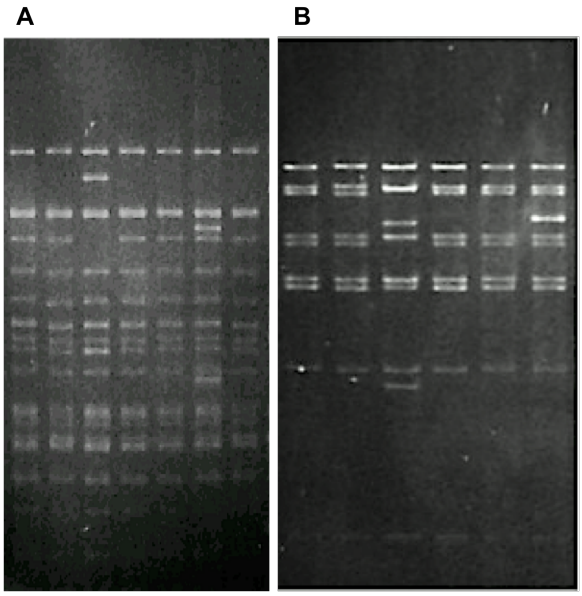


Figure 3. Restriction fragment length polymorphism patterns of plasmid G after digestion with *Cla*I (A) and *Eco*RI (B) restriction enzymes (New England Biolabs Inc, UK). *Cla*I revealed to be the more discriminatory enzyme, as more than 15 bands could be generated after plasmid restriction.

Host range of enterococcal *vanA* plasmids among Gram-positive intestinal bacteria

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Host range of enterococcal *vanA* plasmids among Gram-positive intestinal bacteria

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Objectives: The most prevalent type of acquired glycopeptide resistance is encoded by the *vanA* transposon Tn*1546* located mainly on transferable plasmids in *Enterococcus faecium*. The limited occurrence in other species could be due to the lack of inter-species transferability and/or stability of Tn*1546*-containing plasmids in other species. We investigated the *in vitro* transferability of 14 pre-characterized *vanA*-containing plasmids hosted by *E. faecium* ($n=9$), *Enterococcus faecalis* ($n=4$) and *Enterococcus raffinosus* ($n=1$) into several enterococcal, lactobacterial, lactococcal and bifidobacterial recipients.

Methods: A filter-mating protocol was harmonized using procedures of seven partner laboratories. Donor strains were mated with three *E. faecium* recipients, three *E. faecalis* recipients, a *Lactobacillus acidophilus* recipient, a *Lactococcus lactis* recipient and two *Bifidobacterium* recipients. Transfer rates were calculated per donor and recipient. Transconjugants were confirmed by determining their phenotypic and genotypic properties. Stability of plasmids in the new host was assessed in long-term growth experiments.

Results: In total, 282 enterococcal matings and 73 inter-genus matings were performed and evaluated. In summary, intra-species transfer was far more frequent than inter-species transfer, if that was detectable at all. All recipients of the same species behaved similarly. Inter-genus transfer was shown for broad host range control plasmids (pIP501/pAMβ1) only. Acquired resistance plasmids remained stable in the new host.

Conclusions: Intra-species transfer of enterococcal *vanA* plasmids was far more frequent than transfer across species or genus barriers and may thus explain the preferred prevalence of *vanA*-containing plasmids among *E. faecium*. A reservoir of *vanA* plasmids in non-enterococcal intestinal colonizers does not seem to be reasonable.

Keywords: vancomycin resistance, *repA*, relaxases

Introduction

The rapid global emergence of transferable high-level glycopeptide resistance in enterococci in the late 1990s has challenged the already limited therapeutic options in the treatment of severe enterococcal infections. Different types of acquired vancomycin resistance have been described in enterococci based on phenotypic and genotypic characteristics.[1,2] By far the most prevalent type is *vanA* followed by *vanB*. The *vanA* gene is contained in Tn*1546* or its derivatives usually located on transferable plasmids. The *vanB* gene is part of Tn*1547* or the conjugative transposon CTn*1549/5382*, mainly chromosomally located and less frequently found on plasmids.[2,3] The main clinically relevant reservoir of *vanA* and *vanB* is in *Enterococcus faecium*, at least in Europe, Northern and Latin America and Southeast Asia.[2,4] Nowadays, acquired *vanA*-type vancomycin-resistant *E. faecium* (VREF) are widely prevalent in hospitals around the world.[5] A distinct *vanA* reservoir exists also in

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commensal *E. faecium* isolates from commercial animal husbandry, which differ from hospital strains,[6–10] and in *Paenibacillus* strains from environmental samples.[11–13] Intestinal commensal bacteria such as *Ruminococcus*, *Rhodococcus* and others could serve as a reservoir for *vanB* and other *van* gene clusters.[11,14,15] It has also been discussed whether commensal intestinal colonizers such as bifidobacteria, lactobacilli and lactococci may participate in lateral gene transfer of vancomycin resistance plasmids.[16]

A few plasmids containing Tn*1546* or derivatives have been fully sequenced or partially characterized,[17–19] including transferable plasmids of various types, such as pHTβ1, pIP501-/pRE25-like (Inc18 family) and pRUM-like.[17–23] However, it is not clear whether these plasmids represent the most common plasmid variants carrying *vanA* since many investigated *vanA* plasmids remain non-typeable.[23–25] The mosaic-like composition of enterococcal plasmids leads to problems in typing them when typing is performed only on single plasmid marker genes.[18,21,22,26–28] The putative plasmid/gene reservoir that exists in related non-enterococcal species could supply resistance genes for future spread.[16,29]

Transfer of *vanA* (and *vanB*) clusters has been demonstrated in several studies[30–35] including broad host range plasmid-mediated inter-genus transfer of Tn*1546* into methicillin-resistant *Staphylococcus aureus* (MRSA).[33,36,37] Transfer of *vanA* plasmids into other non-enterococcal species has also been described previously.[38,39]

Many questions remain unanswered with regard to transferability and host reservoir of the mobile *vanA* resistance cluster, for instance why prevalence of Tn*1546* is mostly limited to *E. faecium*. *Enterococcus faecalis*, for instance, possesses a very efficient pheromone response system allowing plasmid exchange at high rates.[40,41] The mobility of Tn*1546*-like elements might be limited by a preference for specific attachment sites.[42]

In the present study we assessed the role of horizontal plasmid transfer for successful dissemination of *vanA*-type clusters and plasmids among various Gram-positive intestinal colonizers. Several representative VRE clinical (outbreak) and animal strains were selected originating from several countries. VanA transferability and maintenance were assessed within various enterococcal and other Gram-positive intestinal colonizers known to exchange resistance determinants with enterococci (lactobacilli, lactococci, bifidobacteria). Altogether 355 matings were performed and corresponding transconjugants were confirmed by phenotypic and molecular methods.

Materials and methods

Bacterial strains and plasmids

Lists of donor and recipient strains are given in Tables 1 and 2, respectively. A number of non-enterococcal recipient bacteria were considered and marker resistances were introduced as part of this study (for details see Table 2). Project partners were asked to send VRE donor isolates that were representative of regional or countrywide outbreak strains, historical VRE from human infections or from colonizations in animals from the early 1990s until now (Table 1). For reasons of comparison *E. faecalis* RE25::pRE25 harbouring a completely sequenced Inc18-prototype plasmid (non-*vanA*) was included. Donor strains had tested positive in previous intra-species filter matings.

Antibiotic susceptibility testing

Enterococcal strains were examined by microbroth dilution according to the German standard DIN 58940 on antibiotic susceptibility testing. MICs for lactococcal, lactobacterial and bifidobacterial isolates were determined as described previously.[43,44]

Introducing marker resistances into selected recipients

Marker resistances for new enterococcal and non-enterococcal recipients were introduced randomly by streaking out exponentially grown cells ($>10^{10}$ cfu) on agar plates with stepwise increasing concentrations of the selective antibiotics (not described in detail). MICs were determined as described above using microbroth dilution and Etest® (bioMérieux, Marcy L'Etoile, France). All recipient candidate strains were plasmid free, except *E. faecium* AK-EM40, which contained several plasmids (see the Results section).

Filter-mating protocol

Seven partners shared their filter-mating procedures on the basis of which a harmonized protocol was established. All experiments were performed on three filters in parallel. For details including concentrations of used antibiotics please see the Supplementary data (available at *JAC* Online). Due to weak growth on selective agar plates, we decided to reduce the rifampicin concentration from 30 to 10 mg/L for matings with recipient GE-1 (MIC=32 mg/L). Brain heart infusion (BHI) broth and agar plates were used for matings with enterococcal recipients. When using lactococcal, lactobacillus and bifidobacterial recipients conditions and media favouring growth of the recipients were considered, such as strict anaerobic growth conditions in or on lactic acid bacteria susceptibility medium (LSM) or deMan, Rogosa-Sharp (MRS) media (for bifidobacteria: +0.3 g/L cysteine) or M144 agar medium (for bifidobacterial matings).[43,44] All transconjugants were confirmed by phenotypic species identification (inter-species matings) and antibiotic susceptibility testing (see next paragraph).

Phenotypic characterization of transconjugants

One cfu per filter ($n=3$) was again separated on selective agar plates. Species and antibiotic susceptibilities were determined. Transconjugants of recipient 64/3 were analysed in follow-up experiments investigating the stability of acquired resistance plasmids by long-term growth experiments over 4 weeks in BHI. Samples were passaged daily (0.1 mL of overnight culture in 5 mL of BHI). At the end of each week samples were taken and stored at −70°C. Colony counts were measured on selective (vancomycin/fusidic acid/rifampicin or erythromycin/fusidic acid/rifampicin) and non-selective agar plates without antibiotics after each week. Generation times were calculated based on Sandegren *et al.*[45] Transfer of broad host range plasmids pIP501 (*E. faecalis* OG1X::pIP501) and pAMβ1 (OG1X::pAMβ1) was used as a control for combinations that revealed no detectable *vanA* plasmid transfer (see the Results section).

Bacteriocin assays

Susceptibility of recipients to bacteriocin(-like) or other inhibitory substances released from donor strains was assayed in BHI agar plate assays. Recipient cells were grown for 3–4 h in BHI broth to reach cell densities equivalent in turbidity to that of a 0.5 McFarland standard. They were diluted 1:100 in saline and streaked onto the entire agar plate expecting semi-confluent growth after overnight incubation at 37°C. Donor cells were spotted onto the agar (~10^9 cells) directly after recipient strains were inoculated and plates were incubated overnight at 37°C. Bacteriocin activity was assessed quantitatively by measuring the diameter of growth inhibition around donor cell colonies.

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Table 1. List of donor strains

Study/strain no.	Species	Strain name	Origin	Country of origin	Year	Plasmid characteristics (van, rep, PSK, tra)	No. of plasmids (sizes in kb)[a]	MLST	Selected donor resistance marker (other resistances)	Remarks/other features	References
1	*E. raffinosus*	TUH 50-23 (UW7358)	faecal	Canada	2006	170 kb *vanA* plasmid; PSK: *axe-txe*, *rep*(pRUM)	1 (**170**)	—	VAN	plasmid transfer previously detected between various enterococcal species	this study (Sollid/Sundsfjord)
2	*E. faecium*	70/90	clinical	France	1990	100 kb; *vanA*, PSK: *axe-txe*(+); (*rep*-Inc18)	2 (**100**, 45)	ST25	VAN (ERY)	strain from France, similar to BM4147::pIP816, but with transferable *vanA* plasmid	[68]
3	*E. faecium*	UW931	BAL	Germany	1996	100 kb; pEF1-like *vanA-tet*(M) plasmid; *rep*(pEF1), T4SS, tra(Tn*925*); *pilA*; + 6 kb pCIZ2-like bacteriocin plasmid; + 4 kb pRI1-like plasmid + 6 kb unknown plasmid[b,c]	4 (**100**, 6, 6, 4)	ST117	VAN (ERY)	epidemic VRE strain with countrywide spread 1995–99 in Germany	[52]
4	*E. faecium*	UW3540	urine	Germany	2002	80 kb *vanA-erm*(B)-*vatD* plasmid (*rep*-Inc18); *axe-txe*(+), but not on *vanA* plasmid	3 (**80**, 30, 10)	ST145	VAN (ERY, QD)	Q/D-resistant outbreak strain with *vanA* and *vatD* resistance cluster on a transferable plasmid: TC only acquired the 80 kb plasmid	[69]
5	*E. faecium*	H182 (UW7353)	hepatic fluid	Portugal	2002	92 kb *vanA* plasmid (*rep*-pRUM)[c]	5 (12, 40, **92**, 140, 230)	ST18	VAN (ERY, KAN)	Tn*1546*-type: PP-4; widespread and persistent plasmid type (2001–07)	[70]
6	*E. faecium*	H311 (UW7483)	urine	Portugal	2002	60 kb *vanA* plasmid (rep-Inc18, *rep*-pRUM; PSK: *ω-ε-ζ*)	4 (10, 50, **60**, 200)	ST132	VAN (ERY, GEN)	epidemic plasmid from Portugal (2001–02)	[70]
7	*E. faecium*	E0292 (UW7486)	urine	USA	1992	200 kb *vanA* plasmid (*rep*-Inc18)	6 (5, 8, 10, 22, 60, **200**)	ST20	VAN (ERY, GEN, STR)	hospital outbreak strain in Detroit/USA	this study (Freitas/Coque)
8	*E. faecium*	H74 (UW7490)	hepatic fluid	Portugal	2001	50 kb *vanA* plasmid (*rep*-Inc18, *rep*-pRUM; PSK: *ω-ε-ζ*)	7 (12, 30, **50**, 60, 170, 200, 350)	ST132	VAN (ERY, GEN)	[d]	[70]
9	*E. faecium*	E0013 (UW7492)	urine	UK	1992	50 kb *vanA* plasmid, *rep*-pHTβ, *rep*-Inc18; PSK: *ω-ε-ζ*	2 (25, **50**)	ST18	VAN (ERY, TET, STR)	one of the first VRE identified in Europe	this study (Freitas/Coque)
10	*E. faecium*	UW261	food	Germany	1995	172 kb*vanA* plasmid; unknown *repA* type (*rep*-pAD1/-Inc18/-RUM-negative); PSK: *ω-ε-ζ*	1 (**172**)	ND	VAN (ERY, TET)	isolate from pork	[68]
11	*E. faecalis*	Val-1 (UW7489)	blood	Spain	1999	100 kb *vanA* plasmid (*rep*-pAD1)[c]	2 (75, **100**)	ST6	VAN (ERY, GEN)	epidemic plasmid from Spain and Italy; larger plasmid in TC	this study (Freitas/Coque)
12	*E. faecalis*	229710 (UW7770)	clinical	Portugal	1996	85 kb *vanA* plasmid (*rep*-pAD1, *rep*-Inc18; PSK: *par*)	1 (**85**)	ST6	VAN (GEN, TET, ERY)	clone B (ST6/CC2); strain widely disseminated all over Portugal and other EU countries (1996–2008)	[46]
13	*E. faecalis*	536540 (UW7771)	clinical	Portugal	2000	85 kb *vanA* plasmid (*rep*-pAD1; PSK: *par*)	2 (**85**, 145)	ST21	VAN (GEN, ERY, TET)	clone X (CC21)[d]	[46]
14	*E. faecalis*	H143 (UW7766)	wound	Portugal	2001	100 kb *vanA* plasmid *rep*-pFC10[b]	1 (**100**)	ST30	VAN (ERY, TET)	clone K	[46]
15	*E. faecalis*	RE25::pRE25	food	Switzerland	<1995	50 kb plasmid pRE25; *repR*(pRE25 = Inc18); tra(T4SS), PSK: *ω-ε-ζ*	1	ND	ERY (STR, CHL, KAN)	similar to pIP501	[71]

BAL, broncheoalveolar lavage; urine, from urinary tract infection; TC, transconjugant; ND, not determined; CHL, chloramphenicol, ERY, erythromycin; GEN, gentamicin (high-level); KAN, kanamycin (high-level); Q/D, quinupristin/dalfopristin; STR, streptomycin (high-level); TET, tetracycline; VAN, vancomycin; tra(Tn*925*), cluster of conjugative genes similar to Tn*916* prototype of conjugative transposons; tra(T4SS), transfer region for type 4 secretion systems involved in horizontal gene transfer.
repA classification according to Jensen *et al.*[48]
PSK systems: *ω-ε-ζ*, *axe-txe* and *par*.
[a]The *vanA* plasmid is shown in bold.
[b]UW931: plasmid data were obtained from Southern hybridizations and 454 plasmid sequencing (not described in detail; G. Werner, unpublished data). Isolate also contained the following plasmids: 6 kb pCIZ2-like bacteriocin plasmid; 4 kb pRI1-like plasmid; and 6 kb unknown cryptic plasmid.
[c]Negative in PCR screenings for PSK systems *ω-ε-ζ*, *axe-txe* and *par*.
[d]Plasmids rearranged when initial mating experiments were performed in the primary laboratory; plasmid rearrangements were not confirmed in experiments performed as part of this study (see also Figure S3).

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Table 2. List of candidates considered as recipients

Species	Strain no.	Wild-type MICs		Mutant MICs		MLST type/comments
		RIF	FUS	RIF	FUS	
E. faecalis	JH2-2			128	128	ST8
E. faecalis	OG1RF			>32	>32	ST1
E. faecalis	UV202 (JH2-2::*recA*-)			64	128	ST8[72]
E. faecium	64/3			>128	>128	ST21
E. faecium	GE-1			32	256	ST515
E. faecium	BM4105-RF			>256	256	ST172
E. faecium	AK-EM40**RF**	4	2	**256**	**256**	ST18[a,b]
L. acidophilus	UW3767**RF**	1	32–128[c]	**256**	32–128[c]	[d]
L. acidophilus	UW5688**RF**	≤0.008	32–128[c]	**256**	32–128[c]	
L. lactis	UW4127**RF**	16	4/2	**(>)1024**	**128**	
B. bifidum	UW4268**R**	≤0.008	4/1	**256**	**NS**	
B. breve	UW4272**R**	≤0.008	16[e]	**256**	**NS**	[d]
B. longum	UW4385**R**	≤0.008	8/2	**256**	**NS**	

FUS, fusidic acid; RIF, rifampicin; NS, not successful [several failed attempts were performed to select fusidic acid-resistant bifidobacterial mutants (concentrations of 20 and 30 mg/L)].
Characteristics marked in bold represent features introduced during this study.
[a]ST18 is MLST clonal complex CC17.
[b]*rep* gene PCRs revealed pRE25-like and pVEF1-like plasmids in AK-EM40, but were negative for *rep*(pRUM) und *rep*(pAD1).[48]
[c]Varying MICs in repeated tests.
[d]Not considered for mating experiments.
[e]Unstable fusidic acid resistance.

Plasmid analysis

Plasmids were isolated using a modified mini-preparation based on phenol/chloroform purification.[32] Alternatively, a modification of the protocol by Barton *et al.*,[46,47] describing S1 nuclease treatment of genomic DNA and resolution of the linearized plasmids in PFGE, was performed (see below). *repA* homologues were identified according to a recent protocol.[48] Primers for gene clusters encoding several post-segregational killing (PSK) systems of the *E. faecium* pRUM plasmid *axe-txe*,[22] the *E. faecalis* pAD1 PSK *par-fst*[49] (primer sequences by K. Weaver, personal communication) and the streptococcal pSM19035 or enterococcal pVEF1-3 ω-ε-ζ type[7] were as follows: axe-txe-F (5′-CTGACCCTTTCCTTA CTTCCG) and axe-txe-R (5′-GGTGAAAGGAATGGAAGCAG) (size 557 bp); par-fst-F (5′-TAATGCGGCAGCTCGCCTCGATT) and par-fst-R (5′-AGCAGCTT ACTTTCGGCTATCGTCT) (size 120 bp); and ε-ζ-F (5′-GTGGTTTAGGTGGCTG CAAG) and ε-ζ-R (5′-TTAACGAATTATCGGCAAGC) (size 1063 bp).

DNA isolation, PCR, sequencing and multilocus sequence typing (MLST)

Genomic DNA was prepared using a DNA extraction kit (DNeasy Tissue Kit; Qiagen, Hilden, Germany).[50] PCR was performed with Illustra® puRe Taq Ready-To-Go PCR beads (GE Healthcare, Freiburg, Germany) as described previously.[50] MLST was carried out according to the reference MLST database (http://efaecium.mlst.net/). Sequencing reactions were performed according to the manufacturer's recommendations for cycle sequencing (Applied Biosystems, Darmstadt, Germany). Sequence files were read, evaluated, aligned and compared with the reference set of alleles using DNASTAR's Lasergene 8.0 software, TraceEditPro v. 1.1.1 from Ridom (http://www.ridom.de) and via the official MLST web site (http://efaecium.mlst.net/).

SmaI- and S1-PFGE

Genomic DNA of transconjugants was isolated, digested with SmaI and resolved in PFGE using 1% agarose gels and a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA, USA).[50] For sizing large plasmids genomic DNA was treated with S1 nuclease[47] from Takara (TaKaRa Bio, Inc., Shiga, Japan) and conditions and criteria set by a protocol described recently,[46] including ramped pulse times of 5–35 s for 22 h at 14°C. SmaI-digested *S. aureus* NCTC 8325 was used as a size marker. Data were analysed and plasmid sizes were calculated using a Dice coefficient and UPGMA clustering (BioNumerics v. 5.1; Applied Maths, Sint-Martens-Latem, Belgium).

Results

Introducing marker resistances into recipients

Most enterococcal recipients were already high-level resistant to rifampicin and fusidic acid (Table 2). We introduced strain AK-EM40, representing an antibiotic-susceptible MLST sequence

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type (ST)18 strain to test whether isolates belonging to the clonal complex (CC) of hospital-associated strains (CC17) might be better candidates for resistance gene acquisition. All descendants of AK-EM40 and lactococcal, lactobacterial and bifidobacterial recipient candidates were selected randomly (Table 2). We were unable to select spontaneous fusidic acid-resistant strains of any of the bifidobacterial recipients in repeated attempts and with varying concentrations. Nevertheless, differentiation between enterococcal donors and putative bifidobacterial transconjugants is possible via phenotypic species and resistance pattern characterization.

Harmonizing the filter-mating protocol

A harmonized mating protocol was established based on the procedures shared by laboratories involved in this study (see the Materials and methods section). All matings described here were performed at a single laboratory according to this document.

Filter mating with enterococcal recipients

Altogether 15 donors were chosen representing (i) major outbreak clones spread in several countries, (ii) specific plasmid-located multiresistance genotypes or (iii) fully sequenced resistance plasmids (pRE25). All donor strains (Table 1) were mated with three to five *E. faecium* and three *E. faecalis* recipients (Table S1, available as Supplementary data at *JAC* Online). Altogether 94 enterococcal matings were performed (in triplicate; $n=282$) and results are summarized in Table 3 (detailed data presented in Table S1). In general, intra-species matings were successful and independent of the respective recipient strain, and the number of transconjugants appeared at comparably high rates ($\geq 10^{-6}$ per donor/recipient). For some *E. faecium* donor × *E. faecalis* recipient matings transconjugants occurred at very low frequencies ($\leq 10^{-8}$ per donor/recipient) only detectable on large agar plates (diameter 150 mm) and when using undiluted filter contents. In contrast, three of the five *E. faecalis* donors transferred their resistance determinants at similar rates into *E. faecium* and *E. faecalis* recipients (Table S1). The *Enterococcus raffinosus* strain TUH50-23 transferred plasmid-encoded vancomycin resistance successfully into the three tested *E. faecium* recipients and at rates ~100-fold lower into *E. faecalis* OG1RF. Transfer rates into other *E. faecalis* recipients were only barely detectable or undetectable (for UV202; Table S1). UW931 previously shown to be capable of transferring its *vanA* plasmid[51,52] was unsuccessful in all attempts. Differences in mating results and supposed host range were not generally congruent with corresponding *repA*-determined plasmid types; e.g. Inc18-type plasmids of *E. faecalis* (RE25) and *E. faecium* (H311, E0292, UW3540) and pAD1-like plasmids in *E. faecalis* (Val-1, 229710, 536540) showed different results (Table 3).

Initial matings with several donor strains and CC17 recipient AK-EMRF all demonstrated no transfer whereas transfer into other *E. faecium* recipients was successful at high frequencies (Table 3 and Table S1). The reasons for this discrepancy were not clear but might be due to various plasmids already present in AK-EMRF leading to plasmid incompatibility or due to the susceptibility of AK-EM40RF to bacteriocins or inhibitory substances released from donor strains (see below). *repA* homologues of the Inc18 type were found by PCR in AK-EM40RF, whereas PCRs for *rep*(pRUM) and *rep*(pAD1) were negative. Due to the initial findings AK-EMRF was excluded from further matings.

Bacteriocin production in donor strains

It was known from previous analyses that donor strain *E. faecium* UW931 is a very potent bacteriocin producer at least when tested against *E. faecium* 64/3 and *E. faecalis* JH2-2.[51,53] Preliminary plasmid sequencing (G. Werner, unpublished results) revealed nucleotide identity with plasmid fragments of pCIZ2 and pEF1 encoding enterocins EntQ and AS-48/EntIJ, respectively.[27,54] Nevertheless, we were able in previous filter-mating experiments to transfer the vancomycin resistance determinant into *E. faecium* 64/3.[51,55] However, we used shorter filter incubation times (4 h). Preliminary analysis revealed bacteriocin production in UW931 during the late exponential growth phase (I. Klare and G. Werner, unpublished results). When counting recipients, donors and transconjugants after filter incubation, we noticed drastically reduced numbers for recipient cell counts (see matings nos 65–67; Table S1). This putative influence of mating time and supposed bacteriocin production on the outcome of mating experiments prompted us to study susceptibilities of the recipients used and putative bacteriocin production in donor strains in agar plate assays [Table S2 (available as Supplementary data at *JAC* Online) and Figure S1 (available as Supplementary data at *JAC* Online)]. Inhibition of growth of recipients appeared frequently and was dependent on the donor strain tested and the recipient used. The effect was specific since certain donor strains (UW261) inhibited growth of only a subset of tested recipients (Table S2). Recipient AK-EM40RF appeared to be more susceptible to inhibitory substances released from donor cells than others. UW931 was again confirmed as a potent bacteriocin producer capable of inhibiting all tested *E. faecium* and *E. faecalis* recipients; however, with variable activity. Inhibition of growth of recipient cells should be considered as a possible reason for undetectable or low resistance gene transfer, which could be missed if only transfer rates per donor are assessed and/or when cell counts are determined only before filter incubation.

Matings with non-enterococcal recipients

To assess the host range of enterococcal resistance plasmids among commensal intestinal colonizers, filter matings with lactobacterial, lactococcal and bifidobacterial recipients were performed using the harmonized protocol (with only one instead of three filters). No transconjugants were obtained with any of the lactobacterial or bifidobacterial recipients (Table 3). Recipient counts of the *L. lactis* recipient were drastically reduced after filter incubation (≤500 cfu/mL) independent of aerobic or anaerobic test conditions (Table S3, available as Supplementary data at *JAC* Online). Counts of the *Lactobacillus acidophilus* recipient cells were not affected, but after mating with *E. raffinosus* TUH50-23 recipient counts dropped to undetectable numbers (<50 cfu/mL). Bifidobacterial recipient counts were not affected by filter incubation, but the *E. raffinosus* donor strain TUH50-23 did not survive filter mating on M144 medium under anaerobic growth conditions.

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Table 3. Results of filter matings with enterococcal, lactococcal, lactobacterial and bifidobacterial recipients

	Donors														
	E. raffinosus	*E. faecium*									*E. faecalis*				
Recipients	TUH50-23	H182	70/90	UW3540	H311	E0292	H74	E0013	UW931	UW261	RE25::pRE25	Val-1	229710	536540	H143
Efm 64/3	+	+	+	+	+	+	+	+	−	+	+	−	+	+	−
Efm BM4105RF	+	+	+	+	+	+	+	+	−	+	+	−	+	+	−
Efm GE-1	+	+	+	+	+	+	+	+	−	+	+	−	+	+	−
Efm AK-EM40RF	ND	−	−	−	−[b]	−[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND
Efs OG1RF	+	−	(+)[a]	−	(+)[a]	−	(+)[a]	(+)[a]	−	−	+	+	+	+	+
Efs JH2-2	(+)[a]	−	(+)[a]	−	(+)[a]	−	(+)[a]	(+)[a]	−	−	+	+	+	+	+
Efs UV202	−	−	(+)[a]	−	(+)[a]	−	−	(+)[a]	−[b,c]	ND	+	+	+	−[d]	(+)[a]
Ll 4127RF A	−[b]	−[b]	−[b]	−[b]	−[b]	−[b]	−[b]	−[b]	−[b]	−[b]	ND	−[b]	−[b]	−[b]	−[b]
Ll 4127RF AA	−[b]	−[b]	−[b]	−[b]	−[b]	−[b]	−[b,d]	−[b]	−[b]	−[b]	ND	−[b]	−[b]	−[b]	−[b]
La 5688RF	−[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Bb UW4268R	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Bl UW4365R	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

AA, anaerob; A, aerob; Bb, *B. bifidum*; Bl, *B. longum*; Efm, *E. faecium*; Efs, *E. faecalis*; La, *L. acidophilus*; Ll, *L. lactis*; ND, not determined.
[a]Only with low efficacy (transconjugants only grew on large selective plates; average rates $<10^{-7}/10^{-8}$).
[b]No or only weak growth of recipient cells on selective plates (<10 cfu/mL filter content).
[c]Performed three times.
[d]No or only weak growth of donor cells on selective plates (<10 cfu/mL filter content).

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We performed control experiments with broad host range plasmids pIP501 and pAMβ1 shown to be transferable into lactic acid bacteria and bifidobacteria.[56,57] We successfully transferred pIP501 into *L. lactis* UW4127RF but were unable to select for pIP501/pAMβ1 transfer into bifidobacterial recipients (*B. bifidum* UW4268R/*B. longum* UW4385R).

Analysis of selected transconjugants

For matings using recipient *E. faecium* 64/3 one transconjugant per mating was confirmed by SmaI macrorestriction of genomic DNA in PFGE. PFGE of transconjugants displayed banding patterns nearly indistinguishable from that of the recipient (Figure S2A, available as Supplementary data at *JAC* Online). The few additional bands in transconjugants compared with the recipient most probably derive from SmaI-digested plasmids. Plasmid acquisition in transconjugants of recipient 64/3 was confirmed by S1 nuclease PFGE (Figure S2B, available as Supplementary data at *JAC* Online). In some matings only the *vanA*-containing plasmid was transferred (H182, E0292, UW3540) while in other matings all plasmids of the donor strain were transferred (H311) (Table 1 and Figure S2). It is important to note that small, cryptic plasmids of ≤10 kb are not well visualized in standard S1 PFGE, so it cannot be excluded that some of the donor plasmids were also mobilized into the transconjugants, while unnoticed in S1 PFGE.

Efficiency of transfer and capability of replication in the new host are important prerequisites for maintenance of resistance plasmids in new hosts. For long-term persistence and stable maintenance on a population scale, stability of plasmid persistence in the absence of selective pressure by antibiotics is another important characteristic. We performed growth experiments on all 12 transconjugants of recipient *E. faecium* 64/3 in non-selective (antibiotic-free) liquid BHI broth for 4 weeks. We did not see substantial differences in the colony counts on selective or non-selective media (Table S4, available as Supplementary data at *JAC* Online). Minor variations, e.g. for H74×64/3 TC1 with a factor of +3 to +3.5 between non-selective and selective growth, were reproducible over weeks and thus reflect less efficient growth of transconjugant cells under stringent selective conditions rather than plasmid loss. S1 nuclease-digested plasmid DNA resolved in PFGE revealed stable plasmid patterns in all transconjugants investigated before and after the growth period (Figure S3, available as Supplementary data at *JAC* Online). In conclusion, newly acquired resistance plasmids were all stably maintained at the population level under non-selective conditions for ∼100 generations.

Discussion

In this study we assessed the role of horizontal plasmid transfer for successful dissemination of *vanA*-type resistance among enterococci and other Gram-positive, commensal, intestinal bacteria known to share a common gene pool and a similar ecological niche with enterococci.

In general, the harmonized protocol worked well and could be recommended, at least for mating experiments among enterococcal strains (see also comments below). Mating time on the filter appeared to be of critical importance. Overnight incubation either on filters or in liquid broth is accepted for conjugation experiments with bacteria in general. Prolonged incubation times may affect survival of recipients and donors on filters and thus reduce transconjugant counts in a qualitative and quantitative manner (Table S3).[56] Transconjugants could also serve as additional donors, being capable of transferring resistance plasmids to further recipients. Due to high cell counts, bacterial growth is not expected to happen during filter incubation. Recipient counts might also drop dramatically due to a supposed production of bacteriocin-(like) substances released from some donor strains (Table S2 and Figure S1). According to all these arguments it may be advisable to reduce the mating time to 2–4 h. Also it appeared beneficial to determine all cell counts after filter incubation and calculate rates per donor and per recipient numbers to visualize and address any effect of cell survival during filter incubation.

Mating results demonstrated a rather limited host range of the *vanA* plasmids examined. As expected, intra-species enterococcal transfer rates were highest, and transfer was less efficient (if demonstrable at all) between different enterococcal species (Table 3). Transfer across species barriers revealed higher rates from *E. faecalis* donors to *E. faecium* recipients than vice versa. This is in agreement with recent reports demonstrating *E. faecalis* pheromone-like plasmid transfer into or prevalence among *E. faecium* isolates;[58] also here all *E. faecalis vanA* plasmids were of the pAD1 or pCF10 type, at least as determined by their replicase genes. Very low transfer efficiencies, e.g. specific for certain *E. faecium vanA* plasmids transferred into *E. faecalis* recipients, were only detectable after a high inoculum on large selective agar plates (plasmid types: Inc18-, pEF1-, pHTβ-like; Tables 1 and 3). Most common mating protocols do not consider using large selection plates to increase detection limit by a factor of 5- to 10-fold, which may change completely the overall outcome of the experiment in the case of very low transfer rates near the detection limit ($<10^{-8}$ cfu/mL). Recipient strains of the same species revealed similar mating results; however, mating rates varied (Table S1). Plasmid transfer rates were independent of *recA* status since mating rates did not differ between isogenic JH2-2 (*recA*+) and UV202 (*recA*−) recipients. Our results are in congruence with results of recent plasmid classification schemes suggesting a narrow host range of some plasmid housekeeping genes determined by replicases (*repA_N*) and relaxases (not documented here).[26,28,48] Some investigated *E. faecium vanA* plasmids contained Inc18-family *repA* genes (70/90, E0292); however, mating results did not confirm a supposed broad host range. Locating these determinants to the investigated *vanA* plasmids does not reliably indicate their functional involvement in plasmid replication and for certain plasmids already several *rep* homologues have been detected by Southern hybridization (H311, H74). Due to the mosaic-like structure of enterococcal plasmids, indentifying *rep* classes like the Inc18-like does not unambiguously allow prediction of the plasmid type and host range since other features like conjugation modules and relaxases may be as important, and little is known as to what extent these determinants or PSK systems determine and influence the host range and stable maintenance of their plasmids in various hosts.

The recipient AK-EMRF representing hospital-associated *E. faecium* (CC17) did not acquire *vanA* determinants. These findings contradict the current hypothesis that members of this clonal complex generally acquire resistance genes, plasmids or

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mobile resistance elements more easily than their colonizing counterparts and question the model that multiresistance genotypes among enterococcal clinical strains derive from an enhanced plasmid acquisition capability.[59] Nevertheless, only a single CC17 recipient was examined and it remains to be elucidated whether these findings are generally applicable.

Recent reports demonstrated a considerable reservoir of *vanB* and other *van* determinants in non-enterococcal intestinal colonizers.[11,14–16,60] In addition, it was speculated that lactobacilli may participate in lateral gene transfer of vancomycin resistance plasmids.[16] Our results would not support this hypothesis. However, it cannot be excluded that results are biased by the corresponding *in vitro* filter-mating model and the experimental setting (conditions, strains, plasmids) used here. Parameters set for our protocol were in congruence with a recent harmonized protocol to assess antibiotic resistance gene transfer among lactococcal species.[56] In that study it was discussed that single parameters, such as donor to recipient ratio, the time of filter incubation and even the donor/recipient strains used influence transfer efficacies and even the overall outcome tremendously.

Using filter mating to determine transfer efficiencies of genetic traits such as resistance plasmids among bacteria generally possesses certain limitations. First, filter mating may not reflect *in vivo* situations. In natural ecosystems such as within the mammalian intestine various stimuli may influence and also trigger horizontal gene transfer.[61] Transfer efficiencies of *vanA/B* gene clusters in mice intestines were higher compared with rates assessed *in vitro*.[30,31,35] Biofilms may also play an important role in lateral gene transfer supporting very tight cell-to-cell contacts between unrelated bacteria[62] also allowing transfer of non-conjugative plasmids.[63]

Long-term *in vitro* growth experiments revealed stable maintenance of the acquired *vanA* plasmids in transconjugants of a single recipient. For some of the plasmids, specific maintenance and PSK systems were already determined (Table 1). It remains to be speculated whether acquisition of plasmids harbouring them reveals stable maintenance also in the new host or if this comparably stable persistence is also host dependent, since we tested only transconjugants of a single recipient strain. In this context it is of special interest that despite glycopeptides (avoparcin) having been banned for more than a decade as growth promotors, a reservoir of *vanA*-type resistance and corresponding plasmids in *E. faecium* from commercial animal farming still exists.[6–9,64] Data from the present, recent and previous unpublished (G. Werner) studies suggest a mosaic-like structure of enterococcal resistance plasmids also encoding many functions unrelated to antimicrobial resistance, but associated with virulence and/or primary or secondary metabolism.[24,65–67] Most probably some of these additional features influence persistence and fitness in the plasmid-acquiring hosts; an argument that remains to be investigated in future analyses.

In conclusion, results of *in vitro* filter-mating experiments showed a considerably narrow host range of the investigated enterococcal *vanA* plasmids. Recipient strains of the same species behaved similarly thus allowing general conclusions to be drawn when using only a single recipient per species. Representatives of Gram-positive species like *Lactococcus*, *Lactobacillus* or *Bifidobacterium* known to share a common gene pool with *Enterococcus* do not seem to participate in the dissemination of commonly encountered enterococcal *vanA* plasmids. Acquisition of resistance plasmids in enterococcal recipients was stable over many generations even in the absence of selective antibiotic pressure.

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Transparency declarations

None to declare.

Supplementary data

The harmonized filter-mating protocol, Tables S1–S4 and Figures S1–S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Diversity of Tn*1546* and its role in the dissemination of vancomycin-resistant enterococci in Portugal

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Diversity of Tn1546 and Its Role in the Dissemination of Vancomycin-Resistant Enterococci in Portugal

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We characterized the molecular diversity of vanA vancomycin-resistant enterococci (VRE; 176 isolates/87 pulsed-field gel electrophoresis types) from different sources and cities in Portugal (1996 to 2004): (i) food animals (FA; n 38 isolates out of 31 samples), hospitalized humans (HH; n 101/101), healthy human volunteers (HV; n 7/4), and environmental sources (n 30/10). Some strains were isolated from different hosts and persistently recovered for years. Twenty-four Tn1546 variants were identified, all located on plasmids (30 to 250 kb). Some Tn1546 variants were associated with specific sources such as FA (3 types), HH (11 types), or HV (1 type), while others were recovered from isolates of different origins (8 types). Polymorphisms in the central vanRSHA region of Tn1546 were scarcely detected, while alterations upstream of vanR and downstream of vanA were frequently identified involving mutations (vanS and vanX), deletions (vanY), insertions (IS1216V, ISEf1, and IS19; sequences with or without homology with others available in GenBank databases), and different genetic rearrangements. Most Tn1546 variants contained IS1216V (14 types) or ISEf1 (6 types). IS1216V was found alone or associated with an IS3-like element at different orientations and positions in Tn1546 from human, animal, and environmental samples. ISEf1 was located within vanX-vanY region at nucleotide 9044 of Tn1546 variants mostly associated with clinical isolates, suggesting a common genetic platform. IS19 was observed within the vanX-vanY region in one Tn1546 variant from poultry. Recent spread of VRE in Portugal reflects a complex epidemiology involving both clonal spread and plasmid dissemination containing a variety of Tn1546 types. Apparent Tn1546 heterogeneity among enterococci from human, animal, and environmental sources might reflect frequent genetic exchange events and evolution of particular widely disseminated genetic elements.

Since their first description in 1988, vancomycin-resistant enterococci (VRE) have been isolated from hospitalized patients, healthy humans, and a wide variety of mammals, insects, and environmental sources (8, 40). Five types of acquired glycopeptide resistance are currently recognized in enterococci (VanA, VanB, VanD, VanE, and VanG), of which VanA is the most commonly found (8). Some of them may be transferred in vivo and in vitro to other bacterial genera such as Bacillus, Streptococcus, Clostridium, Oeskorvia, and Staphylococcus (8), highlighting the concern about the spread of glycopeptide resistance to more virulent human pathogens.

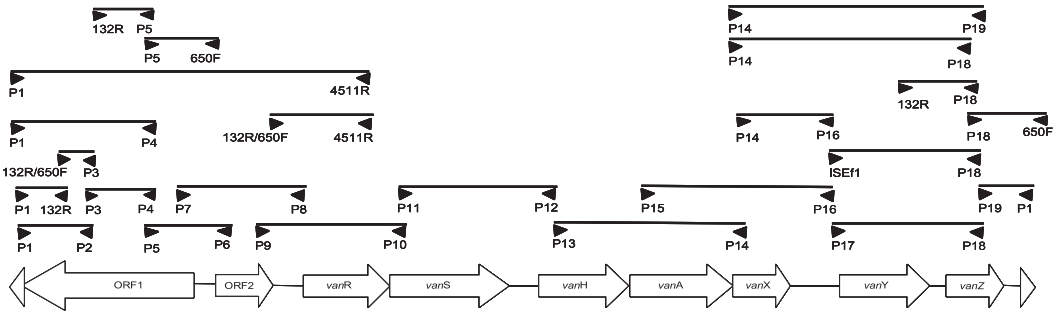
The best-characterized mechanism of resistance is VanA. The genes forming the VanA operon are part of a 10.8-kb Tn3 derivative designated Tn1546, often located on conjugative plasmids (8, 12, 34, 41, 42). Variants of this transposon containing mutations, deletions, and different insertion sequences have been reported, and a presumptive association between specific Tn1546 types and geographical areas and/or environmental sources has been suggested (4, 15, 19, 32, 38, 44, 45). Nevertheless, the epidemiology of VanA is far from being understood as different situations occur in different locations and settings (23). In Europe, the initial VRE scenario consisted of polyclonal enterococcal population with a high diversity of Tn1546 types in the community and with scarce presence in the nosocomial setting (4, 19, 38). However, this epidemiological pattern seems to have changed in the last few years. Five European countries have reported prevalence rates in hospitals of 20%; therefore, frequent reports of nosocomial VRE outbreaks in most countries and a variable presence of these bacteria in community settings have been described (23). Portugal, currently one of the European nations with the highest VRE prevalence rate in invasive isolates in the nosocomial setting (34% in the last 2005 Annual Report of the European Antibiotic Resistance Surveillance System; http//:www.earss.rivm.nl), has a frequent recovery rate in animals, healthy humans, and the environment, as seen in previous surveillance studies from our group, where van genes were screened, between 1996 and 2004, among 1,192 enterococci from different sources and geographical areas in Portugal (28–31, 36). In those studies, we described dissemination of specific vancomycin-resistant Enterococcus faecalis and Enterococcus faecium strains among hospitals of different geographical regions or poultry samples from distinct brands and butcher shops for long periods of time and identified the genetic elements harboring vanA in specific isolates (29, 31, 32).

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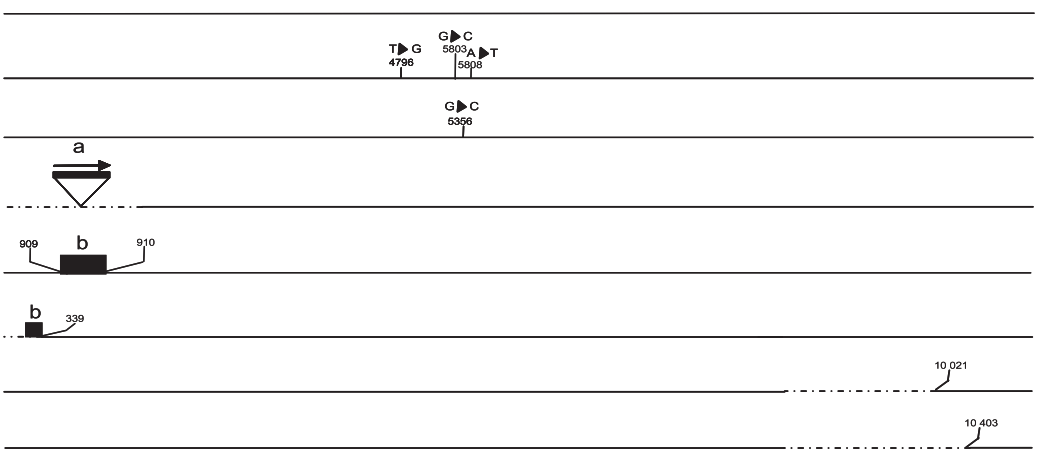
† L.P. and T.M.C. contributed equally to the direction, preparation, and writing of the manuscript.

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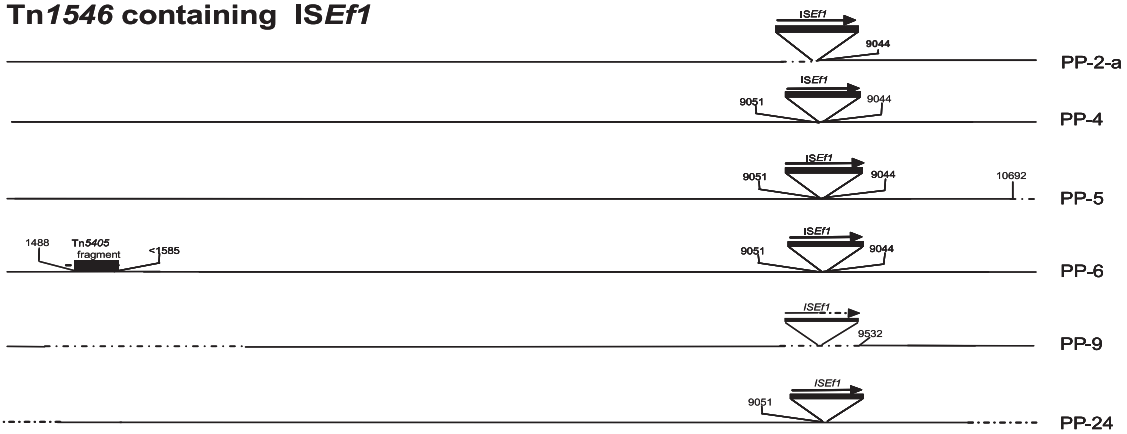
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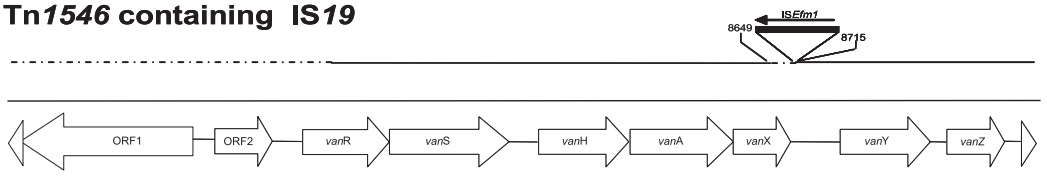
Tn*1546* with no insertion sequences detected



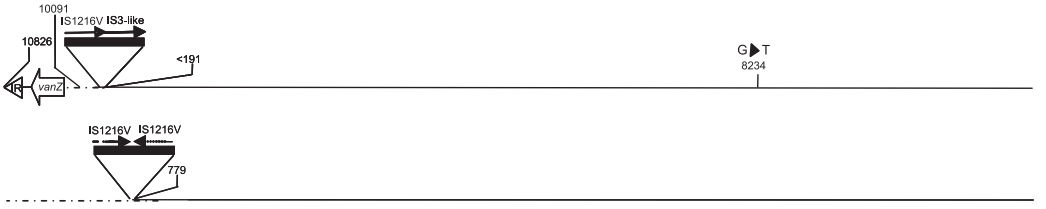
Tn*1546* containing IS*Ef1*



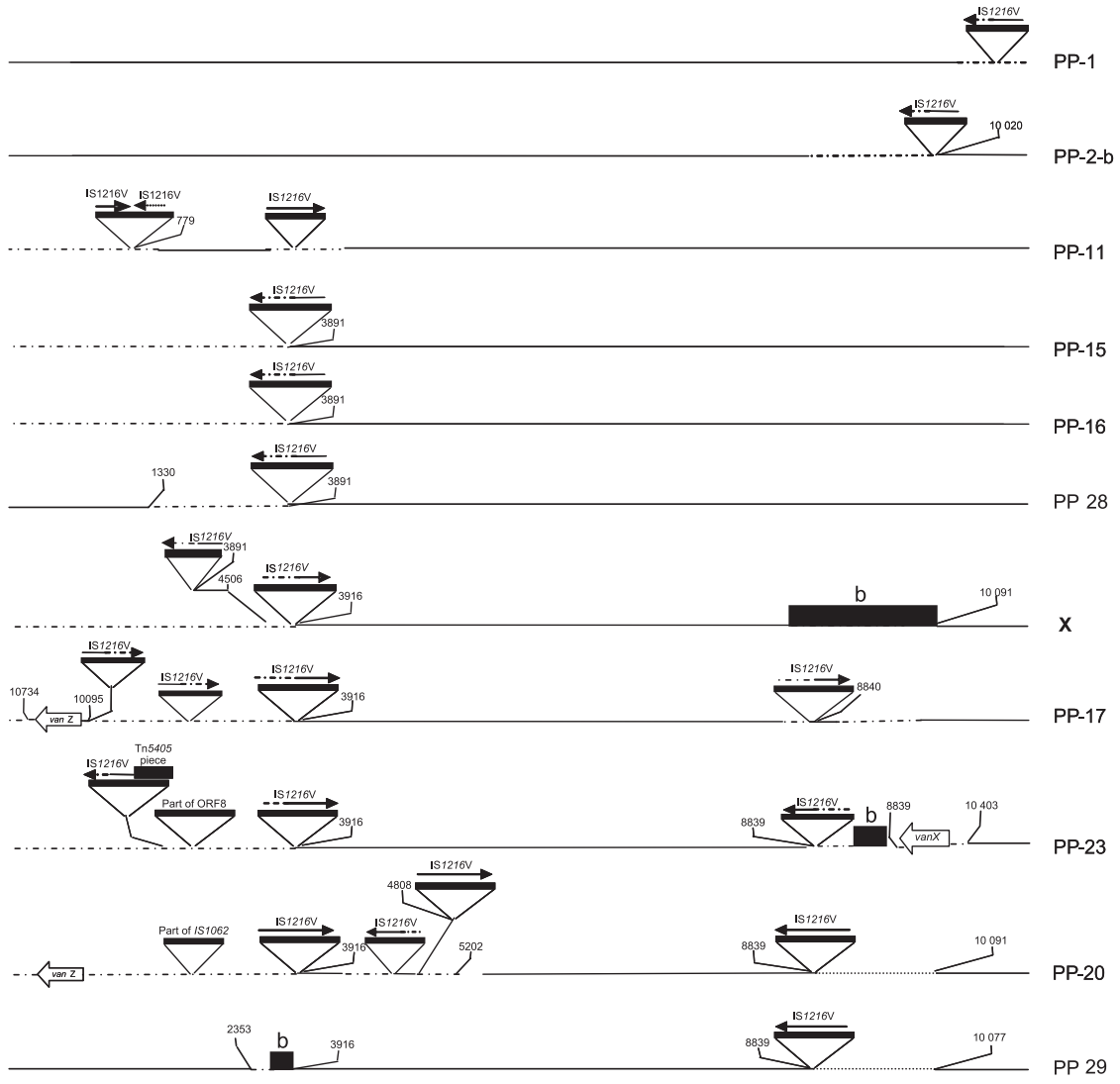
Tn*1546* containing IS*19*



Tn*1546* containing IS*1216*V



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Tn*1546* containing IS*1216*V (cont.)

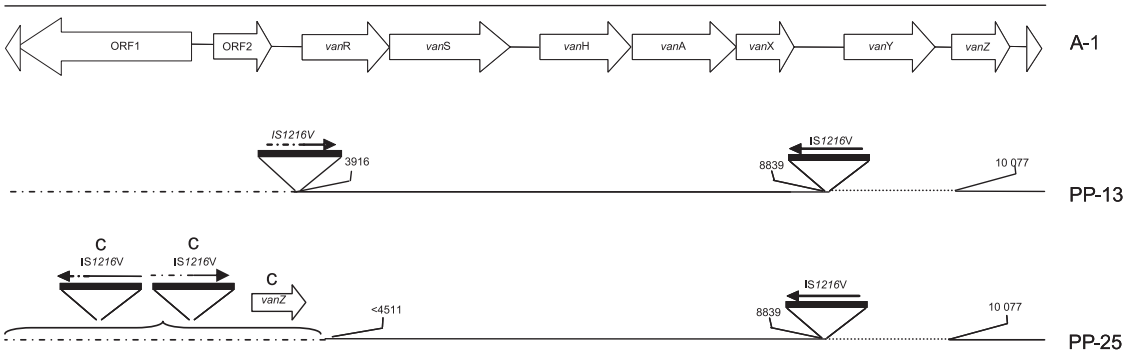


FIG. 1. Primers used for Tn1546 PCR characterization and genetic maps of Tn1546 types grouped by the presence of common insertion sequences. Primers used were based on the scheme described by Arthur et al. (1). Several other combinations of primers and PCRs represented in the scheme were used when necessary with 132R, 650F, 4511R, vanS, vanX, and ISEf1 primers (18, 19, 27, 32). Tn1546 prototype A corresponds to the original sequence described by Arthur et al. (1). The positions of genes and open reading frames and the direction of transcription are depicted with open arrows. IS elements are represented by triangles; other sequences are designated by rectangles. The positions of the first nucleotide upstream and downstream from the DNA insertion sites are depicted. Deletions are indicated by dots, and sequences that were not characterized are indicated by dots and small lines. Mutations are indicated above each represented Tn1546 type. a, acetyltransferase (GenBank accession no. AY887084); b, DNA sequence did not match any sequence available in GenBank; c, the positions of IS1216V and the vanZ gene were not determined.

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The goal of the present study was to analyze in detail the molecular diversity of Tn1546 in our large collection of enterococci from different sources in order to better understand the ecology of VRE and the possible reasons explaining the recent fast spread of these microorganisms in Portugal.

MATERIALS AND METHODS

Bacterial strains. Tn1546 was characterized in 176 vanA isolates corresponding to 133 E. faecium, 38 E. faecalis, and 5 Enterococcus sp. isolates recovered from the following samples: (i) swabs or meat samples from the inner carcass of retail poultry of nine different commercial brands sold throughout the country, which were collected from two different butcher shops (34 isolates from 27 samples; n 34/27); (ii) fecal swine samples from Figueira da Foz (center of Portugal; n 4/4); (iii) human fecal samples from healthy volunteers living in the center and northern part of the country (n 7/4); (iv) hospital sewage water from the Porto area (n 22/6) and from urban sewage water draining directly to the sea in a recreational bathing area (n 2/2); (v) river water samples from the Porto area (n 6/2); and (vi) human clinical samples from different patients at three hospitals in the center (Coimbra and Viseu) and north of Portugal (Porto) (n 101 isolates). All isolates were resistant to vancomycin (MIC range, 32 to 256 g/ml), and 155 out of 176 (88%) were resistant to teicoplanin. Eighty-seven pulsed-field gel electrophoresis (PFGE) profiles were included and were observed among 173 isolates tested (three isolates were not typed): 49 from clinical isolates (49/101 isolates), 24 from poultry (24/34), 2 from swine (2/4), 2 from the river (2/5), 5 from healthy humans (5/7), and 8 from hospital sewage (8/22). Some of the clinical PFGE types (HB, H70, H71, H76, H78, H88, and H100) were isolated from hospitals of different geographical regions and sometimes persisted for long periods of time (clinical PFGE types HB, H70, H78, and H88 and poultry PFGE types P1, P2, P3, P4, and P22) (29, 31, 32). Transference of vancomycin resistance to E. faecalis JH2-2 or E. faecium GE1 was achieved by conjugation in 90% of the strains included in the present study (28–32).

Characterization of Tn1546 elements. The backbone structure of Tn1546 was determined by the PCR-overlapping scheme described by Woodford et al. (45) using other combinations of primers and PCRs when necessary (Fig. 1). Some Tn1546 types from human samples and sewage have previously been characterized by using this PCR-overlapping assay (29, 30, 32). In this study, we analyzed and compared in detail Tn1546 from different sources. Specific mutations at vanX were searched by analysis of restriction fragment length polymorphism patterns of vanX digested with DdeI (35). Mutations in vanS among strains exhibiting teicoplanin susceptibility were analyzed by sequencing. Characterization of PCR-overlapping fragments of unusual size was performed by sequencing in representative strains harboring different Tn1546 sequences or isolated from different animal, human, or environmental sources. PCR products were purified with the GFX PCR purification kit (Amersham Biosciences) and sequenced on an ABI PRISM 377 automated sequencer (PE Biosystems, Foster City, CA). Nucleotide sequences were compared to those in the GenBank databases by using the BLASTN local alignment search tools.

Location of Tn1546. Plasmid location of vanA genes was assessed by the method of Barton hybridizing S1 nuclease-digested genomic DNA with a vanA probe following standard procedures (2, 37). Labeling and detection were carried out with ECL enhanced chemiluminescence kits following the manufacturer s instructions (Amersham Life Sciences, Uppsala, Sweden).

RESULTS

Characterization of Tn1546 elements. The PCR-overlapping assay revealed the presence of 24 variants of Tn1546 (Table 1 and Fig. 1) (28–30, 32). Some Tn1546 variants were only recovered from samples of specific sources such as poultry (types S, PP-1, and PP-11), hospitalized humans (types PP-2, PP-4, PP-9, PP-10, PP-13, PP-23, PP-24, PP-25, PP-27, PP-28, and PP-29), or healthy humans (type PP-6). Other Tn1546 types were detected among isolates of different origins, such as types A, D, X, PP-3, PP-5, PP-15, PP-16, and PP-20 (Table 1).

We did not find any polymorphism or size variation in the central regions of Tn1546 (vanRSHA) for any of the enterococci studied, with the exception of types A and PP-20. However, alterations upstream of vanR and downstream of vanA were frequently detected (Fig. 1).

Different insertion sequences were identified among Portuguese Tn1546 variants: IS1216V, IS3, ISEf1, and IS19. IS1216V was found alone or associated with an IS3-like element at different orientations and positions in 14 Tn1546 variants from human, animal, and environmental samples. It was located at nucleotide (nt) 3916 and/or 8839 of Tn1546 in seven variants (types X, PP-13, PP-17, PP-20, PP-23, PP-25, and PP-29). This insertion sequence was associated with deletion of vanY (types PP-13, PP-20, PP-25, and PP-29), with duplication of the vanZ region in the 5 end of the transposon (types D1, PP-17, PP-20, and PP-25), or with duplication of vanX genes in the right side of Tn1546 (PP-23). ISEf1 was located within the vanX-vanY region at nt 9044 of Tn1546 in six variants (types PP-2, PP-4, PP-5, PP-6, PP-9, and PP-24), suggesting a common origin. Two nucleotide changes within the right inverted repeat and transposase regions of ISEf1 were identified in relation to previously detected sequences (changes G3 A and C3 T at positions 269144 and 269763, respectively; GenBank accession no. AE016830). The insertion of ISEf1 was associated with the duplication of GACTGAAA sequence at bp 9044 to 9051 of Tn1546 (GenBank accession no. EF064799). IS19, also known as ISEfm1, was also observed within the vanX-vanY region in Tn1546 type S recovered from poultry isolates (Fig. 1).

Other sequences were identified within transposase or vanS regions. Some of them showed homology with known sequences as Tn5405 is widely distributed among enterococcal plasmids (41), with a putative resolvase from streptococcal plasmid pRE25, with IS1062 found in the genome of E. faecalis strain V583, or with a putative N-acetyltransferase found in the genome of E. faecium strain DO (GenBank accession no. SAU73027, X92945, X96976, and EAN11147, respectively). Other regions identified within Tn1546 types did not match any sequence available in the GenBank database (within Tn1546 types D4, X, PP-10, PP-23, and PP-29; Fig. 1).

Mutations were also identified among different Tn1546 types. Two variants of Tn1546 type A (this type is identical to the original Tn1546) from single swine and poultry isolates showed mutations in vanS which were susceptible to teicoplanin (MIC, 4 mg/liter) and were designated as types A2 and A3, respectively. Type A2 contained the changes G4796T, C5803G, and T5808A, previously described by Hashimoto et al. in Tn1546 from Japan (16), and type A3 harbored a silent mutation, C5356G. Some isolates carrying type D from swine and healthy humans contained the nucleotide change G8234T within vanX, which was also described in Tn1546 from animals of different European countries such as the United Kingdom, Denmark, Finland, The Netherlands, and Norway (35).

Location of Tn1546. Hybridization of S1 nuclease-digested genomic DNA with a vanA probe showed that this gene was located on plasmids ranging from approximately 30 to 250 kb.

DISCUSSION

VRE spread in Portugal is associated with both epidemic strains and horizontal transfer of Tn1546 (28–30, 32; this study). In contrast with other European studies from Italy and Denmark (3, 14), we found an apparent high diversity of Tn1546 in Portugal. Tn1546 heterogeneity has been previously

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TABLE 1. Tn1546 types, PFGE type, date of isolation, source, city, species, range of MICs for glycopeptides, and conjugation frequency of the VRE characterized

Tn1546 type	No. of isolates (PFGE type s)	Date (mo/yr)	Source[a]	City in Portugal	Species (no. of isolates)	MIC (g/ml) of:		Conjugation frequency[b]
						Vancomycin	Teicoplanin	
A	3 (HK, H22, H98)	10/99–9/01	HP (2)	Porto, Coimbra	E. faecalis (1), E. faecium (2)	256	128– 256	10 ^{4}–10 ^{8}
	14 (P1, P2, P3, P4, P5, P6, P8, P9, P15, P16, P22, P25)	9/99–2/01	RP (6)	Porto	E. faecalis (1), E. faecium (12), Enterococcus (1)	256	128– 256	(10 ^{9})
	1 (S70)	12/97	S	Figueira da Foz	E. faecium (1)	256	4	10 ^{9}
D	3 (HV58, HV60, HV69)	1/01–6/04	HV	Porto	E. faecium (1)	256	64– 256	(10 ^{5}–10 ^{8})
	1 (H76)	6/02	HP (1)	Porto	E. faecium (1)	256	32	10 ^{3}
	2 (P10, P13)	10–11/99	RP (1)	Porto	E. faecium (1)	256	64–128	
PP-10	1 (H83)	?/96	HP (1)	Coimbra	E. faecium (1)	256	256	10 ^{7}
	1 (H94)	3/02	HP (1)	Coimbra	E. faecium (1)	256	64	10 ^{5}
PP-3	1 (H106)	4/03	R	Porto	E. faecium (1)	256	32	
	1 (P11)	11/99	RP (1)	Porto	E. faecium (1)	256	4	
PP-27	1 (H87)	3/02	HP (1)	Coimbra	E. faecium (1)	256	128	
Tn1546 containing IS1216V								
D	1 (P7)	9/99	RP (1)	Porto	E. faecium (1)	256	32	(10 ^{8})
	1 (ND)[c]	7/04	US	Porto	E. faecium (1)	256	256	ND
	3 (SA)	12/97–1/98	S	Figueira da Foz	E. faecium (1)	256	64– 256	
X	1 (H84)	3/00	HP (1)	Coimbra	E. faecium (1)	256	64	
	1 (H106)	4/03	R	Porto	E. faecium (1)	256	32	10 ^{7}
PP-1	4 (P21, P27, P29, P31)	10/00–7/00	RP (3)	Porto	E. faecalis (1), Enterococcus (3)	256	32	
PP-2	10 (H70 H92, H93, HB)	6/01–10/02	HP (2)	Viseu, Coimbra	E. faecalis (1), E. faecium (9)	256	32– 256	(10 ^{8})
PP-11	1 (P19)	10/99	RP (1)	Porto	E. faecalis (1)	256	128	
PP-13	6 (H78, H85, H86, H88, H91)	7/01–12/02	HP (1)	Porto	E. faecium (1)	256	32–64	(10 ^{5}–10 ^{8})
PP-15	8 (HB, HC, H96)	3/01–8/02	HP (1)	Porto	E. faecalis (7), E. faecium (1)	256	128–256	10 ^{3}–10 ^{8}
	1 (P1)	11/99	RP (1)	Porto	E. faecalis (1)	256	128	
PP-16	3 (HB, H77)	6/01–4/02	HP (1)	Porto	E. faecalis (2), E. faecium (1)	256	256	10 ^{6}–10 ^{7}
	19 (SWA, SWE, SWC, SWD, HB, SWM)	2/01–2/02	HS (2)	Porto	E. faecalis (4), E. faecium (15)	256	256	(10 ^{1}–10 ^{8})
PP-17	2 (SWF, SWH)	5/01	HS (1)	Porto	E. faecium (1)	256	32	(10 ^{3})
PP-20	2 (H85, H109)	7/01	HP (1)	Porto	E. faecium (1)	256	16–32	
	1 (SWH)	10/01	HS (1)	Porto	E. faecium (1)	256	16	
PP-23	1 (H119)	9/02	HP (1)	Porto	E. faecium (1)	256	16	10 ^{7}
PP-28	1 (H110)	?/01	HP (1)	Porto	E. faecium (1)	256	128	
PP-29	1 (H102)	3/01	HP (1)	Porto	E. faecium (1)	256	256	
Tn1546 containing ISEf1								
PP-2	10 (H70 H92, H93, HB)	6/01–10/02	HP (2)	Viseu, Coimbra	E. faecalis (1), E. faecium (9)	256	32– 256	10 ^{6}

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TABLE 1—Continued

Tn1546 type	No. of isolates (PFGE type s)	Date (mo/yr)	Source[a]	City in Portugal	Species (no. of isolates)	MIC (g/ml) of:		Conjugation frequency[b]
						Vancomycin	Teicoplanin	
PP-4	28 (HG, HX, HB, HA, HN, HQ, H75, H76, H78, H80, H81, H82, H88, H90, H95, H97, H108, H111, H112, H117, H122)	?/96–09/03	HP (3)	Porto, Coimbra, Viseu	E. faecalis (13), E. faecium (15)	256	32– 256	(10 ^{4}–10 ^{8})
PP-5	22 (HF, HB, H70, H71, H72 H78, H99, H100, H103, H104, H105)	8/99–3/03	HP (3)	Porto, Coimbra, Viseu	E. faecalis (3), E. faecium (19)	256	32–128	10 ^{1}–10 ^{7}
	3 (HV59)	2/01	HV	Porto	E. faecium (1)	256	128	10 ^{8}
	4 (H78, H106)	3–4/03	R	Porto	E. faecium (1)	256	64	(10 ^{4}–10 ^{5})
	1 (ND)	7/04	US	Porto	E. faecium (1)	256	256	ND
PP-6	1 (HV68)	6/04	HV	Porto	E. faecium (1)	256	256	10 ^{6}
PP-9	2 (H74, H106)	1/00	HP (1)	Coimbra	E. faecium (1)	256	64–128	10 ^{4}
PP-24	1 (ND)	3/03	HP (1)	Coimbra	E. faecium (1)	256	32	10 ^{2}
PP-25	1 (H88)	7/01	HP (1)	Porto	E. faecium (1)	256	32	ND
Tn1546 with IS19 S	7 (P1, P8, P12, P17, P20)	9/99–3/01	RP (5)	Porto	E. faecium (4), E. faecalis (2), Enterococcus (1)	32– 256	4–128	(10 ^{7})

[a] HP, hospitalized patients; HV, healthy volunteers; RP, retail poultry; S, swine; R, river; US, urban sewage; HS, hospital sewage. The number of hospitals associated with a particular sewage plant or the number of poultry brands from which Tn1546 types were recovered appears in parentheses.
[b] , negative mating; parentheses, variable success in mating among the represented isolates; , positive mating but conjugation frequency not determined.
[c] ND, not determined.

described (18, 19, 38, 44, 46), but only a few works included a representative number of strains from different origins in a particular area, thus precluding conclusions about local epidemiological dynamics (3, 45). Difficulties also arise from the different methods used for this purpose, which complicates comparison among studies (14, 44, 45).

Tn1546 may be located in conjugative plasmids (14, 20, 25, 43, 47) or in larger composite transposons, as documented for isolates with chromosomally located vanA (15). Horizontal transfer of conjugative plasmids containing Tn1546 seems to have played a relevant role in the recent increase of VRE in Portugal, as vanA was plasmid located in most VRE isolates studied and specific plasmids containing the most prevalent Tn1546 variants were identified in representative distinct clones collected from different cities and years (13). Nevertheless, the role of prevalent and persistent E. faecalis and E. faecium strains in the maintenance of the resistance by acquiring different genetic elements must not be underestimated, highlighting the complex epidemiology associated with successful spread of multidrug-resistant enterococci, as illustrated in Poland and the United States, where both successful plasmids and prevalent clones contributed to endemicity (11, 21, 22). Plasmid persistence has been described in areas with both high and low antibiotic selective pressure, as in the United States versus Norway or Denmark, suggesting that factors other than antibiotic pressure might be important in VRE maintenance and epidemiology (17, 20).

The diversity of Tn1546 observed in the present study was attributed to the occurrence of insertions, deletions, and point mutations. Two insertion sequences, IS1216 (IS6 family) and ISEf1 (IS30 family), were the most frequently detected insertion sequences within Tn1546. IS1216V has been extensively found among European transposons containing vanA from different sources and detected at different positions and orientations, as in the present study (18, 38, 44, 46). We identified known insertions of IS1216V such as that associated with a deletion of vanY, which is believed to affect the transcription of vanZ, thus explaining a decrease in MICs of teicoplanin (24, 44), or that detected within vanS in PP-20 (10, 26). In addition, we observed different rearrangements resulting in the duplication of vanZ (at the 5 end) or vanX (at the 3 end) of Tn1546 variants containing IS1216V, some of them previously identified among English isolates (9). IS1216V is one of the main insertion sequences found in the E. faecalis genome and also among enterococcal plasmids widely disseminated in the community (39) (GenBank accession no. AF408195). The diversity found among Tn1546 variants containing this IS might be explained by genetic exchange between sequences containing IS1216, as IS6 family members are involved in mechanisms associated with cointegrative processes and replicative transposition (6, 33). Interestingly, Tn1546 types containing IS1216 (D, X, PP-1, PP-2, PP-11, PP-13, PP15, PP-16, PP-17, PP-20, PP-23, PP-25, PP-28, and PP-29) were isolated from different sources, which might mirror wide dissemination of vancomycin resistance plasmids which have evolved by different recombinatorial events throughout different hosts. Transposons with the same structure in animals and human enterococci have also been described in the United Kingdom and The Netherlands

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(35, 44), also suggesting horizontal transfer of widespread conjugative plasmids, as recently demonstrated (25).

ISEf1 was first identified within Tn1546 among clinical Portuguese strains (32), but these Tn1546 variants might be currently dispersed among European hospitals as they have recently been identified in hospital outbreak enterococcal strains from Spain and Germany (43; V. Francia, personal communication). In contrast to IS1216V, ISEf1 was always detected at the same position (within the vanX-vanY region at nt 9044) and Tn1546 variants containing ISEf1 were mostly associated with clinical isolates, which may reflect successful spread of particular plasmids in the clinical setting. Although hospitalized patients seem to constitute the main reservoir of these Tn1546 types, the isolation from a single healthy human without previous contact with the hospital setting, from hospital residual waters, and from river waters indicates that a potential dissemination of genetic elements harboring these transposon types in the Portuguese community cannot be discarded.

IS19 (also called ISEfm1 and belonging to the IS982 family) has been found in clinical enterococcal isolates from different continents and located in a variety of genetic elements, including VanD and VanA clusters (5, 18) (GenBank accession no. AF169185 and AY887084). We first detected this insertion sequence in enterococci from poultry and in a different position from that described in Tn1546 from Korea (within vanX-vanY instead of vanS-vanH). This IS family is spread among gram-positive organisms, and its presence among a variety of enterococcal genetic elements and strains might indicate genetic exchange among gram-positive bacteria sharing the same ecological compartment.

Some studies including isolates from different areas have suggested certain host and geographical specificity of particular Tn1546 variants (4, 38, 44). Diverse insertion sequences have been identified within Tn1546, but this heterogeneity has perhaps been overestimated as the genetic background of transposons has not been analyzed in most studies. Particular prevalent plasmids may contain different Tn1546 variants as a consequence of recombinatorial events or genetic exchange with genetic elements of microorganisms belonging to the same exchange communities in particular areas, as we demonstrated for prevalent Tn1546 combinations (7, 13).

In summary, both clonal dissemination and horizontal transfer of particular genetic elements seem to play a relevant role in the recent wide dissemination of VRE in Portugal. The high diversity of Tn1546 types among enterococci isolated from human, animal, and environmental sources may reflect frequent genetic exchange events within particular widely disseminated genetic elements and microorganisms. Detailed characterization of enterococcal plasmids becomes relevant in order to understand differences in the epidemiology of VRE in different geographical areas and settings.

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3.3. Integration of all data and multilayered analysis of the relative contribution of epidemic clones and mobile genetic elements in the current expansion of specific clonal lineages through the analysis of temporal and spatial differences of that biological units.

3.3.1. Mobile genetic elements and Lateral Genetic Transfer in Enterococci

Mobile Genetic Elements and Lateral Genetic Transfer in Enterococci

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Book Chapter (*In press*)

ABSTRACT

Enterococci are commensals of most mammals and opportunistic pathogens of humans and animals that are widely used in industry as starter cultures for dairy products (cheeses, fermented milk products), as animal feed additive and as probiotics. The wide natural distribution and extensive applications of enterococci in veterinary and dairy industry and their ability to survive in diverse ecological conditions (pH, temperature, salts, presence of antibiotics, biocides and metals), enable them to contaminate soil, sand, water, plants and foods for long periods of time. In the last years, these organisms have focused the interest of professionals from medical, veterinary and food industry fields because of the difficulties of treating human and animal infections caused by multidrug resistant enterococci, the risk of transmission of clones carrying virulence and antibiotic resistant traits among different hosts and the risk of dissemination of certain transferable antibiotic resistances genetic elements to more pathogenic bacteria as *Staphylococcus aureus*. Despite of the outstanding knowledge gained from studies on physiology, population structure, and genetics, we are far from understand the factors related to the acquisition, expression and persistence of different adaptive genes to different environments, and the influence of different hosts and settings as reservoirs of highly transmissible clones and mobile genetic elements. This chapter analyzes the role of lateral gene transfer in the diversification and evolution of enterococci in an attempt to better understand its consequences in the ecology of these microorganisms.

INTRODUCTION

Enterococci are commensals of the intestinal tract of humans and animals (1) and one of the most common nosocomial pathogens in the USA and in several EU countries (88, 239). They are frequently isolated from farm animals, pets, and retail food products (1, 48, 49, 59, 84). In addition, they can be used as starter cultures for the production of certain cheeses and other fermented milk products, as animal feed additive and as probiotics (1, 5). The ability of enterococci to survive in harsh conditions of pH, temperature and salt as well as their intrinsic resistance to different antibiotics, biocides and metals enable them to contaminate soil, sand, water, plants and foods for long periods of time (1, 52, 139).

As in the case of human enterococcal infections, the most predominant species recovered from foods and food borne animals are *Enterococcus faecalis* and *Enterococcus faecium,* although *Enterococcus durans, Enterococcus hirae* and pigmented enterococci are often identified in samples from these sources (1, 56, 78, 124, 150).

Despite the wide distribution of enterococcal species among animals and foods, there is limited evidence about the influence of the food chain in the dissemination of multidrug resistant and pathogenic enterococci among humans. Currently, the role of non-human hosts as reservoirs of highly transmissible clones and mobile genetic elements (MGE), the transient or permanent human faecal carriage of enterococci from animal origin, and the consequent risk of gene transfer to resident human flora are still highly discussed (4, 59, 156, 157, 199).

In this chapter, we will discuss the impact of the lateral genetic transfer (LGT) in the diversification, selection and spread of different enterococcal lineages (*section 1*), the opportunities and constraints of LGT processes among enterococci of different origins and species belonging to the same communities of *genetic exchange* (*section 2*), and we will analyze the diversity and the influence of different MGE belonging to the enterococcal mobilome in the transfer of adaptive traits between different hosts (*section 3*).

1. Lateral Genetic Transfer, bacterial diversification and Enterococci

The involvement of LGT in the neo-Darwinian paradigm of the evolution has extensively been discussed since the reconsideration of the *biological species concept* by Ernst Mayr in 1942 (18, 131, 132). Modern concepts about the evolution of bacterial species are based on the analysis of different mechanisms that drive such evolution (107). These studies have allowed to divide prokaryotes in ‘closed lineages’ (in which most of the

evolutionary changes occurred by mutation) and 'open lineages' (in which most of the evolutionary changes occurred by horizontal gene transfer) (11). Classic microbial systematics is being currently questioned under the light of these novel standpoints that highlight the need to redefine either natural groups and evolutionary units considering the assymetrical prevalence of LGT (19) and *ecotypes* based on environmental preferences (34, 232). Besides the relevance of the *biological species concept* in the evolutionary theory, the establishment of proper bacterial designations is essential for implementing accurate interventions related to medical diagnosis, veterinary, food safety, public health or biotechnology.

The emergence and increase of enterococcal strains with new adaptive traits (inferred by their antibiotic resistance, colonization, or virulence phenotypes and genotypes) illustrates the role of LGT in the combinatorial evolution of these microorganisms. Species definitions on enterococci are currently based on the similarity at 16S rRNA and also on phenotypic traits (*species phenetic definition*). Population structure analysis based on multilocus sequence typing (MLST) data using the algorithm eBURST is currently available only for the predominant species, *Enterococcus faecium* and *Enterococcus faecalis.* This tool has revealed the presence of expanded specific clonal lineages adapted to different hosts as *E. faecium* clonal complex (CC)17 or *E. faecalis* CC2, causing most of the nosocomial infections worldwide, or *E. faecium* CC9 and *E. faecium* CC5, which are predominant among poultry and swine, respectively (59, 61, 90, 112, 182, 246). Nonetheless, phylogenetic inferences by using eBURST may result unreliable for species with high recombination rates and a relatively low mutation rate as shown for *E. faecium* (228, 246). Combination of MLST analysis using algorithms other than eBURST (247) and phylogenomic analysis (229) now suggest different evolutionary trajectories for STs within major clonal complexes as *E. faecium* CC17 and also show the influence of LGT on closely related strains in determining strain-specific properties. These studies also establish an open genome for *E. faecium* and a high genomic diversity among strains of these species (229). Other enterococcal species have not been studied in such detail.

The dynamic interplay between groups of bacterial species, genetic elements and environments constructs *communities of genetic exchange* (GEC, *genetic exchange community*) (202). Environments under frequent selective pressures as dairy products, foodstuffs, biofilms on food-processing and medical equipment, gastrointestinal tract and oral cavity of animals and humans favour LGT among specialized GECs, which constitute high-efficiency *recombination spots* (176, 202). In this scenario, we can consider each enterococcal species as constituted by locally variable populations of clones with specific properties enabling adaptation to particular conditions and therefore diversely influenced by LGT with microorganisms of different GECs.

2. Opportunities and Constraints for LGT in Enterococci

The concept of LGT (also called Horizontal gene transfer, HGT) is defined as *the process of physical transference of DNA from one cell to another without cell division requirement (acquisition of foreign DNA) and its incorporation into the recipient´s genome such as to allow stable inheritance (stable incorporation)* (13, 207). These two processes (acquisition and incorporation) are often studied separately in molecular biology and ecology. However, in the scope of this review they will be discussed together as highly-related evolutionary processes which equally contribute to the diversification of bacterial lineages by adaptation to new ecological niches, metabolic network expansions and even speciation (72, 110, 247). The propensivity or *tendency* of genes to be successfully transferred and retained, thus the tendency to LGT, is called *gene transferability* in evolutionary biology (35). In this section we will analyze the processes and the "*transferability"* of LGT in enterococci.

2.1. Acquisition of foreign DNA

Physical movement of DNA from one cell to another occurs by conjugation, transformation or transduction. In enterococci, it mainly occurs by conjugation although recent reports suggest that transduction may also be frequent (30, 133). These processes require *compatibility* between donor and host and also between incoming and resident MGE, as plasmids or certain transposons. Compatibility between bacterial strains players is determined by surface proteins on the recipient (e.g. transfer of pheromone-responsiveness plasmids between enterococci requires recognition of surface proteins on the recipient), but that between MGE players comprises a diversity of mechanisms. They include different *entry exclusion strategies* that limit the conjugative transfer of MGE carrying transfer systems similar to those already carried by the recipient cell (69). Once the DNA has entered in the cytoplasm, plasmids that use mechanisms of replication and inheritance similar to those of the recipient strains cannot proliferate in the same cell line and they are called *incompatible* (180). Similarly, Tn3-like elements as Tn*1546* responsible for the VanA phenotype cannot be transposed to a genome that already carries another Tn3 element (*transposition immunity*) (74).

Two main post-mating barriers protect the host cell from invasion by foreign dsDNA, *restriction-modification (RM) systems* and *clustered, regularly interspaced short palindromic repeats (CRISPR).* As DNA acquired by conjugation or transduction enter in the cell as ssDNA, these systems will act against plasmids and phages once the dsDNA structure of these elements is re-established. *RM systems* recognize specific sequences

of foreign DNA that are rapidly cleaved after triggering an endonuclease activity and further degradated by exonucleases (101). Anti-RM systems that avoid this host defence mechanism are often located within mobile elements as phage, plasmids and transposons. Little is known about the diversity of RM and anti-RM systems linked to enterococci and the enterococcal mobilome. To date, the presence of anti-RM systems is confined to analogs of ArdA (alleviation of restriction of DNA A) proteins that act against type I restriction systems (detected in CTn*916* and CTn*6000, see CTn section*) and other genes predicted to be involved in methylation in CTn*6000* (23, 189). The presence of different anti-RM systems in the same element might confer inhibition of a broader range of type I restriction systems, thus favouring the promiscuity of certain wide spread enterococcal MGE and consequently, the influence of LGT process in the GECs to which enterococci belongs.

CRISPRs are defence systems that provide a type of acquired inmmunity against specific sequences (for review see (129). The presence of complete CRISPR loci seems to be inversely related to the presence of MGE. This led to hypothesize about the selection imposed by CRISPRs on clonal complexes with impaired host defence systems, often with a diversity of MGE carrying different antibiotic resistance genes as has been demonstrated for enterococci recently (152). RM and CRISPR systems can be transferred to other species which would help to construct specialized GECs.

2.2. Incorporation of DNA

Mechanisms for *incorporation* of DNA include homologous and non-homologous recombination, as in transposition and site-specific recombination, all them being frequent among enterococci (for reviews see 162, 214; for analysis of elements involving in these process, see section 4). The efficiency of such processes depends on the sequence divergence, the length of the fragment to be transferred and the location in the host genome (some examples are given for particular elements in the section 4). It is of note that recombination can take place between a wide diversity of sequences ranging from a few nucleotides (e.g. fragmens of different size and nature found in different plasmids) to entire large chromosomal fragments (e.g. large genomic fragments containing *vanB*, 160). The high variety of mosaic platforms observed among all known enterococcal MGEs (plasmids, transposable elements, genetic islands) and enterococcal genomes highlights the high frequency of occurrence of all these processes among these microorganisms. Besides the acquisition of new genetic material by lateral gene gains, drastic genetic erosion by gene losses may also influence the dynamics of predominant enterococcal MGE (e.g. Inc18 plasmids) and lineages (strains belonging to the CC17 *E. faecium* polycluster) (215, 229, 254).

Although it is thought that any gene can undergo LGT, their stable incorporation to the host varies among families of genes. The *complexity hypothesis* established that the *transferability* of a gene depends on the biological process in which it is involved (where *"informational genes"*, as genes involved in transcription and translation, are less likely to be transferred than *"operational genes*", as genes involving in metabolisms and virulence or encoding antibiotic resistance) (97). This theory has just been revisited and it seems that the type of biological function does not influence *transferability* of genes with similar degree of connectivity (ability to enter in genome/population networkings), while connectivity greatly influences the *transferability* of genes with similar biological function (35).

Traits carried on enterococcal MGEs can be kept within a host cell ("private" traits) or secreted outside the host cells ("public" traits") (161). "Private" traits comprise a wide diversity of adaptive (e.g. antibiotic resistance) and non-adaptive genes (e.g. replication, repair, conjugation). "Public traits" comprise secreted proteins involving in capturing resources (e.g. siderophors), metabolic expansion (e.g. catabolism of emerging energy resources as complex carbohydrates), biofilm formation (traits enhancing adhesion), killing of competing lineages (e.g. bacteriocins), exploitation of hosts (e.g. virulence factors), or addictive systems (e.g. toxin-antitoxin systems). While "public traits" are likely environment specific, "private traits" are probably environment independent. Despite the clear cost imposed by proteic "public" traits to a given host, more than 15% of plasmid genes coding for extracellular proteins highlighting the role of LGT in the social evolution of microbes (142). These traits are particularly relevant in the evolution of microorganisms with "open" genomes and belonging to GECs in variable environments (e.g. animal and human gut and others within the food chain) as enterococci.

3. The Enterococcal Mobilome

The *mobilome* is the repertoire of mobile genetic elements in a cell or in all genomes of a given species (197). The enterococcal mobilome comprises four broad categories: i) transposons or transposable elements, ii) plasmids, iii) bacteriophages and iv) self-splicing elements (35a, 170). The mobilome size is determined by the habitat range and susceptibility to different LGT mechanisms. The type of genes who recently joined the accessory genome reflects the adaptive needs experienced by clones of a given enterococcal species that have recently confronted with particular environmental stresses.

3.1. Transposable elements

Transposable elements (TE) are widely distributed among enterococci and comprise insertion sequences, composite transposons, Tn*3*-derivatives transposons, and conjugative transposons. TE of different classes are found in species of different genera, highlighting the *transferability* of these elements in the GECs to which enterococci belong. Tables 1, 2 and 3 comprise a list of the TE associated with enterococcal species.

3.1.1. Insertion sequences

An insertion sequence (IS) is the simplest TE, consisting of a short DNA sequence ranging from 700bp to 2,500bp (for review and detailed description of known ISs see the ISfinder database http://www-is.biotoul.fr/ and 45, 126, 198). ISs corresponding to 10 families widespread among *Firmicutes* have been identified in enterococci from different ecological niches (Table 1). Specific types are largely represented in genomes and MGE of both *E. faecalis* and *E. faecium* such as IS*16* and IS*256* (IS*256* family), IS*1216* (IS*6* family), IS*1476* and IS*1251* (IS*L3* family), and IS*Ef1*/IS*6770* (IS*30* family) (26, 93, 123, 146, 167, 172, 240). Members of these families are involved in the formation of novel IS-based composite transposons (17, 165, 170).

The wide distribution of particular ISs among the genomes of some enterococcal species has been exploited for typing purposes. ISs used as epidemiological markers included IS*Ef1*/IS*6770* for *E. faecium* and *E. faecalis* (216), IS*16* for CC17 *E. faecium* strains (113, 240), and miniature insertion sequences (EFAR repeats) for *E. faecalis* isolates (230). Finally, the involvement of IS*1191* in the formation of functional promoters for biofilm production has been described in virulent *E. faecalis* strains (33).

3.1.2. Composite transposons

Composite transposons consist on a variable number of genes, mainly encoding antibiotic resistance or catabolic functions, which are flanked by terminal IS elements (137). A wide diversity of enterococcal composite transposons (4.7 - 65.0-kb) associated with antibiotic resistance has been described (Table 2). Widespread representative examples of these elements are Tn*1547* (*vanB*) conferring vancomycin resistance; Tn*5405* (*aadE-sat4-aphA-3*) encoding aminoglycoside-streptothricin resistance; and highly related Tn*5281/5384/5385* encoding resistance to high-levels of gentamicin (86, 159, 241). Most of these transposons are disseminated among strains of different species and origins due to their location on transferable broad host plasmids. For instance, Inc18 plasmids carrying Tn*5405* have been recovered from different enterococcal species from pets, foods, foodborne animals and wastewaters (212, 241, 242). The wide spread of ISs and

transposable sequences in both genomes and MGE of enterococci facilitates recombination between related sequences, and the acquisition of different adaptive traits as antibiotic resistance, virulence or bacteriocin production (73, 102, 154, 204, 205).

3.1.3. Tn*3* derivatives

The Tn*3* family of transposons is divided in two main subfamilies depending on whether their transposase and resolvase genes are transcribed oppositely (Tn*3* subfamily) or in the same direction (Tn*21* subfamily) (74). In enterococci, Tn*1546* and Tn*917*/Tn*3871* represent Tn*3* and Tn*21* subfamilies, respectively. The widespread of Tn*3*-transposons in enterococci is facilitated by their location on conjugative plasmids (7, 194).

Tn*1546* (10.8-kb) is a cluster of 7 *van* genes (*vanRSHAXYZ*) coding for resistance to vancomycin and teicoplanin (7, 244). Different Tn*1546* variants have emerged as a result of mutations, deletions, rearrangements and insertions within and between regions not essential for resistance (*orf1, orf2, vanY*, and *vanZ*) (98, 146, 151, 153, 245, 248). Tn*1546* is the predominant genetic element coding for resistance to glycopeptides among enterococci from different hosts (29, 155, 237). It has also been found in other species recovered from gastrointestinal flora and from soil DNA, mirroring a wide environmental distribution (76, 77, 127).

Tn*917* (5.3-kb) contains the *ermB* gene which codifies for macrolides-lincosamide-streptogramin B (MLS_B) resistance (194, 219). Tn*917* is often located on hemolysin-bacteriocin plasmids of *E. faecalis* such as pAD1 and its transposition, as well as the expression of erythromycin resistance, is inducible by erythromycin (220). Tn*3871* (5.1-kb) is closely related to Tn*917* (10). These elements seem to be widely distributed among humans, food and food borne animals (114, 178).

3.1.4. Conjugative Transposons /Integrative Conjugative Elements

Conjugative transposons (CTns) are self-transmissible elements that move from the chromosome from donor bacteria to the chromosome of recipient bacteria by a process that requires intercellular contact. Once in the recipient, they integrate in a site identical to that in which reside in the donor and they are capable of independent replication (for reviews see 25, 163, 175, 177, 249). Because of these properties, they are also called ICEs *(integrative and conjugative elements).*

A diversity of enterococcal CTns has been described (25, 177, 249) (Table 2). Despite the synteny observed for these elements, they differ in the integrase/excisionase sequences (serine and tyrosine recombinase families) and the specificity for the site of insertion (elements that integrate in a single or in multiple sites) which can determine their

promiscuity (175, 177). They have an extraordinary ability to undergo genetic exchange processes within a diversity of genomes or MGEs, which often result in mosaic platforms carrying fragments of transposons, plasmids, RM systems and self-splicing elements (e. g. CTn*6000,* (23, 144). In addition, the independent expression of the modules involved in different basic functions, enable the conjugation system to mobilize a wide variety of non-conjugative MGE as plasmids, integrative and mobilizable elements (IMES) and *cis*-mobilizable elements (CIMES) into which they often insert (25, 144, 163, 165, 235, 249).

CTns greatly contribute to the dissemination of antimicrobial resistance among a diversity of environmental, commensal and pathogenic bacteria including enterococci (3, 143). Most enterococcal CTns carry tetracycline resistance genes (*tetM* or *tetS*) although they can also harbor genes coding for kanamycin and erythromycin (CTn*1545*), or glycopeptide resistance (CTn*1549* and its close homolog CTn*5382*) (27, 71, 163, 235). In addition, there are modular transposable structures containing CTn*916* transposons as CTn*5385* (CTn*5381*-Tn*5384*-Tn*552*), or CTn*5382* (*pbp5*-CTn*1549)* coding for resistance to multiple antibiotics (27, 165). Enterococcal CTns lacking antibiotic resistance genes have also been described (24, 25, 166). Although specific function has not been assigned for them, interplay between different CTn*916* elements might also contribute to the genome evolution through catalysing large genomic deletions (166).

Elements belonging to the CTn*916*/Tn*1545* family (*tetM*) are the most frequently isolated among enterococci (163, 175, 177). They have a very low integration specificity, which may explain the wide dissemination and broad host range of Tn*916*-like elements (163, 175, 249). CTn*6000* (*tetS*), CTn*5801* (*tetM*) and CTn*5397* (*tetM*) have been recovered from isolates of different origins, some of them being more prevalent among specific enterococcal species associated with particular hosts. Its occurrence among non-human and non-human hosts might still be underestimated (23, 143, 144).

Analysis of available sequenced elements and genomes revealed the wide spread of particular CTn among many gram positive microorganisms, and a high degree of module exchange among them, thus highlighting relevant genetic interplay among GECs (3, 177, 249) (Table 2). CTns have been recovered from gram-positive bacteria colonizing the gastrointestinal tract and oral cavity of different humans and food animals with and without tetracycline exposure, indicating a probable high transferability of these elements in natural conditions (106).

3.1.5. Genomic islands (GIs)

GIs are large non-transmissible chromosomal DNA regions (10 - 500-kb) with aberrant nucleotide composition that can be mobilized by the conjugation modules of plasmids, CTns or phages (21, 47, 147). They code for specific integrase and very often an

excisionase, and are flanked by direct perfect repeats. They often exhibit a variable content that includes sequences from different MGE (21, 66, 185). GIs may derive from ICEs that for different reasons are defective of self-transfer (25, 231). A few GIs have been described in *E. faecalis* and *E. faecium* which are associated with virulence genes (*pathogenicity islands*, PAIs) or metabolic traits. While the PAI of *E. faecalis* is widely distributed among isolates from human and animal origin (188, 192), the GIs of *E. faecium* remain confined to lineages mainly recovered from hospitalized patients (2, 51, 58, 64, 80, 87, 111).

The $PAI_{E.faecalis}$ (154-kb) carries several putative virulence traits such as *cyl* (encoding a cytolysin active against Gram-positive bacteria and eucaryotic cells), *esp* (encoding a surface protein which contributes to biofilm formation and persistence of urinary tract infections in an animal model), unknown functions and sequences related to enterococcal pheromone and non-pheromone responsive plasmids (62, 128, 191, 193, 211, 218). The presence of highly similar $PAI_{E.\ faecalis}$ variants from human and animal isolates confirms the successful *transferability* of this element previously demonstrated *in vitro* (135, 149, 192). A PAI that encodes an Esp protein similar to that of *E. faecalis* has been identified in *E. faecium* (111, 229). This element is now called ICE*Efm1* and considered as an ICE element as it has recently been shown that it is self-transmissible and contains an integrase and a portion of Tn*916* (226). GIs related to metabolism include an 8.5-kb element recovered from isolates of the CC17 lineage which is linked to a novel metabolic pathway possibly involved in carbohydrate transport and metabolism (87, 117). Finally, a putative 17.8-kb GI harbouring the hyl_{Efm} gene has been associated with increased colonization in a mouse peritonitis model (6, 109, 169). It is a highly conserved genetic structure located within megaplasmids ranging from 150 to 350-kb with a potential role in carbohydrate metabolism that has been exclusively associated with *E. faecium* clinical/outbreak strains in several countries (36, 64, 67, 164).

3.2. Plasmids

Enterococcal plasmids were first described in 1972 and evidence for conjugative transfer among humans and among animals was reported in the early 80´s (30). To date, different enterococcal plasmid types have been described which differ in the mechanism of replication, conjugation abilities, and host range. According to the mechanism of replication, enterococcal plasmid families replicate by a rolling-circle (pRI1-like family) or a theta mechanism (RepA_N, Inc18-type, pHTβ-like) (68, 99, 179, 205, 233, 235). Diversity in the transfer system has also been reported (63, 70). Plasmid free strains seem to be

rare among enterococci (63, 235) (see Table 6 in section 1.3.3.1 of the introduction of this thesis).

3.2.1. Rolling-Circle Replicating plasmids (RCR)

RCR plasmids constitute a large group of small (<10kb), high-copy number plasmids, able to replicate in a broad range of gram-positive bacteria and gram negative bacteria (for reviews see 100, 235). To date, seven different families of RCR plasmids have been described based on the sequence of replication initiator proteins and the double strand origin of replication (*dso*). Enterococcal RCR plasmids belong to the family pRI1 (pRI1-like) and have only been detected among *E. faecium* isolates. Similarly to other families of RCR plasmids, that represented by pRI1 and derivatives comprises plasmids cryptic (pRI1, pJS42, pEFNP1) and conferring resistance to tetracycline (pKQ10::*tetU*). These plasmids are widely spread since they have been detected in isolates from farm animals (poultry, turkey and pigs) and hospitalized patients of different countries in the five continents (63, 68, 99). They can be mobilized by conjugative theta plasmids resistant to glycopeptide and streptogramin of different origins (68).

3.2.2. Theta-replicating plasmids

Enterococcal theta-replicating plasmids comprise small mobilizable (ca. <15 kb) and conjugative plasmids (>30 kb) of different families. They may encode a wide diversity of adaptive functions as resistance to antibiotics, biocides, metals, metabolic functions and virulence traits (Table 3).

3.2.2.1. *Small theta-replicating plasmids* (<15 kb) are widely distributed among enterococci, frequently associated with the production of bacteriocins with activity against a variety of gram-positive genera (Table 4). The replication proteins of these plasmids are highly similar to theta-type gram-positive plasmids belonging to the pUCL287 family from lactic acid bacteria (e.g. pIP501, pAMβ1) (14, 38). However, they may contain mobilization systems commonly associated with RCR plasmids or theta replicating plasmids (70). These plasmids have been isolated from enterococcal isolates recovered from hospitalized patients, animals (pigs, cow), cheeses, milk and dry-fermented sausage (Table 3).

3.*2.2.2. Inc18 family* (25-50kb). It is a group of low copy number plasmids that exhibit a very broad host range (104, 186, 212). Outstanding representatives of this family include

pIP501 from *Streptococcus agalactiae*, pRE25 and pAMβ1 from *E. faecalis,* and pIP186 from *E. faecium*.

Plasmids of the Inc18 family replicate by an antisense-RNA mediated transcriptional attenuation mechanism, which has only been detected in plasmids of gram positive bacteria (22, 42). Their conjugation system is also unique among Firmicutes (22, 70, 75). Plasmids of this family may eventually carry a toxin-antitoxin system known as ω–ε–ζ locus which contributes to plasmid stabilization and maintenance in pRE25 or pSM19035 (42, 186). They are under a global control exerted by ω on essential functions such as copy-number and stable inheritance (for more basic information see (22, 42, 75, 186).

Inc18 plasmids usually confer resistance to macrolides, lincosamides and streptogramins and often, to kanamycin, streptomycin, cloramphenicol and mercury due to the presence of different transposons or genetic islands (40, 114, 186). They have also been linked to the spread of Tn*1546* (*vanA*) among enterococci from hospitalized and healthy humans, animals and foods (99, 179, 204, 205, 252) (Table 3). Different studies indicate a role of the Inc18 plasmid family in the emergence of vancomycin-resistant *Staphylococcus aureus* by transfer of Inc18::Tn*1546* (*vanA*) plasmids from *E. faecalis* (54, 252, 253).

The size and backbone of the Inc18 plasmids is highly variable due to both acquisition of DNA (insertion sequences, transposons, genetic islands) but also to DNA losses which seem to occur by spontaneous deletion after conjugal transfer to different hosts (215, 254). It is also influenced by cointegration with theta plasmids of other families. The mosaic structure of particular members of the group highlights the relevance of lateral genetic transfer in the evolution of this plasmid family.

*3.2.2.3. RepA_N family***.** The family of RepA_N plasmids comprises different plasmid families of narrow host range which are broadly distributed among low G+C Gram-positive bacteria (233). The RepA proteins from the RepA_N family of plasmids fell into five large clusters with strong correlation with the host genus (*Enterococcus, Streptococcus, Lactobacillus, Lactococcus and Staphylococcus*) suggesting that the replication system has evolved in different host backgrounds. In enterococci, members of this family include pheromone-responsive plasmids from *E. faecalis*, and derivatives of pRUM and pLG1 plasmids from *E. faecium* (73, 109, 233).

Pheromone-responsiveness plasmids from enterococci exhibit a narrow-host range and they are commonly recovered from *E. faecalis* and eventually from *E. faecium* isolates of human and poultry origins. For details about the mechanism of replication and conjugation of these plasmids see reviews (30, 31, 103). More than 20 plasmids ranging from 30 to

90-kb have been identified in this group, pAD1, pPD1 and pCF10 being the most studied (30, 57). They have been sub-grouped in different incompatibility groups on the basis of distinct responses to small peptides called pheromones which are secreted by plasmid-free donors (Table 3). These plasmids may encode putative virulence traits as hemolysin/bacteriocin and/or antibiotic resistance elements as Tn*916*-like (*tetM*), Tn*4001 (aac-aph),* Tn*1456 (vanA)* or Tn*1549* (*vanB*) and are commonly recovered from strains isolated in severe nosocomial enterococcal infections in humans and from chicken feces (85, 95, 119, 195, 251).

Production of pheromones is restricted to *E. faecalis,* although *Enterococcus hirae*, *Staphylococcus aureus*, and *Streptococcus gordonii* secrete cAM373-like peptides with activity to pAM373-specific pheromone (30). One of this cAM373-responsive plasmids (pAM368) conferring high level of resistance to vancomycin (*vanA*) has been recently reported (31, 196). Although pheromone plasmids are unable to replicate in *S. aureus*, their transference and establishment in this host is possible by co-integration with other plasmids able to replicate in this species, which highlights the potential risk of spread of glycopeptide resistant staphylococci in the hospital setting (31, 238). In addition, specific plasmids as pAD1 enhance the rate of transfer of highly promiscuous conjugative transposons as Tn*916* or plasmid mobilization as that of pAMα1 (30, 31).

Derivatives of pAD1 can also contain an unique antisense-regulated toxin-antitoxin system *par* that ensures their stable inheritance. The *par* locus is encoded by the plasmid pAD1 but related loci have been identified on other *E. faecalis* plasmids (e.g. pCF10, pTEF2, pAM373, pAMS1, pMG2200) and chromosomes (*E. faecalis* V583), as well as on plasmids (VRSAp, pRC18) and chromosomes of *Staphylococcus* and *Lactobacillus* species. The occurrence of these *par*-like loci may be relevant in plasmid stability and metabolic regulation in different Gram-positive bacteria (234, 236). The mobilization of pheromone-responsive plasmids has recently been reported to involve transfer of antibiotic resistance and MLST markers, and capsule genes, and thus to promote chromosomal diversification in *E. faecalis* (128).

p*RUM-like derivatives* (e.g. pRUM, pS177) are mosaic plasmids generated by the recombination processes between transposons, ISs and small plasmids. They contain a specific TA system named Axe-Txe that acts as a segregational stability module in a wide spectrum of bacterial hosts (73, 79). Plasmids containing RepA and Axe-Txe from pRUM are widespread among *E. faecium* isolates from different countries, mainly in CC17 of nosocomial origin (63, 179). Vancomycin or tetracycline resistant isolates from humans and poultry frequently harbour pRUM-like plasmids, sometimes carrying Tn*1546* (63, 79, 99, 138, 208).

RepA_N megaplasmids. Megaplasmids are widely distributed among *E. faecium* isolates from different origins (15, 59, 64, 83, 109, 179, 243, 250). To date, the only megaplasmid fully sequenced is pLG1, a ~281-Kb plasmid isolated from a vancomycin resistant *E. faecium* nosocomial strain from Germany (109). It contains a *rep* gene coding for a protein with similarity with $RepA_{pAD1}$ and thus has been consider as a member of the RepA_N family.

Large plasmids are also common among *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* and they confer the ability to utilize complex carbohydrates from the diet, host-derived glycoproteins and glycoconjugates (118, 184). Enterococcal megaplasmids carry genes involved in the sugar metabolism (mannitol, glycerol, sorbitol, and raffinose) (15, 83, 179, 243, 250), in resistance to antibiotics (macrolides, glycopeptides, aminoglycosides) and heavy metals (copper-*tcrYAZB*) and enhanced epidemicity (glycoside hydrolase hyl_{Efm}) (6, 109). Megaplasmids seem to be frequent among *Enterococcus durans* and *Enterococcus hirae* (60, 122) but they have not been detected among *E. faecalis*. To date, the diversity of megaplasmids among *E. faecium, E. durans and E. hirae* remains largely unexplored.

3.2.2.4. pMG1-like family. It includes derivatives of pHT plasmids (pHTα, pHTβ and pHTγ) and pMG1 which have been associated with the spread of resistance to glycopeptides (Tn*1546_vanA*) and high-level resistance to aminoglycosides (Tn*4001*-like elements) among *E. faecium* and *E. avium* in the United States and Japan (210, 224, 225) (Table 3). Plasmids of this family show a high homology with the virulence pXO2 plasmid from *Bacillus anthracis*, suggesting they may have a common ancestor. Nevertheless, many ORFs of pHT and pMG1 plasmids do not show significant homology with any reported proteins, having been categorized as a new type of highly efficient conjugative plasmids. Its transfer system belongs to the MobP family based on the *oriT* and relaxase sequences (70, 222). To date, these plasmids types have been exclusively identified among *E. faecium* recovered from humans.

3.3. Bacteriophages

Most of the bacteriophages currently described in *Enterococcus* spp. belong to tailed families of *Podoviridae*, *Siphoviridae* and *Myoviridae* (134). Data related with the spread of virulence traits or antibiotic resistance by lysogenic bacteriophages among enterococci are limited and the contribution extent of prophages to niche adaptation and to the infectious phenotype of enterococci remains to be clarified (133, 229). Similarly to that happened for other MGEs, the incidence of prophages elements is mainly detected in the

genomes of *E. faecium* and *E. faecalis* (V583) that are poor in CRISPR systems (229) and seem to be more predominant in human isolates that in those from animal origin (116). Nevertheless, the high incidence of phage specific genes in *E. faecium* and *E. faecalis* suggests that they play an important role in the genome diversity observed among members of these species (113, 135, 229).

3.4. Self splicing elements

The group II introns are a family of large self-splicing RNAs wide spread in bacteria and have been considered useful tools for lactic acid microorganisms. They are capable of catalyzing their own splicing reaction, and often function as MGEs able to move by site-specific insertions (retrohoming), or to a lesser extent, by insertions into noncognate sites (retrotransposition). Most bacterial group II introns encode reverse transcriptase ORFs and are retroelements (39, 105).

Putative group II introns are present in the available sequenced genomes of *E. faecalis* and *E. faecium* (GenBank accession numbers AE016830 and NZ_AAAK03000007) encoding reverse transcriptases, in conjugative plasmids from humans and animals (pMG1, pVEF4 from *E. faecium* and pBEE99 from *E. faecalis*) or CTns (CTn*916*, CTn*5397* or CTn*6000*) (http://www.fp.ucalgary.ca/group2introns/) (32, 168, 205). In other species, their presence has been associated with the ability of transfer in conjugative plasmids pXO1 and pXO2 from *Bacillus anthracis*, the last being highly related with pMG1 from *E. faecium* (92, 210), and lactococcal plasmids from food (148). The prevalence of group II introns among enterococci might be underestimated given the LGT that is known to occur between bacteria belonging to the same GECs, corroborating recent data (205).

4. Conclusions

The emergence and increase of enterococcal strains with new adaptive traits (inferred by their antibiotic resistance, colonization, or virulence phenotypes and genotypes) illustrates the role of LGT in the combinatorial evolution of these microorganisms. Future advances in genomics and proteomics, and in the fields of social evolution and ecological genetics will be critical for designing safety interventions in human medicine, veterinary and food industry and also for developing new antibacterial targets under innovative ECO-EVO perspectives (12).

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Table 1. Insertion sequences found among enterococcal mobile genetic elements.

Family group[a]	Nature of the catalytic site	Insertion Sequence
IS*6*	DDE	IS*1216* IS*257*
IS*256*	DDE	IS*256* IS*16* IS*1191*/IS*905*[c] IS*1542*
IS*3*	DDE	IS*3*/IS*911* IS*150* IS*Enfa3* IS*153* IS*1485* IS*981*
IS*982*	DDE	IS*19* (IS*Efm1*)[d]
IS*30*	DDE	IS*6770* *(*IS*Ef1*)[d] IS*1252* IS*1678* IS*1062*
IS*5*	DDE	IS*Efa5*
IS*110*	Site-specific recombinase	IS*110*/*116/902* IS*Enfa110*
IS*605* IS*200*	Complex organization[b]	IS*Efa4* IS*Enfa200*
IS*L3*	Unknown[b]	IS*L3* IS*204*/IS*1001* IS*1096*/IS*1165* IS*1251* IS*1476*
IS*1380*	Unknown[b]	IS*Ecp1*
IS*NYC*	Unknown[b]	IS*1182*

Abbreviations: PAI, Pathogenicity island. [a]An IS family is defined as a group of ISs with related transposases, strong conservation of the catalytic site, conservation of organization and similar IRs (126) (http://www-is.biotoul.fr/). [b]The active sites of IS*200*/IS*605*, IS*L3*, IS*1380*, and IS*NYC* families have not been defined.
[c]IS*905* is an isoform of IS*1191*. [d]IS*19,* IS*Efm1,* IS*6770* and IS*Ef1* are synonyms.

Table 2. Enterococcal transposons[a]

Transposon	Family	Integrase type	Size (kb)	Characterized function[a] (genotype)	Host range	Origin	Integration sites	Reference
Conjugative Transposons								
Tn*916* and Tn*916* variants*	Tn*916*	Tyr	18	AB^R, Tet *(tetM)*	*Enterococcus, Staphylococcus, Streptococcus, Lactococcus, Lactobacillus, Bacillus, Clostridium, Leuconostoc, Listeria, Mycoplasma, Actinobacillus, Acholeplasma, Acinetobacter, Alcaligenes, Butyrivibria, Citrobacter, Erysipelothrix, Escherichia, Fusobacterium, Granulicatella, Haemophilus, Neisseria, Pseudomonas, Thermus, Ureaplasma, Veillonella,* anaerobes	H, HH, FA, WA, F, SW	A+T rich	(30, 143, 163)
Tn*918**	Tn*916*	Tyr	16	AB^R, Tet *(tetM)*	*Enterococcus faecalis, staphylococci*	H	A+T rich	(30)
Tn*919**	Tn*916*	Tyr	15.4	AB^R, Tet *(tetM)*	*S. sanguis, E. faecalis, Lactoccus lactis*	HH	A+T rich	(53)
Tn*920**	Tn*916*	Tyr	23	AB^R, Tet *(tetM)*	*Enterococcus faecalis*	H	A+T rich	(140)
Tn*925**	Tn*916*	Tyr	18	AB^R, Tet *(tetM)*	*Enterococcus faecalis, Leuconostoc*	H, HH, F	A+T rich	(28); Freitas AR *et al.*, unpublished
Tn*3702**	Tn*916*	Tyr	18	AB^R, Tet *(tetM)*	*Enterococcus faecalis*	H	A+T rich	(91)
Tn*5031*, Tn*5032*, Tn*5033**	Tn*916*	Tyr	NK	AB^R, Tet *(tetM)*	*Enterococcus faecium*	H	A+T rich	(55)
Tn*5381**	Tn*916*	Tyr	18	AB^R, Tet *(tetM)*	*Enterococcus faecalis*	H	A+T rich	(165, 171)
Tn*5383**	Tn*916*	Tyr	18	AB^R, Tet *(tetM)*	*Enterococcus faecalis*	H	A+T rich	(171)
Tn*1545* and variants	Tn*916*	Tyr	ca. 25	AB^R, Tet, Erm, Kan (*tetM*, *ermB, aphA-3*)	*Streptococcus, Enterococcus, Staphylococcus, Bacillus, Bacteroides, Clostridium, Lactococcus, Listeria, Escherichia, Eubacterium, Neisseria, Roseburia, Ureaplasma*	H, F	A+T rich	(30, 235)
Tn*5382*	Tn*916*	Tyr	34	AB^R, Van, Amp (*vanB2, pbp5*)	*Enterococcus* (*E. faecium*, *E. faecalis*, *E. gallinarum, E. hirae*), *Streptococcus*, anaerobes	H, A, F	A+T rich	(9, 27, 121, 227)
Tn*1549*	Tn*916*	Tyr	34	AB^R, Van (*vanB2*)	*E. faecium*, anaerobes	H, HH	A+T rich	(71, 108, 190)
Tn*5386*	Tn*916*	Tyr	29	Lantibiotics (*spa*)	*Enterococcus faecium*	H	A+T rich	(166)
Tn*5397*	Tn*916*	Ser	21	AB^R, Tet *(tetM)*	*Enterococcus (E. faecium, E. hirae), Streptococcus, Clostridium difficile, Bacillus subtilis*	H, HH, A, WA, F, SW	Single site	(3, 143, 158)
Tn*5801*, CW*459* (Tn*6086*, Tn*6014*)	Tn*916*	Not related to Tn*916*	25	AB^R, Tet *(tetM)*	*Staphylococcus, Enterococcus, Streptococcus mitis, Clostridium perfringens*	H, HH, A, F, SW	3' end of gene encoding GMP synthase	(43, 44, 143)
Tn*6000*	Tn*916*	Related to that of *S. aureus* PAI SaPIbov and SaPIbov2	33.2	AB^R, Tet *(tetS)*	*Enterococcus (E. faecium*, *E. faecalis*, *E. casseliflavus*)	H, HH, A	3' end of gene encoding ribosomal protein L31	(23, 143, 144, 174)

Table 2. Enterococcal transposons (*Continued*)

Transposon	Family	Integrase type	Size (kb)	Characterized function[a] (genotype)	Host range	Origin	Integration sites	Reference
Conjugative Tranposons *(cont.)*								
Tn*6009*	Tn*916*	Tyr	18	AB^R, Tet *(tetM)* Mercury (*mer*)	*Enterococcus, Streptococcus, Pseudomonas, Klebsiella, Serratia*	HHc	Not identified	(206)
Tn*950*	NI	Not identified	47	AB^R, Erm *(ermB)*	*Enterococcus faecium*	H	Not identified	(209)
EfaC1	NI	Tyr	25.3	None identified	*Enterococcus faecalis*	H	3' end of gene encoding tRNA	(24, 154)
EfaC2	NI	Tyr	32.7	None identified	*Enterococcus faecalis*	H	3' end of gene encoding GMP synthase	(24, 154)
EfaD2	NI	Tyr	ND	None identified	*Enterococcus faecalis*	H	Unknown	(24, 154)
OG1RF-CTn homologue	NI	Putative phage-related integrase	49	Putative adhesin proteins	*Enterococcus faecalis*	H	Unknown	(20)
Tn*3* derivatives								
Tn*917* and variants (Tn*3871*)	Tn*3* (Tn*551*)	Ser	5.1-5.4	AB^R, Erm *(ermB)*	*Enterococcus, Staphylococcus, Lactococcus, Streptococcus, Bacillus, Listeria, Paenibacillus*	H, HH, A, F, SW	A+T rich	(136, 145, 219)
Tn*1546* and variants	Tn*3* (Tn*3*)	Ser	10.8	AB^R, Van *(vanA)*	*Enterococcus, Bacillus, Staphylococcus, Oeskorvia, Streptococcus, Rhodococcus, Arcanobacterium haemolyticum, Paenibacillus*	H, HH, A, WA, P, F, SW	A+T rich	(7, 77, 120, 146, 237)

Table 2. Enterococcal Transposons (*Continued*)

Transposon	Flanking insertion sequences (Tn backbone)	Size (kb)	Characterized function	Host range	Origin	Comments	Reference
Composite Transposons							
Tn*5281* and variants	IS*256*-//-IS*256* (IS*256*-*aac6'-aph2''*-IS*256*)	4.7	AB^R, Gm (*aac6'-aph2''*)	*Enterococcus, Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma*	H, P	Essentially identical to Tn*4001* from *S. aureus* and Tn*3706* from *Streptococcus* Larger elements identified: Tn*5384*, Tn*5385*, *SCCmec*	(89, 115, 200, 201)
Tn*924*	IS*257*-//-IS*257* (IS*257*-*aac6'-aph2''*-IS*257*)	27	AB^R, Gm (*aac6'-aph2''*)	*E. faecalis*	H		(213)
Tn*5384*	IS*256*-//-IS*256* (IS*256*-Tn*4001*-ΔTn*917*-*mer*-IS*256*)	26	AB^R, Gm, Erm (*aac6'-aph2''*, *ermB*) Mercuric chloride (*merX*)	*E. faecalis*	H	Directly repeated copies of IS*256* Larger element identified: Tn*5385*	(17, 167)
Tn*5385*	IS*1216*-//-IS*1216* (IS*1216*-Tn*5381*-*Tn5384*-*Tn552*-IS*1216*)	65	AB^R, Gm, Erm, Sm, Tet, Pen (*aac6'-aph2''*, *ermB, aadE, tetM, blaZ*) Mercuric chloride (*mer*)	*E. faecalis*	H	Directly repeated copies of IS*1216*	(165)
Tn*5405* and variants	IS*1182*-//-IS*1182* (IS*1182*-*aadE-sat4-aphA-3*-IS*1182*)	12	AB^R, Sm, St, Km (*aadE-sat4-aphA-3*)	*Enterococcus, Staphylococcus*, *Streptococcus, Campylobacter*	H, HH, P, A, F, SW	Inverted repeated copies of IS*1182* Larger element identified*:* Tn*4001*-Tn*5405,* Tn*5405*::Tn*5404, ermB*-Tn*5405*	(16, 46, 212, 242)
Tn*1547*	IS*16*-//-IS*256*-like (IS*16*-*vanB1*-IS*256*-like)	64	AB^R, Van *(vanB1)*	*Enterococcus*	H	Direct orientation of IS*16* and IS*256*-like	(159)
Tn*5482*	IS*1216*-//-IS*1216* (IS*1216*-ΔIS*3*-like-Tn*1546*::*IS1251*-IS*1216*)	26-30	AB^R, Van *(vanA)*	*E. faecium, E. faecalis*	H		(82)
Tn*5506*	IS*1216*-//-IS*1252*-*IS1216* (IS*1216V2*-*vanA*-IS*1252*-*IS1216V1*)	39	AB^R, Van *(vanA)*	*E. faecium*	H	IS*1216V2* in inverted direction of IS*1216V1* with IS*1252* insertion	(85)

[a] AB^r, antibiotic resistance; Amp, ampicillin; Erm, erythromycin; Gm, high level of resistance to gentamicin; Km, kanamycin; Sm, streptomycin; St, streptothricin; Tet, tetracycline; Van, vancomycin: Tyr, tyrosine recombinase; Ser, serine recombinase; H, Hospital; HH, healthy humans; HHc, Healthy humans (children); A, animals (farm or healthy animals); WA, wild animals; F, foods; P, pets; SW, sewage.

Table 3. Enterococcal plasmid types.

Plasmid family	Plasmid	Size (kb)	Original host (country)	Origin	Host range[c]	Genetic markers (pheromone response[d])	Reference
ROLLING-CIRCLE REPLICATING PLASMIDS							
pT181	pJS42	4,1	*Enterococcus faecium* JH95		*E. faecium*	cryptic	GenBank acc. no. NC_010291
(pRI1-like)	pRI1	6,0	*Enterococcus faecium* 9631160-1 (UK)	F (chicken), A (poultry), H	*E. faecium, E. hirae*	cryptic	(68)
	pEFNP1	ND	*Enterococcus faecium* N15		*E. faecium*	cryptic	GenBank acc. no. AB038522
	pKQ10	ND	*Enterococcus faecium*		*E. faecium*	*tet(U)*	(173)
THETA REPLICATING PLASMIDS							
Small	pCIZ2	7,4	*Enterococcus faecium* L50 (Spain)	F (sausage, chicken), H	*E. faecium*	cryptic	(37)
mobilizable	p200B	12,5	*Enterococcus faecium*		*E. faecium*	cryptic	GenBank acc. no. AB158402
	pB82	6,2	*Enterococcus faecium* VR82 (USA)	H, F (chicken), SW	*E. faecium, E. hirae, E. durans*	cryptic	(217)
	pEFR	3,2	*Enterococcus faecium*		*E. faecium*	cryptic	GenBank acc. no. AF511037
	pAMα1	9,8	*Enterococcus faecalis* DS5 (USA)	H	*E. faecalis*	*tet(L)*	(50)
	pS86	5,1	*Enterococcus faecalis* S-86 (Spain)	H	*E. faecalis*	cryptic	(130)
	pEF47	5,5	*Enterococcus faecalis* 47 (Slovakia)	A (cow)	*E. faecalis*	cryptic	GenBank acc. no. AY842500
	p703/5	ND	*Enterococcus faecalis* KBL703 (Korea)	F (milk)	*E. faecalis*	NK	AF109375
	pEF1071	9,3	*Enterococcus faecalis* BFE 1071 (Germany)	A (pig)	*E. faecalis*	cryptic	(8)
	Plasmid	>3,2[b]	*Enterococcus faecalis* FAIR-E 309 (Argentina)	F (cheese)	*E. faecalis*	cryptic	GenBank acc. no. AY063485
	pEF418	>15,9[b]	*Enterococcus faecalis* 418	H	*E. faecalis, E. faecium*	*aadE*	GenBank acc. no. AF408195
	pGL	8,3	*Enterococcus durans* 41D (USA)	F (cheese)	*E. durans*	cryptic	GenBank acc. no. HQ696461
Inc18-like	pIP501	30,6	*Streptococcus agalactiae* B96 (France)	H, A, SW	*E. faecalis, E. faecium*	*cat, erm(B)*	(215)
	pRE25	50,2	*Enterococcus faecalis* RE25	F (sausage, chicken), H, HH, A (poultry, pig), SW	*E. faecalis, E. faecium*	*cat, erm(B), sat4, aph(3')-III, aadK*	(186)
	pAMβ1	27,8	*Enterococcus faecalis* DS5	H	*E. faecalis, E. faecium*	*erm(R)*	GenBank acc. no. GU128949

Table 3. *(Continued)*

Plasmid family	Plasmid	Size (kb)	Original host (country)	Origin	Host range[c]	Genetic markers (pheromone response[d])	Reference
Inc18 (cont.)	pTEF1[a]	66,3	*Enterococcus faecalis* V583	H	*E. faecalis*	*Tn4001, erm(B), qacZ* (cAD1)	(154)
	pEF-01[a]	35,9	*Enterococcus faecalis* EF-01 (China)	A (bovine)	*Efs*	*cfr*	GenBank acc. no. CP002208
	pPPM1000	ND	*Enterococcus faecium*		Unknown	Sm^r, Hg^r	(40)
	pIP816	34,6	*Enterococcus faecium* BM4147 (France)	H	*E. faecium*	*vanA, str*	(205)
	pVEF1[a]	39,6	*Enterococcus faecium* 399/F99/H8 (Norway)	HH, F (chicken), A (poultry)	*E. faecium, E. hirae, E. durans*	*vanA*	(204)
	pVEF2[a]	39,7	*Enterococcus faecium* 399/F99/A9 (Norway)	F (chicken), A (poultry)	*E. faecium, E. hirae, E. durans*	*vanA*	(204)
	pVEF3[a]	63,1	*Enterococcus faecium* 399/S99/A7 (Norway)	A (poultry)	*E. faecium*	*vanA*	(203)
	pVEF4[a]	>44[b]	*Enterococcus faecium* 399/F98/A4 (Norway)	A (poultry)	*E. faecium*	*vanA*	(205)
	pEF1	21,3	*Enterococcus faecium* 6T1a (Spain)	F (olive), H, HH, F (chicken), A (pig), SW	*Efm*	*uvrA*	(181)
RepA_N	*Pheromone- responsive plasmids*						
	pAD1	59,3	*Enterococcus faecalis* DS16 (USA)	H, F (chicken), A (pig), SW	*E. faecalis*	*uvrB* (cAD1)	(57)
	pJH2	59	*Enterococcus faecalis* JH1 (UK)	H	*E. faecalis*	(cAD1)	(30)
	pIP964	65	*Enterococcus faecalis*		*E. faecalis*	(cAD1)	(30)
	pAMγ1	60	*Enterococcus faecalis* DS5		*E. faecalis*	*uvr* (cAD1)	(30)
	pBEM10	70	*Enterococcus faecalis* HH2 (USA)	H	*E. faecalis*	*bla,* Tn*4001* (cAD1)	(140)
	pCF10	67,7	*Enterococcus faecalis* SF-7 (USA)	H, F (chicken)	*E. faecalis*	*tet(M)*-CTn*925, uvrA* (cCF10)	(30)
	pTEF2	57,7	*Enterococcus faecalis* V583 (USA)	H	*E. faecalis*	(cCF10)	(154)
	pAMS1	130	*Enterococcus faecalis* MC4 (Sweden)	HH	*E. faecalis*	(cCF10)	(187)
	pMB1	90	*Enterococcus faecalis* S-48 (Spain)	HH	*E. faecalis*	(cCF10)	(30)
	pPD1	59	*Enterococcus faecalis* 39-5 (USA)	HH	*E. faecalis*	(cPD1)	(65)
	pYI14	61	*Enterococcus faecalis* 39-5 (Japan)	H	*E. faecalis*	(cPD1)	(223)
	pMB2	56	*Enterococcus faecalis* S-48 (Spain)	HH	*E. faecalis*	(cPD1)	(30)

Table 3. *(Continued)*

Plasmid family	Plasmid	Size (kb)	Original host (country)	Origin	Host range[c]	Genetic markers (pheromone response[d])	Reference
RepA_N	pEJ97-1	11,3	*Enterococcus faecalis* EJ97 (Spain)	SW	*E. faecalis*	(cPD1)	(183)
	pAM373	36,8	*Enterococcus faecalis* RC73 (USA)	H, F (chicken)	*E. faecalis*	(cAM373)	(41)
	pAM368	107	*Enterococcus faecalis* 368 (USA)	H	*E. faecalis*	*vanA* (cAM373)	(196)
	pBEE99	80,6	*Enterococcus faecalis* E99 (USA)	H	*E. faecalis*	*bee, uvr* (UK pheromone)	(32)
	pMG2200	106,5	*Enterococcus faecalis* NKH15 (Japan)	H	*E. faecalis*	*vanB2, uvr* (UK pheromone)	(251)
	pAM323	66	*Enterococcus faecalis* HH2 (USA)	H	*E. faecalis*	(cAM323)	(140)
	pAM324	53	*Enterococcus faecalis* HH2 (USA)	H	*E. faecalis*	(cAM324)	(140)
	pHKK100	55	*Enterococcus faecalis* 228 (Spain)	H	*E. faecalis, E. faecium*	*vanA* (cHKK100)	(81)
	pOB1	71	*Enterococcus faecalis* 5952 (USA)	H	*E. faecalis*	(cOB1)	(141)
	pYI1/2	58/56	*Enterococcus faecalis*	H	*E. faecalis*	(cOB1/cYI2)	(94)
	pYI17	57,5	*Enterococcus faecalis* YI717	H	*E. faecalis*	(cYI17)	(221)
	pAMγ2	~60	*Enterococcus faecalis* DS5		*E. faecalis*	(cAMγ2)	(30)
	pAMγ3	~60	*Enterococcus faecalis* DS5		*E. faecalis*	(cAMγ3)	(30)
	pSL1/2	128,1	*Enterococcus faecalis* KV1 (Korea)	H/A (chicken)	*E. faecalis*	*vanA, ermB, aac(6)-aph(2), ant(6)-Ia, aph(3')-IIIa* (cSL1)	(119)
	pHKK703	55	*Enterococcus faecium* R7 (USA)	H	*E. faecalis, E. faecium*	(cCF10)	(85)
	pBRG1	50	*Enterococcus faecium* LS10 (Italy)	H	*E. faecalis, E. faecium*	*vanA* (cCF10-like)	(125)
	pRUM	24,9	*Enterococcus faecium* U37 (USA)	H, F (poultry), A (pig)	*E. faecium*	*cat,* ΔTn*5405, erm(B), uvr*	(73)
	pS177	39	*Enterococcus faecium* S177		*E. faecium*	*vanA*	(79)
	p5753cB[a]	56,7	*Enterococcus faecium* 5753c (USA)	H	*E. faecium*	*aac(6')-aph(2"), tetM,* Δ*Tn5405, erm(B)*	GenBank acc. no. GQ900487
	pLG1	281,0	*Enterococcus faecium* UW2774 (Germany)	H, HH, A (pig), F (chicken), SW	*E. faecium*	*vanA, erm(B), tcrYAZB, ctpA*	(109)
pHTβ-like	pHTβ	52,9	*Enterococcus faecium* FH (Japan)	H, HH, F (chicken)	*E. faecium, E. avium, E. durans*	*vanA*	(222)
	pMG1	65,0	*Enterococcus faecium* (Japan)	H, HH, F (chicken)	*E. faecium, E. durans*	Tn*4001*	(210)

Abbreviations: ND, not determined; H, Hospital; HH, healthy humans; A, animals (farm or healthy animals); F, foods; SW, sewage.
[a]Mosaic plasmids containing more than one rep sequence related with different plasmid types in which one of them belongs to the plasmid family alocated in the table.
[b]Only partial sequence of the plasmid is available. [c]*Enterococcus* species where these plasmids have been identified to date or have been shown to be able to replicate in. [d]Only applicable to pheromone-responsive plasmids.

Table 4. Characteristics of bacteriocins previously associated with small theta-replicating plasmids in enterococci[a]

Bacteriocin	Type[b]	Source			Activity spectrum	Observations	References
		Plasmid (size)	Host	Origin			
Bac 32	Class IIa, pediocin-like	pTI1/p200B (12.5 kb)	*E. faecium*	H	*E. faecium, E. hirae, E. durans*	Novel bacteriocin widely disseminated among *E. faecium* (both VRE and VSE)	(96)
Bac 43	Class IIa, pediocin-like	pDT1/pB82 (6.2 kb)	*E. faecium*	H	*E. faecium, E. faecalis, E. hirae, E. durans, Listeria monocytogenes*	Only identified in vancomycin-resistant clinical *E. faecium*	(217)
Enterocin Q	Class IIc, leaderless	pCIZ2 (7.4 kb)	*E. faecium L50*	F (sausage)	*Listeria monocytogenes, Clostridium spp., Streptococcus spp.*	*E. faecium* L50 produces 3 different bacteriocins (EntP, EntL50 and EntQ)	(37)
Enterocin 1071A **Enterocin 1071B**	Class IIb, two peptide bacteriocin	pEF1071 (9.3 kb)	*E. faecalis*	A (minipig)	*E. faecium, E. faecalis, E. durans, Listeria innocua, Lactobacillus salivarius, Streptococcus agalactiae, Peptostreptococcus aerogenes, Micrococcus sp., Propionibacterium*		(8)
Duracin GL	Class II	pGL (8.3 kb)	*E. durans*	F (cheese)	Antilisterial mode of action		GenBank accession no. HQ696461

[a]A, animal; H, hospital; F, foods.
[b]The heat-stable peptide bacteriocins from lactic acid bacteria have so far been grouped into two major classes: class I, the lantibiotics, and class II, the heat-stable nonlantibiotics. The bacteriocins from enterococci belong almost exclusively to the heat-stable, nonlantibiotic class II bacteriocins, with the exception of cytolysin. Class II bacteriocins are usually sub-divided in pediocin-like bacteriocin (IIa), the two-peptide bacteriocins (IIb), the leaderless peptide bacteriocins (IIc), and the circular bacteriocins (IId).

Book outline

Title of the book: "Enterococcus and safety"

Series "Advances in Food Safety and Food Microbiology"

Editors of the series: Anderson de Souza Sant'Ana and Bernadette D.G.M. Franco. (Department of Food and Experimental Nutrition. Faculty of Pharmaceutical Sciences. University of São Paulo. São Paulo, SP. Brazil)

Book Editors: Teresa Semedo-Lemsaddek, Maria Teresa Barreto-Crespo and Rogério Tenreiro

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3.4. Screening of the *in vitro* activity of the new molecule tigecycline against *Enterococcus* spp. from different ecological niches in Portugal

3.4.1. Non-susceptibility to tigecycline in enterococci from hospitalized patients, food products and community sources.

Non-susceptibility to tigecycline in enterococci from hospitalized patients, food products and community sources

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ABSTRACT

We analyzed the in vitro activity of tigecycline against 1,140 enterococci collected from humans, food products, animals and the environment in Portugal (1996-2008). Ten isolates non-susceptible to tigecycline (7 *E. faecalis,* 3 *Enterococcus* spp.; MIC 0.5-1.0 mg/L), which were also resistant to tetracycline and minocycline, were mostly observed in samples collected before the introduction of tigecycline in the therapeutic arsenal. The *E. faecalis* isolates were recovered from hospitalized patients (n=2, ST319/CC2 and ST34), healthy humans (n=2, ST21/CC21), chicken meat (n=1 ST260) and from two swine samples. The remaining isolates were also recovered from chicken meat (n=1 *E. gallinarum*) and swine (n=2, *E. hirae* and *Enterococcus* spp.). The recovery of enterococcal isolates with reduced susceptibility to tigecycline among different reservoirs, including animals for food consumption, suggests that selection of resistant tigecycline isolates by antibiotics other than tigecycline might occur in non-clinical settings.

Keywords: *Enterococcus,* hospitals, healthy humans, animals, tigecycline, tetracycline

The most clinically relevant Gram-positive pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and multidrug-resistant pneumococci, have increasingly acquired resistance to most antimicrobial agents making the nosocomial infections they cause difficult to treat (1). Against this background, there is a clear need for therapeutic alternatives. Glycylcyclines are novel tetracycline analogues with improved ribosomal binding-site affinities which show activity against a broad spectrum of bacteria, including those resistant to tetracycline (2, 3). Tigecycline (9-*t*-butylglycylamido-minocycline), the first glycylcycline approved for clinical use by the FDA in 2005 and registered by the European Medicines Agency in May 2006, is recommended to treat complicated skin and intra-abdominal infections caused by several Gram negative and Gram positive organisms including multidrug-resistant *Enterococcus faecalis* and *Enterococcus faecium*. The World Health Organization has recently highlighted the relevance of tigecycline as one of the last therapeutic options in several human infections (4). A few *E. faecalis* resistant isolates showing MIC values close to the ECOFF (epidemiological cut-off value) for this antibiotic have been reported in large clinical trials although a mechanism of resistance remains to be characterized (5, 6). To our knowledge, the activity of tigecycline against enterococcal isolates recovered from the community has not previously been studied. Moreover, there are no data regarding the activity of this antibiotic against enterococcal isolates from Portugal, one of the countries with highest VRE prevalence (http://www.earss.rivm.nl). The aim of this study was to evaluate the *in vitro* activity of tigecycline, an antibiotic of last resort to treat severe human infections, against enterococci from different sources.

A total of 1,140 enterococci isolates collected during 1996-2008 were studied, most of which have been included in previous studies by our group (7). They were recovered from the following sources: (i) *n*=294 clinical isolates from different patients at six hospitals of different cities in Northwestern, central and Eastern Portugal (1996-2008); (ii) *n*=208 from faecal samples of healthy human volunteers living in the north and centre of the country (2001-2004); (iii) *n*=288 from swine faeces and environmental samples recovered in six piggeries and one slaughterhouse from the north, centre and south of Portugal (1997-2007); (iv) *n*=234 from raw poultry products corresponding to 93 chicken lots and six turkey lots from 10 different commercial brands and purchased at two different butcher shops in Porto area (1999-2001); and (v) *n*=116 from hospitals wastewaters and from the estuary of Douro river in Porto city (2001-2003). Clonal relatedness was established in specific cases by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) as described (http://www.mlst.net) (7). Species identification and vancomycin and tetracycline resistance gene detection were performed by multiplex PCR assays (8).

Susceptibility testing was determined by the standard agar dilution or disk diffusion methods following CLSI guidelines (9). For tigecycline (Wyeth Pharmaceuticals, Hampshire UK), cation-adjusted Mueller-Hinton II agar (BioMérieux, Marcy l'Étoile, France) was always freshly prepared and the antibiotic was incorporated into the media a few hours prior use to avoid oxidation (10). When borderline to resistance MIC values were obtained, susceptibility assays were repeated 5 times in order to evaluate reproducibility of results. The strains *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as control of susceptibility tests. The susceptibility breakpoint value was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) which establishes the clinical criteria for interpretation of tigecycline activity in enterococci (susceptibility ≤0.25 mg/L and resistance >0.5 mg/L) (http://www.eucast.org; last update on January 2011). This committee also recommends an ECOFF (MIC ≤0.25 mg/L) to detect enterococci expressing acquired resistance mechanisms to tigecycline (http://217.70.33.99/Eucast2/SearchController) (11). According to these guidelines, isolates showing MIC ≥0.5 mg/L were considered non-susceptible to tigecycline.

Among 284 clinical isolates and 856 non-clinical isolates, enterococci were identified as *E. faecium* (n=485), *E. faecalis* (n=405) and *Enterococcus* spp. (n=250). Eight hundred and eighty-four isolates (78%) were resistant to three or more antibiotics from different groups; 796 (70%) showed resistance to tetracycline; and 270 (24%) were resistant to vancomycin (83% *vanA*, 16% *vanC1* and 1% *vanB*). Most studied isolates (>99%) were susceptible to tigecycline showing MICs ranging from ≤0.03 to 0.25 mg/L (Table 1). In agreement with other studies, the MIC_{50} and MIC_{90} values for enterococci from different origins were 0.125 mg/L and 0.25 mg/L, respectively (12, 13). Also, *E. faecalis* isolates showed higher MIC values than *E. faecium* and *Enterococcus* spp. isolates (0.25 mg/L vs 0.125 mg/L) as previously reported (12-14). A difference in tigecycline activity was not observed in isolates from different sources or with different antibiotic phenotypes.

Ten tetracycline resistant isolates recovered from hospitalized and non-hospitalized humans, food products and animals (7 *E. faecalis*, 1 *E. hirae*, 1 *E. gallinarum* and 1 *Enterococcus* spp., MIC=32->256 mg/L) were also resistant to minocycline (MIC=32-64 mg/L), and showed non-susceptibility to tigecycline with MICs ranging from 0.5 mg/L (n=7, of which 4 contained *tetM*) to 1 mg/L (n=3, 1 *tetM* and 1 *tetM+tetL*) (Table 2). *E. faecalis* were obtained from: i) hospitalized patients [n=2 isolates recovered in 2002; the new sequence type (ST) 319 belonging to the Clonal Complex (CC) 2 and ST34]; ii) healthy humans (n=2 isolates from 2001; same PFGE type identified as ST21/CC21); iii) chicken meat (n=1 ST260 from 1999); and iv) swine (n=2 isolates from 2006-2007, not typed by MLST). The remaining enterococcal isolates (1999-2007) were recovered from chicken

meat (1 *E. gallinarum*) and swine (1 *E. hirae*, 1 *Enterococcus* spp.). Previous studies have also described a few *E. faecalis* clinical isolates exhibiting MICs against tigecycline of 0.5 mg/L (6, 12, 15) and 1 mg/L (by broth microdilution) (5).

To our knowledge, the only enterococcal isolate showing a MIC value against tigecycline higher than the EUCAST clinical breakpoint was a clinical German *E. faecalis* strain belonging to the prevalent ST6 (CC2) from which resistance was associated with antibiotic treatment comprising multiple courses of different drugs besides tigecycline (5). In this study, we report the lack of susceptibility to tigecycline among *E. faecalis* from hospitalized patients, healthy humans, chicken meat and swine mainly collected before the introduction of tigecycline in the clinical practice. The data suggest that a selection process of resistant tigecycline isolates might occur in non-clinical settings involving antibiotics other than tigecycline.

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Competing interests: None declared

Ethical approval: Not required

Table 1. In vitro activity of tigecycline against enterococci isolated from different species and sources.

Species	Source	Type	*n* tested	Number of isolates inhibited at tigecycline MIC (mg/L) of: ≤0.03	0.0625	0.125	0.25	0.5	1.0	MIC50	MIC90
E. faecalis	ATCC 29212	Control[a]			X	X					
S. aureus	ATCC 29213	Control[a]				X	X				
E. faecium	Hospital	VRE	112	2	36	49	25			0.125	0.25
n = 485		VSE	18		5	10	3			0.125	0.25
	Healthy humans	VRE	6		1	3	2			0.125	0.25
		VSE	80	3	23	42	12			0.125	0.25
	Swine	VRE	7		2	4	1			0.125	0.25
		VSE	98	1	17	50	30			0.125	0.25
	Poultry	VRE	46	3	4	26	13			0.125	0.25
		VSE	42	1	7	23	11			0.125	0.25
	Environment	VRE	26		9	15	2			0.125	0.25
		VSE	50	1	12	23	14			0.125	0.25
E. faecalis	Hospital	VRE	37		5	15	17			0.125	0.25
n = 405		VSE	123	2	20	38	61	**2**		0.25	0.25
	Healthy humans	VRE	0								
		VSE	100	1	7	24	66	**1**	**1**	0.25	0.25
	Swine	VRE	1				1			ND*	ND*
		VSE	52	2	3	8	37	**2**		0.25	0.25
	Poultry	VRE	5	1		2	2			ND*	ND*
		VSE	59	1	1	21	35	**1**		0.25	0.25
	Environment	VRE	3		1	2				ND*	ND*
		VSE	25		5	12	8			0.125	0.25
Enterococcus spp.	Hospital	VRE	2			2				ND*	ND*
n = 250		VSE	2			2				ND*	ND*
	Healthy humans	VRE	0								
		VSE	22		3	9	10			0.125	0.25
	Swine	VRE	0								
		VSE	130	4	10	53	61	**1**[b]	**1**[c]	0.125	0.25
	Poultry	VRE	25		5	12	8			0.125	0.25
		VSE	57	3	9	28	16		**1**[d]	0.125	0.25
	Environment	VRE	0								
		VSE	12		3	7	2			0.125	0.25

Abbreviations: VSE, vancomycin-susceptible enterococci; VRE, vancomycin-resistant enterococci; ND*, not determined due to the low number of isolates; *n*, number of isolates. Numbers in bold correspond to the 10 isolates showing MIC values of 0.5-1.0 mg/L (non-susceptibility).

[a]The MICs (mg/L) of tigecycline varied between 0.125-0.25 mg/L for *S. aureus* ATCC 29213 and 0.06-0.125 mg/L for *E. faecalis* ATCC 29212 control strains, which are consistent with data reported by other groups (5, 12).

[b]Swine isolate identified as *E. hirae.*

[c]Swine isolate that could not be identified by the multiplex PCR assays used which includes *E. faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*, *E. hirae* and *E. durans*.

[d]Poultry isolate identified as *E. gallinarum.*

Table 2.* Features of *Enterococcus* isolates non-susceptible to tigecyline.

Species	Source	Year	PFGE	MLST	*tet*[a]	Antibiotic resistance profiles						
						TIG	TET	MIN	ERY	CIP	STR	GEN
						MIC (µg/ML)						
E. faecalis	Healthy human	2001	A	ST21 (CC21)	M	**0.5**	**32**	**64**	S	S	**R**	S
E. faecalis	Healthy human	2001	A	ST21 (CC21)	M	**1**	**64**	**64**	S	S	**R**	S
E. faecalis	Chicken retail product	1999	B	ST260 (CS)	M	**0.5**	**64**	**64**	S	S	S	S
E. faecalis	Piggery 1 (waste lagoon)	2006	ND	ND	-	**0.5**	**128**	**32**	**R**	S	S	**R**
E. faecalis	Piggery 1 (liquid manure)	2007	ND	ND	-	**0.5**	**>256**	**64**	**R**	S	**R**	S
E. faecalis	Hospitalized patient	2002	C	ST319 (CC2)[b]	-	**0.5**	**>256**	**64**	**R**	S	**R**	S
E. faecalis	Hospitalized patient	2002	D	ST34	M	**0.5**	**128**	**64**	S	S	S	S
E. gallinarum	Chicken retail product	1999	ND	NA	M	**1**	**128**	**32**	S	S	S	S
E. hirae	Piggery 1 (solid manure)	2006	ND	NA	M, L	**0.5**	**>256**	**32**	S	S	S	S
Enterococcus spp.	Piggery 2 (liquid manure)	2007	ND	NA	-	**1**	**64**	**32**	S	**R**	S	S

*Abbreviations: PFGE, Pulsed-Field Gel Electrophoresis; MLST, Multi-locus Sequence Typing; CC, clonal complex; CS, singleton; ST, sequence type; ND, not determined; NA, not applicable; R, resistant; S, susceptible; TIG, tigecycline; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; STR, high-level resistance to streptomycin; GEN, high-level resistance to gentamicin.
[a]*tet* genes sought by PCR included *tetM, tetL, tetO, tetK, tetS* and *tetX*.
[b]ST319 is a novel sequence type clustering in CC2 by eBURST analysis.

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Title: Non-susceptibility to tigecycline in enterococci from hospitalized patients, food products and community sources

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Abstract: We analyzed the in vitro activity of tigecycline against 1,140 enterococci collected from humans, food products, animals and the environment in Portugal (1996-2008). Ten isolates non-susceptible to tigecycline (7 E. faecalis, 3 Enterococcus spp.; MIC 0.5-1.0 mg/L), which were also resistant to tetracycline and minocycline, were mostly observed in samples collected before the introduction of tigecycline in the therapeutic arsenal. The E. faecalis isolates were recovered from hospitalized patients (n=2, ST319/CC2 and ST34), healthy humans (n=2, ST21/CC21), chicken meat (n=1 ST260) and from two swine samples. The remaining isolates were also recovered from chicken meat (n=1 E. gallinarum) and swine (n=2, E. hirae and Enterococcus spp.). The recovery of enterococcal isolates with reduced susceptibility to tigecycline among different reservoirs, including animals for food consumption, suggests that selection of resistant tigecycline isolates by antibiotics other than tigecycline might occur in non-clinical settings.

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- **Transferability of tigecycline resistance and plasmid characterization**

Further molecular analysis were performed in order to evaluate the *in vitro* transfer of tigecycline resistance of the ten enterococci isolates with reduced susceptibility to tigecycline described in the former manuscript (3.4.1.).

Methods:

- ***Transferability of tigecycline and plasmid characterization***

Transfer of tigecycline resistance from the isolates analyzed to *E. faecalis* JH2-2, *E. faecium* GE-1 and BM4105RF, and *S. aureus* NCTC8325 recipient strains (all fusidic acid and rifampicin resistant, plasmid free) was screened by filter mating at a 1:1 donor-recipient ratio using brain heart infusion (BHI) agar supplemented with 0.5 mg/L tigecycline, 30 mg/L rifampicin and 20 mg/L fusidic acid as described (3). Plasmid characterization included determination of plasmid size and number by S1-PFGE, comparison of *Eco*RI, *Cla*I and *Hind*III digested plasmid DNA patterns, and identification of the genes encoding replication initiator proteins by a multiplex PCR method (4).

- ***Characterization of E. faecalis isolates transferring tigecycline resistance***

Transferability was successfully achieved for three *tetM*-carrying *E. faecalis* isolates (2 ST21 and one ST260 isolate from retail chicken) to *E. faecalis* strain JH2-2. *E. faecalis* JH2-2 transconjugants exhibited MIC values to tetracycline of 64-128 mg/L and MIC to tigecycline of 2 mg/L, which are three to seven-fold higher than those of recipient strains.

A single copy of the *tetM* gene located on a 80Kb-conjugative plasmid was identified in all three wild-type isolates while multiple copies located on both plasmid and chromosome were detected in the corresponding *E. faecalis* JH2-2 transconjugants (4, 5 and 7 copies, respectively) (Figure 1). Interestingly, an enhanced MIC value in transconjugants was not observed for minocycline despite *tetM* confers resistance to both tetracycline and minocycline.

These *tetM*-80Kb-plasmids contained a RepA protein displaying $\geq$98% identity at the protein level with RepA_N from plasmids pCF10, pMG2200 and pBEE99 (GenBank accession numbers YP_195765, YP_002333458 and YP_003329050, respectively) and other sequences specific of pCF10 backbone (data not shown) and thus they were considered pCF10 derivatives (GenBank acc. number AY855841). Interestingly, this plasmid was not observed in the 7 enterococci isolates that did not transfer tigecycline resistance (data not shown). Comparison of digested plasmid DNA patterns of the three pCF10 derivatives revealed highly related patterns, those collected from the healthy human showing an identical RFLP profile. Moreover, comparison of *Eco*RI and *Cla*I

enzyme-RFLP patterns of the pCF10 derivatives identified in this study with that of the original pCF10 generated by the NEBcutter programme (http://tools.neb.com/NEBcutter2) also revealed that they were highly similar. A second plasmid of ca. 5-kb lacking *tet* genes containing a RepA protein 100% homologous to that of the theta-replicating plasmids 9,7Kb-pAMα1 and 5,5kb-pEF47 (GenBank accession numbers AF503772 and AY842500, respectively) was identified. These plasmids contain identical replication and transfer machineries although only pAMα1 carries *tetL* gene.

Previous studies have shown that **the** presence of *tetM* in both VRE and *S. aureus* is associated with a slight increase in the MIC values of tigecycline and minocycline, respectively (1, 2). The *tetM* gene is linked to CTn*916* or derivatives which may be located on the chromosome and less frequently on conjugative or non-conjugative plasmids. We identified the *tetM* gene in a 6,125Kb orf12–int_{CTn916} region identical to CTn*925*, a CTn*916* derivative previously identified on pCF10 (GenBank accession number AY855841).

The diversity of *Sma*I profiles observed after hybridization with the *tetM* gene probe in the transconjugants highlights the occurrence of different genetic events linked to CTn*925* during conjugation and it confirms the increased tetracycline resistance by gene amplification of *tetM* copies as described (5).

These preliminar results indicate that event though the mechanism of tigecycline resistance was not elucidated, a pCF10 derivative could be involved in the transfer of an element encoding tigecycline resistance as only the three isolates carrying this plasmid were able to transfer the resistance. Further studies are ongoing to elucidate the gene(s) conferring non-susceptibility to tigecycline in different enterococci species from different origins.

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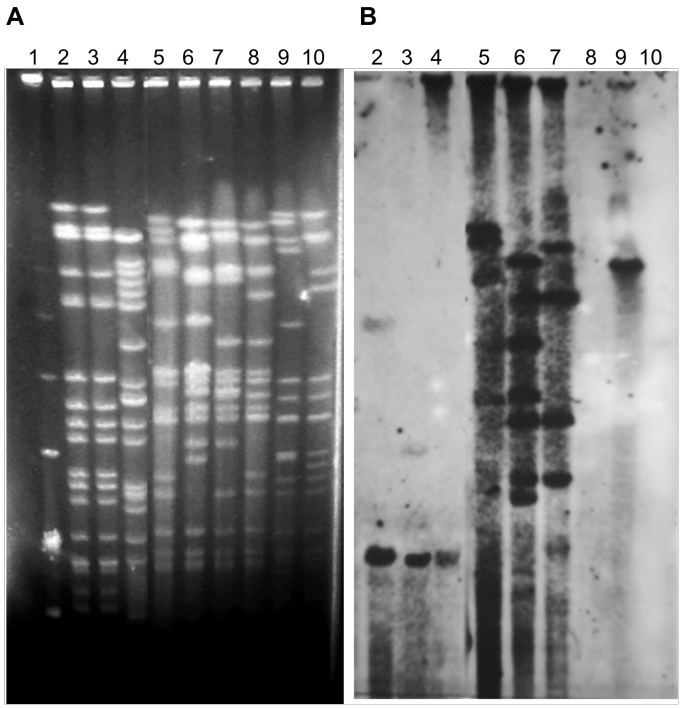


Figure 1. *Sma*I digested genomic DNA of tetracycline resistant *Enterococcus faecalis* (wild-type and transconjugants) from different sources (A) and hybridization with a *tetM* probe (B).
Lane 1, Low Range PFGE marker (New England Biolabs, La Jolla CA); Lane 2, C7 (tigecycline resistant strain from a healthy human, ST21); Lane 3, C11 (tigecycline resistant strain from a healthy human, ST21); Lane 4, 31F6 (tigecycline resistant strain from retail chicken, ST260); Lane 5, TC C7 (JH2-2 transconjugant of C7); Lane 6, TC C11 (JH2-2 transconjugant of C11); Lane 7, TC 31F6 (JH2-2 transconjugant of 31F6); Lane 8, JH2-2 (recipient strain); Lane 9, HVR10 (tigecycline susceptible clinical strain; positive control for *tetM*); Lane 10, HVR14 (tigecycline susceptible clinical strain; negative control for *tetM*).

Chapter 4

General Conclusions

The first rule of intelligent tinkering is to save all the parts.

Paul R. Ehrlich

The overall results of this thesis indicates that LGT contributes to the adaptation of *E. faecalis* and *E. faecium* to different hosts and it influences the selection of particular clonal lineages in which the acquisition and persistence of adaptive traits as antibiotic resistance are favoured. The diversity of plasmids (*plasmidome*) encompassing different replication, transfer and maintenance systems highlights the wide possibilities of adaptation of these microorganisms.

This general statement is based on the conclusions established after the accomplishment of the specific aims. They can be summarizes as follows:

1. The population structure of vancomycin-resistant *Enterococcus faecium* inferred by the analysis of isolates from different origins and locations confirms the ***selection and expansion of specific E. faecium clonal lineages*** in particular hosts as ***CC17*** in hospitalized humans; ***CC5*** in swine and ***CC9*** in poultry. The selection and further diversification of particular clones belonging to these CCs (e.g. ST5/CC5, specific strains of different STs in CC17) reflects the role of LGT in the combinatorial evolution of these microorganisms.

2. The population structure of nosocomial vancomycin-resistant *Enterococcus faecalis* inferred by the analysis of isolates causing hospital outbreaks in different countries revealed the ***worldwide dissemination of clonal complexes CC2, CC9 and CC87*** which, in contrast with that observed for *E. faecium*, lack host homogeneous distribution. However, globally spread *E. faecalis* strains were detected (e.g. ST6/CC2), similarly to that observed for *E. faecium*. Geographical uneven distribution of main CCs might indicate the influence of different selection pressures or local hosts factors in the expansion of these lineages.

3. The interhost (Human-swine) transmission of enterococcal clones belonging to host-adapted clonal complexes of *E. faecium* (CC5, CC17) and *E. faecalis* (CC2), confirms the relevance of reverse and alternative routes for dissemination of opportunistic bacteria. Multilayered ecological studies will be necessary to understand the factors enabling these clones and MGE to overcome the species barriers between humans and animals.

4. The analysis and characterization of the ***plasmidome*** of *E. faecium* and *E. faecalis* isolates from different origins and geographical areas reflect a ***species specific plasmid pool***. Within the same species, differences in the plasmid content among strains from different hosts (humans and animals), or different habitats (different body

sites, ecological conditions) are mostly due to the presence of broad host plasmids (e.g. Inc18) or narrow host plasmids (megaplasmids and small plasmids) encoding traits associated with specialized environments (e.g. megaplasmids in host specific CC17 and CC5 differ in genes coding for copper resistance; isolates of CC17 show a wide diversity in traits coding for resistance to antibiotics, bacteriocins traits enhancing colonization or enabling hydrolysis of complex carbohydrates) .

5. The ***global spread of vancomycin resistance*** among enterococci is greatly caused by the successful dissemination of ***vanA*-Tn*1546*** *on* ***transferable plasmids*** carrying replication, conjugation and/or maintenance systems related to narrow host plasmids belonging to the family RepA_N (pRUM in *E. faecium* and pheromone-responsive in *E. faecalis*) and the broad host Inc18. The diversification of narrow host plasmids, inferred by the mosaicism and the rearrangements observed, reflects the impact of LGT in the combinatorial evolution of these elements and it would explain why the glycopeptide resistance remains confined to this genera. In addition, the cumulative acquisition of adaptive traits by these species-specific plasmids would have contributed to the successful expansion of certain lineages in the predominant enterococcal species.

6. The narrow host range of pheromone plasmids and the frequent rearrangements observed within *E. faecalis* plasmids might explain the maintenance of pAD1-Tn*1546* within this species. The ***transferability of these plasmids to Staphylococcus aureus*** seems to be associated with the presence of the broad host conjugative module of Inc18 plasmids.

7. The recovery of ***enterococcal isolates from different origins with reduced susceptibility to tigecycline***, a glycylcycline recently introduced as a therapeutical option in the treatment of Gram-positive diseases, highlights the possibility of selection of resistance to this new antimicrobial agent by different antibiotics in non-clinical settings.

Chapter 5

References

Science is organized knowledge.

Herbert Spencer

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Chapter 6

Appendix

"Repetitio Est Mater Studiorum"

Repetition is the mother of learning

6.1. Material and Methods

6.1.1. Bacterial isolates

The isolates analysed in this thesis included diverse *Enterococcus* isolates (n=1306) obtained during 1986 and 2009 from humans (431 hospitalized patients and 205 healthy human volunteers), swine and piggeries (n=320), poultry (n=234), and the environment (n=116).

6.1.1.1. *Enterococci isolates from humans*

a) Isolates from Northern and Central hospitals of Portugal

This group includes 325 clinical isolates from different clinical specimens obtained since 1996 to 2008 from hospitalized patients in six different cities covering the North and Centre of Portugal: Hospital de S. Teotónio (HST; n=140) in Viseu; Hospital de Santo António (HSA; n=76) in Porto; Hospital Universitário de Coimbra (HUC; n=75) in Coimbra; Hospital de S. Pedro (HSP; n=22) in Vila Real; Hospital Pedro Hispano (HPH; n=7) in Matosinhos; and Centro Hospital Cova da Beira (CHCB; n=5) in Covilhã. The isolates were identified as *E. faecalis* (n=191), *E. faecium* (n=130) and *Enterococcus* spp. (n=4). All were included in an *in vitro* tigecycline susceptibility surveillance study. A subgroup of VRE (61 *E. faecium*, 27 *E. faecalis*), representative of disseminated and persistent strains, was selected for a detailed analysis of the population structure (PFGE and MLST) and mobile genetic elements (transposons and plasmids).

b) Vancomycin-resistant isolates representative of clinical outbreaks in different countries

This group includes 106 vancomycin-resistant enterococci (70 *E. faecium*, 36 *E. faecalis*), collected from 27 countries in Europe (Cyprus, Denmark, Finland, France, Germany, Great Britain, Greece, Hungary, Italy, Poland, Serbia, Spain, The Netherlands), North America (Canada, USA) and South America (Argentina, Brazil, Chile, Colombia, Ecuador, Paraguay, Peru, Venezuela), Africa (Tunisia), Australia and Asia (Singapore, South Arabia) during a 24-year period (1986-2009). These isolates are representative of nosocomial VRE strains in these areas, which have been associated with hospital outbreaks in most of the cases.

c) Isolates from healthy human volunteers

This group includes 202 enterococcal isolates from feaces of healthy individuals living in northern and central Portugal (5 cities; 2001-2004). They were included in an *in vitro* tigecycline susceptibility surveillance study and a subgroup of 7 vancomycin-resistant *E. faecium* was also included in studies to assess the population structure (MLST) and the characterization of mobile genetic elements (transposons and plasmids) carrying resistance genes to glycopeptides. Three vancomycin-resistant *E. faecium* recovered from 525 of community-dwelling human samples (2002-2006) in Denmark were included in a joint international study for comparison with enterococci isolates from different origins and countries.

6.1.1.2. *Enterococci isolates from swine and piggeries*

This group includes 288 enterococci isolates obtained from diverse samples (pig faeces, food/medicines, residues, piggeries facilities and a river receiving piggeries effluent) of five intensive and one extensive pig production farms in the North, Centre and South of Portugal between April 2006 and December 2007, and one slaughterhouse in the Centre (1997). They were identified as *E. faecium* (n=104), *E. faecalis* (n=53), *E. hirae* (n=41) and *Enterococcus* spp. (n=90) and were included in an *in vitro* tigecycline susceptibility surveillance study and a subgroup of 23 *E. faecium* and 12 *E. faecalis* was selected for MLST typing.

An additional group of 32 VREfm recovered from swine in different national surveillance studies conducted in Spain, Denmark, Switzerland and the USA was included in a joint international study for evaluating their clonal diversity and relationship with human clinical isolates. These isolates included: i) 15 VREfm from healthy pigs among 1,594 Efm fecal isolates in Denmark (DANMAP, 1995-2006); ii) 2 VREfm recovered from 900 pig's fecal samples at slaughterhouses (9.7% of all pig slaughtered in 1998) in Valencia and Murcia, Spain; iii) 1 VREfm recovered from samples of pig's feces amongst 155 *Enterococcus* isolates obtained in 16 Swiss farms during 1999-2000; and iv) 6 VREfm recently isolated from 55 swine (10.9%) in three Michigan counties (2008, USA).

6.1.1.3. *Enterococci isolates from retail poulry*

This group includes enterococci isolates (n=234) obtained during September 1999 and March 2001 from raw meat samples or swabs from the inner carcass of retail raw poultry (chicken and turkey lots) reared in intensive production farms and of 10 different commercial brands sold throughout Portugal, which were collected from two different butcher shops and one market in the Porto area. They were identified as *E. faecium*

(n=88), *E. faecalis* (n=64) and *Enterococcus* spp. (n=82), and included in an *in vitro* tigecycline susceptibility surveillance study. A subgroup of 9 enterococci (6 *E. faecium* and 3 *E. faecalis*) was selected for MLST typing, and a second subgroup of 43 VRE (26 *E. faecium*, 7 *Enterococcus* spp., 5 *E. hirae*, 4 *E. durans* and 1 *E. faecalis*) was selected for characterization of mobile genetic elements (transposons and plasmids) carrying resistance genes to glycopeptides.

6.1.1.4. *Enterococci isolates from the environment*

This group includes 116 enterococci isolates recovered during 2001-2004 from: i) samples of sewage waters from four Porto hospitals obtained upstream, flowing from residential neighbourhoods, and downstream the hospital setting (n=103); ii) the shore of the river Douro estuary and the Coura and Sousa rivers, tributaries of the Douro before the estuary (n=11); iii) and from urban sewage waters draining directly to the sea in a recreational bathing area of Porto area (n=2). They were identified as *E. faecium* (n=76), *E. faecalis* (n=28) and *Enterococcus* spp. (n=12), and were included in an *in vitro* tigecycline susceptibility surveillance study. A subgroup of 17 VRE representing disseminated or persistent environmental strains (14 *E. faecium*, 3 *E. faecalis*) was selected for analysis of the population structure (MLST) and/or characterization of mobile genetic elements (transposons and plasmids).

6.1.2. Media, sample processing and growth conditions

Different selective and non-selective media were used aerobically at 37ºC (8-24h) for the isolation and growth of enterococci isolates.

Brain-Heart Infusion (BHI) Agar or Broth medium (Oxoid Ltd, Basingstoke, Hants, UK) is a non-selective and highly nutritious medium suitable for the cultivation of several fastidious microorganisms and was routinely used for bacterial growth. *Tryptone Soya Broth* (Oxoid Ldt.) with 15% glycerol was used to stock enterococci cultures at -80ºC. The *Mueller Hinton II* (MH) Agar (BioMérieux, Marcy l'Étoile, France), a non-selective and non-differential medium, was used for antimicrobial susceptibility testing (16-24h). Mueller-Hinton II enables the growth of non-fastidious bacteria as enterococci contains low levels of thymine and thymidine and controlled levels of Ca^{2+} and Mg^{2+} divalent ions, guaranteeing minimum interference from the constituents of the formula with the result of the antimicrobial susceptibility test and ensuring accurate susceptibility determinations. *Buffered Peptone Water* (Oxoid Ldt.) was used as a non-selective pre-enrichment medium for enterococci sample processing during 16h.

Slanetz & Bartley Agar (Oxoid, Ldt.) was used as a selective medium for detection and quantification of enterococci. The sodium azide present in this medium inhibits growth of aerobic bacteria, particularly Gram-negatives. Enterococci produce reddish-pink to reddish-brown colonies because they reduce tetrazolium chloride to formazan, but this reaction is not exclusive to enterococci and the count at this stage should be considered presumptive. Furthermore, the selection of antibiotic-resistant enterococci was performed by aliquoting plates of Slanetz & Bartley Agar with 6 μg/ml vancomycin, 16 μg/ml ampicillin, 8 μg/ml erythromycin, 16 μg/ml tetracycline, 1000 μg/ml streptomycin or 125 μg/ml gentamicin and without antibiotics during 48h.

6.1.3. Identification of *Enterococcus*

Genus identification was performed by Gram stain, catalase test, bilis-esculin hydrolysis and growth in NaCl 6,5% (117, 118). The identification of the genus was confirmed by a PCR targeting the elongation factor EF-Tu when necessary (204).

Species identification was performed by amplification of species-specific genes. Two multiplex PCR schemes able to amplify intragenic fragments of $ddl_{E.\ faecium}$, $ddl_{E.\ faecalis}$, *vanC-1*, *vanC-2, murG* and $mur\text{-}2_{ed}$ genes were specific of *E. faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*, *E. hirae* and *E. durans*, respectively (9, 61, 110, 362).

6.1.4. Antimicrobial susceptibility tests

Susceptibility tests define the *in vitro* activity of an antibiotic against a microorganism and are used to predict its *in vivo* efficacy. They are used to determine the minimal inhibitory concentration (MIC) of antimicrobial agents (192). Susceptibility of enterococci against different antibiotics was determined by three methods: the disk diffusion test, the agar dilution test and/or the Épsilon-test (E-test). The inoculum consisted on a direct colony bacterial suspension in 0.9% sterile saline equivalent to 0.5 McFarland turbidity (the same as 1.5 x 10^8 colony-forming units (CFU)/ml) and Mueller Hinton 2 agar plates (90 mm diameter, 4 mm agar depth) were incubated at 37ºC (ambient air) for 16-24h. The standard control strains *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were always included.

Different committees and regulatory authorities define bacterial MIC breakpoints for guidance of therapy dividing bacteria into three categories of susceptibility: *susceptible* (S - where the antimicrobial activity is associated with a likelihood of therapeutic success), *intermediate* (I - where the antimicrobial activity is associated with an indeterminate or uncertain therapeutic effect), or *resistant* (R - where the antimicrobial activity is associated with a higher than expected likelihood of therapeutic failure). The epidemiological cut-off

value (ECOFF) separates microorganisms without (wild type) and with acquired resistance mechanisms (non-wild type) to a specific antibiotic (199).

The activity of different antibiotic families against enterococci was determined for penicillins (ampicillin), glycopeptides (vancomycin, teicoplanin), macrolides (erythromycin), tetracyclines (tetracycline, minocycline), fluoroquinolones (ciprofloxacin), nitrofurantoins (nitrofurantoin), ansamycins (rifampin), phenicols (chloramphenicol), streptogramins (quinupristin-dalfopristin), lipopeptides (daptomycin) and oxazolidinones (linezolid) accordingly to standard reference methods described in the CLSI guidelines (66), and for glycylcyclines (tigecycline) following EUCAST criteria (htpp://www.eucast.org/).

6.1.4.1. *The disk diffusion test*

This test is a semi-quantitative method based on Kirby-Bauer studies (17). It consists on the use of a dried filter paper disc impregnated with a defined concentration of antibiotic in the agar used to culture the microorganisms. The susceptibility to the antibiotic is indicated by a clear zone of inhibition around the disc and the diameter of the zone of inhibition is proportional to the susceptibility of the organisms tested. The zone diameters for individual antibiotics are translated in terms of susceptible (S), intermediate (I) or resistance (R) categories by referring to an interpretative chart from CLSI standard guidelines. The rationale of these interpretative charts assumes that the relationship between the diameter of the zone and the MICs is known and that the outer limit of the zone of inhibition contains an antibiotic concentration which is similar to the MIC of that antibiotic to a specific organism.

Antibiotic susceptibility to different antimicrobial families was performed by the Kirby-Bauer method with test disk concentrations as follows: vancomycin (30μg), teicoplanin (30μg), amoxycillin (10μg), erythromycin (15μg), ciprofloxacin (5μg), tetracycline (30μg), minocycline (30μg), nitrofurantoin (300μg), rifampin (5μg), chloramphenicol (30μg), quinupristin-dalfopristin (15μg), linezolid (30μg), gentamicin (120μg) (Oxoid Ltd, Basingstoke, Hants, UK), and streptomycin (300μg) (Becton Dickinson, France).

6.1.4.2. *The agar dilution method*

The agar dilution method is one of the reference methods for the quantitative determination of MIC against a specific antimicrobial agent (435). Microorganisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution) containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent that completely inhibits the visible growth of a microorganism is known

as the MIC. Agar dilutions were prepared in 90 mm diameter Petri dishes which allowed testing several organisms on each plate. Antibiotic susceptibility to vancomycin (4mg/L≤MIC≤256mg/L), kanamycin (500mg/L≤MIC≤2000mg/L) and tigecycline (0.03mg/L≤MIC≤4mg/L) was performed by the agar dilution method.

6.1.4.3. *The Epsilon test (E-test)*

The Epsilon Test is an "exponential gradient" quantitative method combining the principle of the agar diffusion test with the determination of the MIC value. The E-test utilises a thin inert strip impregnated with a predefined stable antimicrobial agent present on a variable range of concentrations. A lawn of bacteria is inoculated onto the surface of a Mueller Hinton agar plate and the E-test strip is laid on top; following incubation the antibiotic diffuses out into the agar producing a symmetrical clear inhibition zone (in the form of ellipse) of the antibiotic to be tested. The intersection of the inhibitory zone edge and the calibrated carrier strip indicates the MIC value over a wide concentration range (>10 dilutions) with inherent precision and accuracy. This technique was performed to determine the minimum inhibitory concentration of tetracycline and tigecycline accordingly to the manufacturer's recommendations (AB BIODISK, Solna, Suece).

6.1.5. Molecular techniques / DNA methodology

6.1.5.1. *DNA extraction*

Different methods have been described for the isolation of chromosomal or plasmid DNA but all of them include the disruption of the cell, the removal of cell debris and proteins, and the selective precipitation of double-stranded DNA of high molecular weight and purity suitable for several applications on molecular biology. Total DNA extraction was achieved by conventional techniques (heat, alkaline solutions and/or detergents) or by commercial kits, which are based on the conventional methods combined with the use of specific columns to purify the DNA.

a) Genomic DNA

In most cases, genomic DNA extraction was simply achieved by heat or the *boiling* method in which the double-stranded DNA is melted to generate two single strands resulting in DNA denaturation. The bacterial cells grown overnight on BHI plates were suspended in a lysis buffer (0.25% SDS, 0.05 N NaOH) and incubated at 100ºC for 15

minutes. The cell lysate was diluted (10 mM Tris-HCl, pH=8.5), spinned and stored at -20ºC.

In particular cases (long PCR products), DNA was extracted by commercial columns of the *commercial kit* QIAamp® DNA Mini Kit (DNA Purification from Tissues - Qiagen GmbH, Hilden, Germany). Briefly, from an overnight culture (in BHI broth) 1.5 ml was spin, suspended in 10 mM Tris, 1 mM EDTA pH=8.0 and 50 mg/ml solution of lysozyme and incubated at 37ºC for 30 min. The bacteria was then lysed by the addition of 10% SDS and a 20 mg/ml proteinase K solution (supplied with the kit) and incubated at 65ºC for 2 hours. After centrifugation, DNA binding onto a silica membrane was successively spinned and washed to eliminate contaminants (proteins or enzymatic inhibitors), and finally eluted and stored in a low salt buffer (10 mM Tris-Cl, 0.5 mM EDTA pH=9.0) or water at -20ºC.

Extraction of genomic DNA by the *in situ* lysis of bacteria cells embedded in agarose was achieved according to reference methods (201, 296). It requires the preparation of unsheared DNA by the incorporation of organisms in agarose, the lysis and disruption of the cells and the washing of cellular debris. Briefly, a single colony enterococci was grown overnight in 5 ml of brain heart infusion broth at 37ºC and the cells are harvested and suspended in 1.25 ml of PIV buffer. A portion (0.4 ml) of this suspension was mixed with 0.4 ml of 2% agarose (*SeaKem Gold Agarose*) in water at 50 to 60°C and then pipetted into a plug mold allowed to solidify at 4ºC. For lysis, one to five plugs were placed in 4 ml of fresh lysis solution (6 mM Tris-HCl pH=7.6; 1 M NaCl; 100 mM EDTA pH=7.5; 0.5% Brij 58; 0.2% deoxycholate; 0.5% sodium lauroyl sarcosine; 20 ug/ml of RNase; 1 mg/ml of lysozyme). Following incubation overnight at 37°C with gentle shaking, this solution was replaced with 5 ml of ESP solution and then incubated overnight at 50°C with shaking. The plugs were finally washed three times for 30 min each with 5 ml of TE 1X and then stored in this buffer at 4°C (see preparation of solutions in 6.2.2.a).

b) Plasmid DNA

Plasmids are extrachromosomal genetic elements which molecular typing is relevant for epidemiological purposes due to the variability of genes they may carry (variable region with e.g. antibiotic resistant genes). Most plasmids exist as supercoiled double-stranded DNA molecules with a covalently closed circular (**CCC**) topology. Plasmid DNA can also appear as damage structures generated after preparation by conventional methods during bacterial lysis or the removal of chromosomal DNA, as open circular (**OC**) or linear forms (**L**) depending if one or both strands have been broken, or even in larger multimeric forms of CCC or OC forms. The relative positions of these three configurations can change dramatically on a conventional agarose gel depending on the gel and the

conditions of electrophoresis. This clearly complicates the interpretation of plasmid banding patterns in agarose gels, but the mobilities are usually in the order OC DNA (slowest), linear and CCC DNA (fastest) (Figure 1).

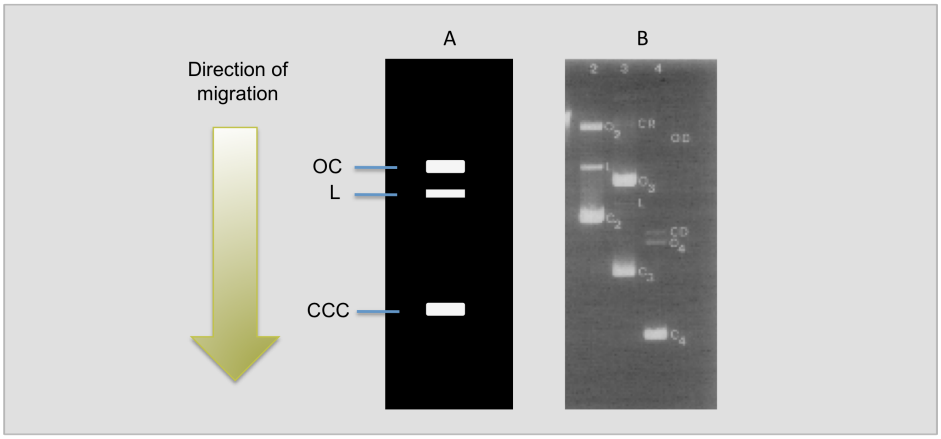


Figure 1 – **A.** Schematic representation of plasmid DNA electrophoresis in an agarose gel. CCC, covalently closed circular; L, linear; OC, open circular. **B.** Agarose gel electrophoresis of different plasmid DNA preparations (adapted from 175). C, CCC DNA; O, OC DNA; L, linear DNA; OD, OC dimers; CD, CCC dimmers; CR, chromosomal DNA. Numbers assigned to O and C only refer to the corresponding gel slots.

Plasmid DNA extraction was achieved by different alkaline lysis based methods of Birnboim and Doly (plasmids <150 kb) and by the Barton's method (plasmids 10->300 kb).

The **alkaline lysis method of Birnboim and Doly** (24) is based on the differences of resistance to strongly anionic detergent at high pH between plasmid and chromosomal DNA. The use of a SDS-NaOH solution induces the cellular lysis and the denaturation of plasmid and chromosomal DNA. Further neutralization with acetate potassium allows the reconfiguration of plasmid DNA to the soluble CCC form while chromosomal DNA and proteins precipitate in a complex formed by potassium and SDS which is eliminated by centrifugation. The closed circular plasmid DNA is released into the supernatant, is concentrated by precipitation with ethanol and can be then purified in many different extents according to the need of the experiment.

A number of modifications of Birnboim and Doly's method have been optimized during the last years accordingly to the final purpose. Small plasmids (2-150 kb) were extracted by the fast *Kado & Liu* method (198) which combines denaturation of chromosomal DNA with high pH and temperature values (see protocol 1 in section 6.3.). This method was only used to visualize supercoiled (CCC) plasmids as phenol-chloroform used to remove proteins and cell debris can inhibit further digestion with restriction enzymes. A modification of the method described by *Handwerger et al.* (164), which is generally similar to the Kado & Liu method although extended in order to achieve a higher

purity in the final extracted plasmid DNA (see protocol 2 in section 6.3.), was used when extracting DNA for RFLP profiling purposes. Finally, a commercial version of the alkaline lysis method allows the achievement of greater DNA volumes with higher purity. We adapted a published protocol (347) using the *QIAGEN*® Plasmid Midi kit (see protocol 3 in section 6.3.). However, the chromosomal contamination was significantly higher with this method in comparison to that adapted from Handwerger *et al.* which worked perfectly for plasmid typing in both *E. faecium* and *E. faecalis* isolates and was largely used in this thesis.

The **Barton's method** (16) consists on the analysis of genomic DNA by Pulsed-Field Gel Electrophoresis (PFGE) after S1 nuclease selective treatment that completely digests chromosomal DNA and converts the supercoiled plasmids into full-length linear molecules. The migration of the linear DNA is directly proportional to their size which can be estimated by comparison with linear DNA markers. This method is extremely useful to visualize multiple large plasmids (30->300 kb) as bands in a faint genomic background (see protocol 4 in section 6.3.).

6.1.5.2. *DNA digestion*

Comparison of RFLP patterns was used as a tool to further characterize genetic islands obtained by long PCR products and to analyse similarities among genomes or plasmids. The restriction enzymes and reaction conditions were selected depending on each experiment and were established according the manufacturer instructions. The incubation time was overnight (16-24h) at the optimal temperature for each enzyme (generally 37ºC) with the exception of the advanced line of *FastDigest* enzymes for which incubation periods of 5-15 minutes were sufficient.

Genomic DNA embedded in agarose molds was digested similarly to liquid-phase reactions but using higher enzyme concentrations due to limited diffusion of enzyme. A thin slice of agarose-embedded DNA (3mm wide) is usually preequilibrated in water or in the appropriate restriction enzyme buffer for 10-30 minutes and then digested (1X restriction enzyme buffer, 10-30U of enzyme and/or BSA) at the appropriate temperature (25ºC or 37ºC) and time period (15 min-24 hours).

The characteristics of the restriction enzymes used are described in section 6.2.1.b.

6.1.5.3. *DNA amplification*

The PCR (Polymerase Chain Reaction) is a standard laboratory technique with numerous applications in molecular biology (214, 290, 359). In this thesis, PCR was

mainly used for the detection and identification of numerous sequences both by simplex and multiplex schemes. Also the backbone structure of *vanA* and *vanB* transposons was determined by PCR mapping and long PCR, respectively (see section 6.1.7.2.).

All PCR reactions were performed in termocyclers Perkin Elmer Gene Amp 2400 or BioRad iCycler with appropriate controls (positive, negative and blank) and reagents [DNA polymerase, oligonucleotide primers, deoxynucleoside triphosphates (dNTPs), divalent cations ($MgCl_2$), reaction buffer (Tris-Cl pH=8-9), and/or bovine serum albumin solution (BSA)]. BSA was added in specific cases as an adjuvant of PCR keeping DNA intact, unsheared, and improving specificity in amplification of DNA/primers secondary structures (see reagents in section 6.2.1.a).

Amplification conditions were adjusted to each experiment depending on the size of the expected amplicon:

a) PCR products < 3 kb. PCR assays were performed in volumes of 25µl or 50µl under the following conditions: 1.5 mM $MgCl_2$, 1x reaction buffer, 0.2 mM of each deoxynucleoside triphosphate, 1 µM of each primer, 10µg/µl of BSA when necessary, and 1.5 units of *Taq* DNA polymerase with amplification programs of 15 min at 94°C; 30-35 cycles of 30 sec. to 1 min at 94°C, 30 sec. to 1 min at 45 to 70°C, and 30 sec. to 3 min at 72°C, and a final extension step of 10 min at 72°C.

b) PCR products > 3 kb (long PCR). PCR assays were performed in volumes of 50µl or 100µl under the following conditions: 2.5 mM $MgCl_2$, 1x reaction buffer, 0.4 mM of each deoxynucleoside triphosphate, 1 µM of each primer, and 2.5 units of LA *Taq* polymerase with amplification conditions of 1 min at 94°C and 35 cycles of a denaturation step of 30 s at 96°C, annealing at 52°C to 59°C for 1 min, and an extension step at 72°C for 3 to 7 min, followed by a final step of 10 min at 72°C.

The primers and conditions of all PCR reactions performed during this thesis are described in Table 3 at the final of material and methods.

PCR products were purified directly from the PCR reactions with the Wizard® SV Gel and PCR Clean-Up System commercial kit. After binding to a silica membrane in the presence of high salt concentration, the DNA is washed with an etanol-based solution and eluted in nuclease-free water. This is required to remove excess nucleotides and primers before DNA sequencing, labeling or restriction enzyme digestion.

6.1.5.4. *DNA separation and detection*

The separation of DNA fragments was achieved by electrophoresis using continuous-our pulsed-field current systems.

a) Electric continuous-field agarose gel electrophoresis

The separation of DNA was achieved by conventional horizontal agarose gel electrophoresis in an electric continuous-field. Digested or non-digested DNA (0.1-10kb) obtained by PCR was separated in mini-medium agarose gels in different concentrations (0.8-2%) depending of the amplicon size, and in the presence of the running TAE 1X buffer (see description of buffers in 6.3.2.b). DNA fragments were separated by applying 80-100V during 30-120 minutes. Plasmid DNA (0.1-150kb) was separated in large agarose gels (0.8-1.5%) in the presence of the TBE 0.5X buffer with different running conditions depending if the plasmid was digested (90V, 5-6h) or non-digested (120V, 4h).

The loading buffer (tracking dye) is mixed with the samples before loading onto the gel to increase the density of the sample, to simplify the loading process by adding colour to the sample and to follow the progress of the electrophoresis based on the movement of the dye. The molecular markers used to determine the molecular weight of the bands are described in section 6.2.1.c.

b) Pulsed-field gel electrophoresis

The resolution of large DNA molecules was achieved by an alternative method based on the principle of an electrophoretic current “pulsed” in different directions over a gradient of time intervals (201, 368). The method includes the genomic DNA extraction in agarose molds (6.1.5.1), the DNA digestion with appropriate restriction enzymes (6.1.5.2) and the separation of a range of DNA molecules, including those of megabase size. The pulse-switching increments and the total electrophoresis run time are influenced by the range of DNA fragment sizes that must be separated as a result of specific chromosome-restriction enzyme interaction (146, 147, 359).

The gel was prepared with the same agarose used for preparing the DNA molds (SeaKem Gold agarose). Its high quality, purity and characteristics (high gel strength with standard gelling temperature and low electroendosmosis coefficient) allow an optimal separation of DNA fragments of high molecular weight (50 kb - 10 Mb) as well as a great electrophoretic mobility adequate to further DNA transfer into membranes. Electrophoresis of digested genomic DNA was carried out on a CHEF-DRIII (Countour-clamped Homogeneous Electric Field) device using the running TBE 0.5X buffer maintained at 14ºC with standardized conditions (6V/cm, 120º). In CHEF gels, the electric field is

generated from multiple electrodes that are arranged in a square or hexagonal contour around the gel and are clamped to predetermined potentials (60). We used different running settings depending of the size of the fragments to be separated or expected by using different restriction enzymes: *Sma*I [1-20s, 38h (*E. faecalis*) or 1-20s, 26h (*E. faecium*)], I-*Ceu*I (5-30s, 22h) and S1 nuclease (5-25s for 6 h followed by 30-45s for 18 h). The molecular weight markers used were the *λ-ladder PFG marker* and the *Low Range PFG marker* (see box 6.2.1.c.).

c) DNA visualization

DNA was detected by staining with the fluorescent dye ethidium bromide and visualized by illumination with 300-nm UV light. Ethidium bromide contains a tricyclic group that intercalates into double-stranded DNA forming a red fluorescent complex which is visualized with UV transillumination (235). After electrophoresis, the agarose gels were stained by immersing the gel in electrophoresis buffer or water containing ethidium bromide for 10-30 minutes at room temperature. Destaining in water was performed when the background of fluorescence was high. During UV illumination, images of stained gels were captured by an appropriate camera integrated system (see box 6.2.3.).

6.1.5.5. *Identification and typing*

Advantages, drawbacks and features of different molecular typing methods are included in Table 1. Clonal relatedness among enterococcal isolates was established by Pulsed-Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST).

a) *Pulsed-Field Gel Electrophoresis (PFGE)*

PFGE has been considered the “gold standard” of the molecular typing methods since its first description in 1983 (368). However, it is restricted to analyze small sets of isolates recovered during a limited time frame (271, 282, 296).

Restriction of DNA is performed with enzymes chosen to yield a relatively small number of large restriction fragments. These enzymes recognize specific 8-base sequences (*Not*I, *Sfi*I) or selected 6-base sequences (*Sma*I, *Apa*I). *Sma*I is the most commonly used enzyme for digestion because it has a G+C rich recognition site, as is the G+C content of enterococcal species. Clonal relationships among enterococcal isolates were established by comparison of *Sma*I-digested genomic DNA which typically resolves 15-20 restriction fragments ranging from 5 to 400 kb (73).

The criteria proposed by Tenover *et al.* (410) were applied to distinguish among isolates. Accordingly to these guidelines, isolates were considered indistinguishable (if the restriction patterns have the same number of bands – isolates are considered to be the same strain), closely related (restriction patterns differ in 2 or 3 bands), possibly related (restriction paterns differ in 4-6 bands) and unrelated (restriction patterns differ in ≥ 7 bands).

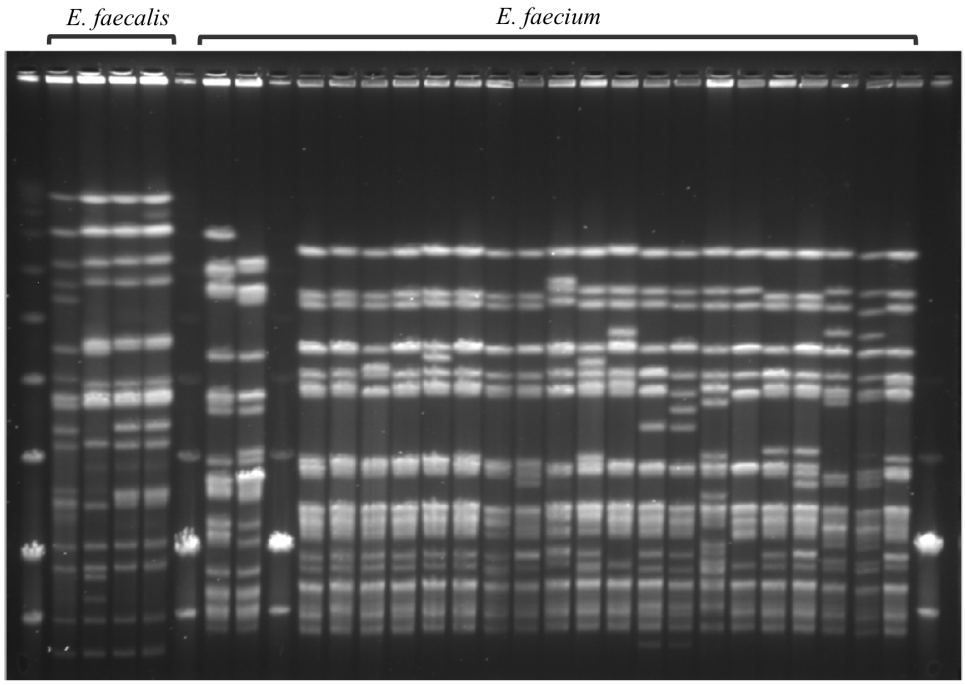


Figure 2. PFGE of *Sma*I digested genomic DNA of enterococcal isolates recovered from humans and swine of different countries (*Freitas AR, Doctoral thesis*).

b) *Multilocus Sequence Typing (MLST)*

MLST is a nucleotide sequence-based typing scheme where multiple genes (loci) are sequenced to measure genetic relatedness and analyze sequence variation between alleles from many strains (259, 436).

Both *E. faecium* and *E. faecalis* MLST schemes include the identification of alleles from DNA sequences of seven housekeeping genes internal fragments. The choice of these housekeeping genes is based on their putative function, on their location within the chromosome and on the availability of sequence data from each enterococci species.

Table 1. Features, advantages and drawbacks of molecular methods used in the typing of *Enterococcus* spp.

METHOD	DESCRIPTION	ADVANTAGES	DISADVANTAGES	REFS
PFGE (Pulsed Field Gel Electrophoresis)	DNA macrorestriction analysis of bacterial genomic DNA by restriction endonucleases with few recognition sites. The digested fragments are resolved by constantly changing the direction of the electrical field during electrophoresis. Is considered the gold standard for tracing the transmission of strains in hospital outbreaks.	* High degree of strain differentiation * Reproducible * Standard criteria for interpretation are available	* Expensive * Time consuming * Requires high level of skill * Difficult interlaboratory comparison of data * Difficult to establish relationships between epidemiological non related strains * Limited to strains recovered during short periods of time	(45, 72, 146, 148, 288, 296, 410)
AFLP (Amplified Fragment Length Polymorphism)	DNA fingerprinting technique that detects digested genomic DNA by selective PCR amplification of conserved and variable DNA sequences using adapter-specific primers.	* Easy performing; faster than PFGE * More reproducible and robust than RAPD analysis * High power of discrimination * Overcomes PFGE by analyzing both conserved and variable DNA sequences	* Non-reproducible * Difficult interlaboratory comparison of data * No information on evolutionary descendents	(25, 83, 189, 364, 446)
MLST (Multi Locus Sequence Typing) (www.mlst.net)	Nucleotide sequence-based typing method based on the identification of alleles from DNA sequences of internal fragments of seven housekeeping genes.	* Highly discriminative and unambiguous * Highly reproducible * Suitable for data exchange	* Expensive * Time consuming * Less sensitive than PFGE * Requires technical skill and knowledge in sequence analysis	(1, 103, 113, 120, 258, 399, 433)
MLVA (Multiple Locus Variable-number of tandem repeat Analysis) (www.mlva.umcutrecht.nl)	Based on differences in the variable number of tandem repeats (VNTR) in multiple loci on the chromosome, which can rapidly be detected by PCRs with specific primers based on the flanking regions of the tandem repeats.	* Easy performing; Fast * Not expensive * Reproducible * Unambiguous assignment of genotypes * Suitable for data exchange * Same discrimination as AFLP and MLST	* Less discriminative than PFGE and MLST * Comparison of fragments sizes from different platforms requires rigorous standardization	(245, 426, 427, 439, 463)

For *E. faecium* MLST typing (177), internal fragments of the following seven genes were amplified by PCR: *adk* (adenylate kinase), *atpA* (ATP synthase, alpha subunit), *ddl* (D-alanine:D-alanine ligase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *purK* (phosphoribosylaminoimidazol carboxylase ATPase subunit), and *pstS* (phosphate ATP-binding cassette transporter). For *E. faecalis* (354) they were: *gdh* (glucose-6-phosphate dehydrogenase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *pstS* (phosphate ATP-binding cassette transporter), *gki* (putative glucokinase), *aroE* (shikimate 5-dehydrogenase), *xpt* (xanthine phosphoribosyltransferase), and *yiqL* (acetyl-coenzyme A acetyltransferase).

The primers and PCR conditions used are detailed in the MLST database (http://efaecium.mlst.net and http://efaecalis.mlst.net) hosted at Imperial College with funding from the Wellcome Trust. PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System purification kit and sequenced with PCR forward and/or reverse primers on an ABI PRISM 377 automated sequencer (PE Biosystems, Foster City, CA USA). The chromatograms obtained were analysed by using the software Chromas Version 2.32 (Technelysium Pty Lda. Tewantin, Australia) and each *loci* sequence was compared with that available in the database. Different sequences were assigned allele numbers, and different allelic profiles were assigned sequence types (STs) by interrogating the MLST database (http://www.mlst.net). Single-locus variants (SLVs) differ in only one of the seven housekeeping genes and double-locus variants (DLVs) differ in two of the seven housekeeping genes. Clusters of related STs differing in not more than in two of the seven loci that were thought to be descendants from a common ancestor were grouped into clonal complexes (CCs) by using eBURST.

c) *DNA transfer and hybridization*

Southern blotting was developed in the early 1970s and basically involves the transfer of DNA from an electrophoresis gel to a membrane and its subsequent hybridization with a DNA probe (396).

The identification of sequences or genes in bacterial genomes and/or mobile genetic elements (plasmids, transposons) was performed by hybridization of DNA with specific probes generated by PCR. DNA transfer and hybridization were performed by standard procedures (359) and optimized throughout the time frame of this work (see description of solutions in section 6.2.2.c):

I. DNA separation. Total or digested DNA (genomic or plasmids) was separated by conventional electrophoresis or by PFGE as described before (6.1.5.4.).

II. DNA transfer and fixation. After gel visualization and the image captured (6.1.5.4.c.), DNA was exposed on UV light for 2 minutes and flip upside down to submerge it in a NaOH 0.4N solution at room temperature for 1.5 hours. The gel must be totally submerged in this denaturing alkaline solution during this time, which allows excellent transfer of DNA onto a positively charged nylon membrane. Southern transfer was carried out by conventional upward capillary blotting of DNA to a positively charged nylon transfer membrane. Briefly, the following components of the southern system are added by this order: i) a large blotting paper wet with NaOH 0.4N is placed on a solid support leaving its ends submerged in the transfer buffer (NaOH 0.4N); ii) the gel is placed upside down; iii) a nylon membrane exactly of the same size of the gel is then carefully positioned eliminating all air bubbles; iv) two blotting papers of the gel size, a stack of paper towels and enough weight (glass plate with weights or books) to guarantee a good capillary flow throughout the stack. The negatively charged DNA is transferred onto the positively charged nylon membrane during 24-36h (total or digested plasmids) or 48-72h (genomic DNA fragments obtained by PFGE). After DNA transfer, the membrane is washed with a Tris 0.5M pH=7 solution during 5 minutes followed by a 2X SSC solution during 2 minutes and is allowed to dry in a filter paper at room temperature. Finally, the membrane is fixed to the nylon membrane by UV cross-linking for 3 minutes, wrapped in SaranWrap and stored at -20ºC.

III. DNA labelling and hybridization. Both hybridization and labelling experiments were performed by using the *Gene Images AlkPhos Direct* labelling and detection commercial kit which is based on a dioxetane chemiluminescence system. Labelling of DNA probes (purified PCR product at 10ng/µl) was performed with thermostable alkaline phosphatase enzyme by complete denaturation to the single-stranded form (boiling bath at 100ºC for 5 minutes) followed by the addition of the cross-linker to covalently couple the enzyme to the nucleic acid probe. This reaction is incubated at 37ºC during 30 minutes and afterwards the DNA probe can be used immediately or kept on ice for up 2 hours. The volume of *hybridization buffer* was calculated and optimized in function of the bottle and membrane sizes (0.125-0.25 ml/cm^2). The membranes are placed into the pre-warmed hybridization buffer and after a short prehybridization incubation (>15 minutes) the labelled probe is added to the buffer. Hybridizations were performed overnight (>16h) in an appropriate hybridization oven at stringent temperatures (55-70°C). The hybridization temperature was based on the DNA probe size and G+C content.

IV. Post hybridization washes. These washes are made with specific buffers in an excessive amount and at a variable temperature depending on the stringency required. The membrane is washed twice with a *primary wash buffer* prepared for the moment of use at 55-70ºC for 10 minutes. The blots are then placed in a clean container and washed

twice with the *secondary wash buffer* at room temperature for 5 minutes (see below for buffers preparation).

V. Signal generation and detection. Chemiluminescence detection was carried out using a dioxetane substrate (*CDP-Star reagent*) which is catalyzed by the probe-bound alkaline phosphatase generating a rapid light output. This reagent was added in a sufficient amount (30-40 µl/cm^2) to the membrane for 5 minutes and the wrapped membrane was exposed during 2-6h at room temperature in closed film cassettes. The signal was detected by developing the autoradiography films in automatic film processors.

VI. Stripping. The DNA immobilized on a nylon membrane can be hybridized with different DNA probes several times without significant loss of signal strength. Between each assay, the DNA probes must be eliminated by washing the nylon membrane with a denaturing and boiling solution (SDS at 1%) during 1-2h followed by a brief wash with 2X SSC solution at room temperature.

d) *Detection and location of resistance/virulence genes*

I) Detection. Genes coding for resistance to particular antibiotics were confirmed by PCR: vancomycin (*vanA, vanB, vanC1, vanC2*; 110) erythromycin [*ermA, ermB, ermC*; (242, 398)], tetracycline (*tetM, tetL, tetO, tetK, tetS*; 2). The presence of putative virulence genes previously shown to be associated with increased colonization/epidemicity in enterococci were identified by PCR and sequencing: enterococcal surface protein (*esp*), hyaluronidase (*hyl*), aggregation substance (*agg*), cytolysin/hemolysin (*cyl*), gelatinase (*gelE*), and a collagen-binding adhesin gene (*acm*) (299, 443).

II) Location. I-*Ceu*I digestion followed by pulsed-field gel electrophoresis is a powerful tool for determining genome structure as it cleaves the circular chromosomes at single sites in the rRNA operons thereby generating DNA fragments (6 in *E. faecium* and 4 in *E. faecalis*) suitable for the rapid mapping by PFGE (246). This method also easily distinguishes between plasmid and chromosomal localizations, as I-*Ceu*I only cuts chromosomal DNA. Nevertheless, S1-PFGE has been considered the gold method to identify large plasmids and we assessed the genomic location of different genes by both I-*Ceu*I- and S1-digested genomic DNA PFGE with further hybridization. Chromosome and/or plasmid location of antibiotic resistance and virulence genes was assessed under high stringency conditions with the genes to be tested and 23S rDNA intragenic probes (86). Genomic DNA from different *Enterococcus* strains was used as positive controls for hybridization (see Table 2 at the end of material and methods).

6.1.5.6 *Bioinformatic analysis*

The establishment of computerized dynamic databases is crucial to the combination, standardization and interpretation of molecular typing data obtained from different laboratory methods.

a) *Analysis of DNA sequences*: the sequences obtained from different PCR assays were analysed by using the software Chromas Version 2.32 and were further compared to those available in GenBank databases by using the BLASTN or BLASTP local alignment search tools for the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov). The programme ClustalW2 was used to produce multiple sequence alignments of DNA or protein sequences and analyse identities, similarities and differences between them (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Identification and assignation of different alleles from conserved housekeeping genes and sequence types were accomplished by interrogating the MLST database (http://www.mlst.net) and using the eBURST algorithm (http://eburst.mlst.net/).

b) *Analysis of agarose electrophoresis gels* (PCR, PFGE, RFLP): fragments sizes of DNA derived from PCR products, plasmids or RFLPs were estimated with the software Gel Doc XR camera by comparison of their mobility with standard molecular weight markers. Data of restriction digested enzyme patterns of plasmids or genomic DNA were exported into Fingerprinting II Informatix software (version 3.0; Bio-Rad Laboratories, Hercules, CA, USA) for further cluster analysis. Data was analysed by clustering Dice similarity coefficients using the unweighted-pair group method with arithmetic averages (UPGMA; tolerance and optimization values) algorithm.

Restriction maps of plasmids with published sequences were generated by using the NEBcutter programme (http://tools.neb.com/NEBcutter2).

6.1.6. Conjugation assays

The most established method for assessing DNA genetic exchange in enterococci is by conjugation (transfer of DNA between bacteria involving direct contact).

Conjugation experiments to study the *in vitro* transfer of genetic resistant elements were performed by the *filter mating* method (169) at 1:1 donor-recipient ratio using different *E. faecium* (GE-1, BM4105RF, 64/3) and *E. faecalis* (JH2-2) strains as recipients (all are fusidic acid- and rifampicin-resistant, plasmid-free). Briefly, 500 µl of exponentially grown donor and recipient cells are placed onto a nitrocellulose filter and incubated overnight at 37°C; the filter is harvested and dilutions in physiological saline are streaked onto single selective agar plates. Transconjugants were selected on Brain Heart Infusion

agar plates containing the selection antibiotic [vancomycin (6 mg/L), tetracycline (8 mg/L) or tigecycline (0.5 mg/L)], fusidic acid (20mg/L) and/or rifampicin (30mg/L), and incubated at 37ºC for 24h-48h. The transconjugants were confirmed by susceptibility tests and *Sma*I-PFGE electrophoresis analysis by comparison of recipient and donor strains.

6.1.7. Analysis of Mobile Genetic Elements

Analysis of mobile genetic elements (MGEs), mainly associated with vancomycin resistance, included the identification and detailed characterization of insertion sequences, transposons and plasmids, and corresponded to the most significant part of the present thesis.

6.1.7.1. *Insertion Sequences*

Specific insertion sequences (ISs), which have been greatly associated with vancomycin resistant genetic elements (VanA: IS*Ef1*, IS*1216*, IS*1251*; VanB: ISEnfa*200,* ISEnfa*110*), were sought by PCR and further sequencing (Table 3).

6.1.7.2. *Transposons*

The analysis of transposons included Tn3 derivatives (Tn*1546*) and composite transposons (Tn*1549*/Tn*5382*) coding for vancomycin resistance.

a) Tn*1546* (typing of *vanA* transposons). The backbone structure of Tn*1546* was determined by PCR mapping (10 amplification reactions) and further sequencing of fragments of unusual size as previously described (309, 472). Specific insertion sequences were sought as previously described.

b) Tn*1549/5382* (typing of *vanB* transposons). The *vanB* gene cluster was amplified by a long PCR that covers 5,959-bp (*van*RSYWHBX) of *vanB* transposon from reference strain V583 (GenBank accession number AE016830) and further RFLP analysis after digestion with *Bsp*HI/*Dra*I enzymes (85). Discrimination between Tn*1547* and Tn*1549*/Tn*5382* elements was established by analysing specific sequences corresponding to the right junction of Tn*5382*, the $vanS_B$-$vanY_B$ intergenic region flanking coding sequences and the relaxase of Tn*1549* (50, 85). Also, the link between *pbp5* and Tn*5382* was investigated accordingly to Carias *et al*. (50).

6.1.7.3. *Plasmids*

Plasmids are extrachromosomal molecules of DNA ranging in size from 1 kb to more than 400 kb. The knowledge on their presence, number and size is of great importance due to their capacity of dissemination among different cells and because they frequently contain genes coding for traits advantageous to the bacterial host. Plasmid typing included determination of number, size, content and comparison of enzyme restriction patterns.

a) Plasmid extraction. Plasmids were extracted by different modifications of the **Birnboim and Doly** method or by **S1-PFGE** depending on the objective of the experiment and the purity of DNA required, if only for visualization and estimation of sizes or for downstream applications as RFLP or hybridization (see 6.1.5.1.b.).

b) Plasmid number and size. The number and size of plasmids were determined using the method of **Barton** *et al.* (for plasmids from 10 kb to >300 kb) and Kado & Liu (for plasmids from 2 kb to 20 kb) (see also 6.1.5.1.b.) by comparison with molecular weight markers or reference strains, respectively.

c) Plasmid content. The classification of *E. faecium* plasmids was based on the presence of specific modules for the basic plasmid functions as replication (Rep initiator proteins), mobilization (relaxases) and stability (toxin-antitoxin systems and bacteriocins) using different PCR typing schemes (Table 3) that allowed the identification of the different plasmid families identified in enterococci and other Gram-positive genera: i) Inc18-like plasmids; ii) pMG1/pHTβ-derivatives; iii) Rolling-circle replicating plasmids (pT181, pE194/pLS1, pC194/pUB110, pSN2, pIJ101/pJVI subfamilies and pRI1-like); iv) low size theta replicating plasmids (pCIZ2, p200B, pEF418, pB82 and others); v) RepA_N plasmids which include pheromone-responsive plasmids, pRUM, and pLG1; and vi) other conjugative plasmids from *S. aureus , Bacillus* and *Lactococcus lactis*.

- *Rep initiator proteins.* The PCR typing assay for gram positive rep-initiator proteins (19 replicon families) developed by Jensen *et al.* was applied to discriminate among 111 DNA sequences from 102 published plasmids of different Gram-positive genera (191); unique rep sequences were additionally tested (*Francia M.V., personal data*) making a total of 23 different amplification reactions.
- *Relaxases.* We used the multiplex-PCR-based relaxase typing method described by Goicoechea *et al*. (149) that enable the identification of 27 sequences corresponding to 22 plasmids (pEF1, pRE25, pIP501, pAMβ1, pSM19035, pRUM, pHTβ, pMG1, pCIZ2, pB82, pEF1071, pMV158, pVA380, pAMα1, pS86, pCF10, pOB1, pTEF1, pTEF2, pRS01, pAD1, pAM373), one transposon type (Tn*1549*), the *E. faecalis* pathogenicity island and diverse relaxases present in the genome

of *Enterococcus* strains (BM4518, *E. faecium* DO1887 and *E. hirae* JM89) through the amplification of 12 relaxase types. Samples with negative or multiple bands results in the multiplex PCR were subsequently assayed using primers for individual relaxase genes.

- *Toxin-Antitoxin systems (TA).* The presence of TA identified in streptococci and enterococci (Axe-Txe, ω–ε–ζ and *par* of pAD1), and some from Gram negative bacteria (*maz*EF, *rel*BE) was analyzed by using primers and conditions previously described (193, 286; *Weaver K.E., personal data*).
- *Bacteriocins.* Search of bacteriocins found on enterococcal plasmids (A, B, P, Q, L50A, L50B, Bac32 and Bac43) was performed by simplex PCR using primers previously described or designed for this study (81, 185, 398, 417) (Table 3).
- *Virulence.* The presence of the putative hyaluronidase gene hyl_{Efm}, known to be located on plasmids, was investigated by PCR as described (443).

PCR products of each *rel/rep/TA/bac/hyl* gene type were sequenced in order to confirm the specificity of the method and to analyze similarities with available sequences. Correlation of the genes amplified with vancomycin-resistant plasmids was confirmed when probes for *van* genes and genes encoding relaxases, replication, TA systems, bacteriocins or *hyl* hybridized in the same plasmid band (by Southern blot – see 6.1.5.5.c). Plasmid and/or chromosomal location of sequences corresponding with these genes was determined by hybridization of S1- and/or I-*Ceu*I-digested genomic DNA with probes obtained by PCR using DNA from reference plasmids (see Table 2).

d) RFLP profiling. Restriction Fragment Length Polymorphism (RFLP) has been considered a useful tool to discriminate among plasmids isolated from different strains. After extraction of plasmid DNA (6.1.5.1.b), the method involves the digestion of a sample of DNA by a restriction endonuclease which can recognize and cleave double-stranded DNA at precise sites defined by a sequence of nucleotides. The resulting DNA fragments are then separated by length through agarose gel electrophoresis (6.1.5.4.a) resulting in a plasmid fingerprinting. In specific cases, enzyme digested plasmid DNA separated on agarose gels were Southern blotted onto nylon membranes (6.1.5.5.c) and the blots were hybridized with intragenic probes to further characterize plasmid content.

Plasmids of similar size and gene content and obtained from different isolates were digested with different restriction enzymes in order to establish a possible relationship (see protocol 5 in 6.3.). *Eco*RI, *Hind*III and *Cla*I corresponded to the restriction enzymes used for this task. They generated different profiles, from 5 to 25 DNA fragments, and interpretation of results was difficult due to the lack of standard criteria for definition of

similar or different plasmids as exists for PFGE (410). The computerized analysis (6.1.5.6) of restriction enzyme plasmid patterns was extremely useful in this context by grouping plasmids in different clusters accordingly to their profile similarities.

Table 2. Strains used as recipients in conjugation or controls for PCR and hybridization.

Strain / Plasmid	Control of / Applicability	Main features	Reference
***Enterococcus faecium* GE-1**	Conjugation recipient strain	ST515, Rif^R, Fus^R (plasmid free)	(281)
***Enterococcus faecium* BM4105RF**	Conjugation recipient strain	ST172, Rif^R, Fus^R (plasmid free)	(331)
***Enterococcus faecium* 64/3**	Conjugation recipient strain	ST21, Rif^R, Fus^R (plasmid free)	(461)
***Enterococcus faecalis* JH2-2**	Conjugation recipient strain	ST8, Rif^R, Fus^R (plasmid free)	(187)
***Enterococcus faecium* UA2-1**	*vanA*	Vm^R	From B. E. Murray
***Enterococcus faecalis* V583**	*vanB*, Esp-PAI pTEF1, pTEF2, pTEF3	ST2/CC2, Vm^R (1^{st} clinical VRE from USA)	(323)
***Enterococcus faecium* C68**	hyl_{Efm}	Vm^r, Ap^r, Em^r, Gm^r, Sm^r, Tc^r	(50)
Enterococcus faecalis	Plasmid typing - pIP501 (*rep, rel, TA* genes)	Cm^r, Em^r, Lin^r, Pr^r, ω-ε-ζ system (Inc18)	(415)
***Enterococcus faecalis* RE25**	Plasmid typing – pRE25 (*rep, rel, TA* genes)	Km^r, Sm^r, Cm^r, Em^r, Lm^r, Tc^r	(411)
***Enterococcus faecalis* HUMV 14.3**	Plasmid typing – pEF1, pCIZ2 *(rep, rel, bac* genes)	Bacteriocin cluster	From V. M. Francia (81, 353)
***Enterococcus faecium* 399.F99.H8**	Plasmid typing – pVEF1 *(rep, TA* genes)	VanA, ω-ε-ζ system	(388)
***Enterococcus faecium* U37**	Plasmid typing – pRUM *(rep, rel, TA* genes)	Cm^r, Em^r, Sm^r, St^r Axe-Txe system	(151)
***Enterococcus faecium* pMG1**	Plasmid typing – pMG1/pHTβ *(rep, rel* genes)	High-level Gm^r	(184)
***Enterococcus faecalis* DS16**	Plasmid typing – pAD1 *(rep, rel, TA* genes)	*cyl, par*, UV	(130)
***Enterococcus faecalis* S-86**	Plasmid typing – pS86/pAMα1 *(rep, rel* genes)		(270)
***Enterococcus faecium* 9631160-1**	Plasmid typing – pRI1-like *(rep, rel* genes)	$Streptogramin^r$	(141)
***Enterococcus faecium* 64/3xUW2774**	Plasmid typing – pLG1 *(rep, rel* genes)	hyl_{Efm}-PAI, VanA, *tcr*YAZB	(223)

Abbreviations: Rep, replication; Rel, relaxases; TA, toxin-antitoxin; ^{r}, resistant; Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Fus, Fusidic Acid; Gm, gentamicin; Lm, lincomycin; Pr, pristinamicin; Rif, rifampicin; Sm, streptomycin; St, streptothricin; Tc, tetracycline; Vm, vancomycin.

Table 3. Oligonucleotides and PCR conditions used for the characterization of *Enterococcus* isolates

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Identification of bacterial genera								
1	Ent-F	TAC TGA CAA ACC ATT CAT GAT G	Putative elongation factor Tu (tuf) in *E. faecalis* ATCC19433	AF124221	319-340	112 bp	1 cycle 10'-94ºC; 30 cycles 1''-94ºC and simultaneous 30''-55ºC	(204)
2	Ent-R	AAC TTC GTC ACC AAC GCG GAA C	Putative elongation factor Tu (tuf) in *E. gallinarum* ATCC49573	AF124223	430-409			
Identification of Enterococcus species and glycopeptide resistance genes (one multiplex)								
3	E1-F	ATC AAG TAC AGT TAG TCT T	D-Ala:D-Ala ligase (ddl) in *E. faecalis* FA2-2	AB186053	98-116	941 bp	Multiplex PCR	(110)
4	E2-R	ACG ATT CAA AGC TAA CTG			1038-1021		1 cycle 10' -94ºC	
5	F1-F	GCA AGG CTT CTT AGA GA	D-alanine:D-alanine ligase (Ddl; putative) in *E. faecium* BM4147	EFU39790	363-379	535 bp	30 cycles 30''-94ºC,	(110)
6	F2-R	CAT CGT GTA AGC TAA CTT C			897-879		30''-58ºC, 30''-72ºC;	
7	vanA-F	GGG AAA ACG ACA ATT GC	VanA protein of Tn*1546* in *E. faecium* BM4147	M97297	7154-7170	732 bp	1 cycle 10'-72ºC	(110)
8	vanA-R	GTA CAA TGC GGC CGT TA			7885-7869			
9	vanB-F	ATG GGA AGC CGA TAG TC	D-alanine--D-lactate ligase (vanB) in *E. faecalis* V583	AE016830	2213188-2213204	635 bp		(110)
10	vanB-R	GAT TTC GTT CCT CGA CC			2213822-2213806			
11	vanC1-F	GAA AGA CAA CAG GAA GAC CGC	D-alanine-D-alanine (vanC) ligase-related protein in *E. gallinarum* BM4174	AF162694	1322-1342	876 bp		(61)
12	vanC1-R	ATC GCA TCA CAA GCA CCA ATC			2197-2117			
13	vanC2-F	CGG GGA AGA TGG CAG TAT	Putative ligase (vanC-2; intrinsic low level vancomycin resistance) in *E. casseliflavus*	L29638	350-367	484 bp		(362)
14	vanC2-R	CGC AGG GAC GGT GAT TTT			833-816			
Identification of macrolide resistance genes (one multiplex)								
15	ermA1-F	CTT CGA TAG TTT ATT AAT ATT AGT	S-adenosyl-methionine dependent methylase (*ermA*) in *S. aureus* RN1389	X03216	4596-4619	645 bp	Multiplex PCR	(402)
16	ermA2-R	TCT AAA AAG CAT GTA AAA GAA			5240-5220		1 cycle 10'-94ºC; 35 cycles 1'-94ºC, 1'-50ºC, 1'-72ºC; 1 cycle 10'-72ºC	
17	ermB1-F	CAT TTA ACG ACG AAA CTG GC	Erm(B) (rRNA methylase) in Tn*917* of *E. faecalis* DS16	M11180	837-856	424 bp		
18	ermB2-R	GGA ACA TCT GTG GTA TGG CG			1261-1242			

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Reference
Identification of macrolide resistance genes (cont. multiplex)								
19	ermC1-F	TCA AAA CAT AAT ATA GAT AAA	RNA adenine N-6-methyltransferase (ermC) in *S. aureus*	CP000258	7907-7927	642 bp	Multiplex PCR *(cont.)*	(402)
20	ermC2-R	GCT AAT ATT GTT TAA ATC GTC AAT	USA300 FPR3757		8548-8525			
Identification of tetracycline resistance genes								
21	tetM-F	GTT AAA TAG TGT TCT TGG AG	Tet(M) in *Enterococcus faecalis* DS16	M85225	1485-1504	657 bp	Multiplex PCR	(2)
22	tetM-R	CTA AGA TAT GGC TCT AAC AA			2141-2122		1 cycle 10'-94ºC; 25 cycles 30''-94ºC, 30''-53ºC, 30''-72ºC; 1 cycle 10'-72ºC	
23	tetL-F	CAT TTG GTC TTA TTG GAT CG	Tetracycline resistance protein (TetL) in pKKS825 from *Staphylococcus aureus* 825	FN377602	6264-6283	475 bp		
24	tetL-R	ATT ACA CTT CCG ATT TCG G			6738-6720			
25	tetO-F	CAA TAT CAC CAG AGC AGG CT	Tetracycline resistance protein (TetO) in Tn*5252*-like from *Streptococcus pneumoniae*	FM178797	1046-1065	634 bp		
26	tetO-R	GAT GGC ATA CAG GCA CAG AC			1679-1660			
27	tetK-F	TTA GGT GAA GGG TTA GGT CC	Tetracycline efflux protein (TetK) in pT181 from *S. aureus*	S67449	448-467	718 bp	Multiplex PCR	(2)
28	tetK-R	GCA AAC TCA TTC CAG AAG CA			1165-1146		1 cycle 10'-94ºC; 25 cycles 30''-94ºC, 30''-57ºC, 30''-72ºC; 1 cycle 10'-72ºC	
29	tetS-F	TGG AAC GCC AGA GAG GTA TT	Tet(S); tetracycline resistance protein in Tn*6000* from *Enterococcus casseliflavus*	FN555436	26732-26751	661 bp		
30	tetS-R	ACA TAG ACA AGC CGT TGA CC			27392-27373			
31	tetX-F	CAA TAA TTG GTG GTG GAC CC	Tetracycline resistance protein (TetX) in Tn*4351* from *Bacteroides fragilis*	M37699	644-653	468 bp	1 cycle 10'-94ºC; 30 cycles 30''-94ºC, 30''-58ºC, 30''-72ºC; 1 cycle 10'-72ºC	(458)
32	tetX-R	TTC TTA CCT TGG ACA TCC CG			1111-1092			
Characterization of Tn1546 (vanA transposon)								
33	P1-F	GGA TTT ACA ACG CTA AG	Transposase (ORF1) of Tn*1546* in *E. faecium* BM4147	M97297	22-38	1309 bp	1 cycle of 10'-94ºC; 30 cycles 30''- 94ºC, 1'-45ºC, 5'-72ºC; 1 cycle of 10'-72ºC	(472)
34	P2-R	GCC TTT ATC AGA TGC TA			1330-1314			
35	P3-F	GGT TTT CGA TTA TTG GA	Transposase (ORF1) of Tn*1546* in *E. faecium* BM4147	M97297	1222-1238	1132 bp		
36	P4-R	AAA TAA TAG AAC GAC TC			2353-2337			

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Characterization of Tn1546 (cont.)								
37	P5-F	CGG AAT GCA TAC GGC TC	End of transposase and begin of resolvase of Tn*1546* in *E. faecium* BM4147	M97297	2227-2243	1299 bp	1 cycle of 10'-94ºC; 30 cycles 30''- 94ºC, 1'-45ºC, 5'-72ºC; 1 cycle of 10'-72ºC	(472)
38	P6-R	AGC CAT TAC AGT AAT TA			3525-3509			
39	P7-F	GGA TGG ACT AAC ACC AA	End of transposase and entire resolvase (ORF2) of Tn*1546* in *E. faecium* BM4147	M97297	2769-2785	1274 bp		
40	P8-R	TTA AGT ATA ATT CAA CC			4042-4026			
41	P9-F	GTG AAG GGA TTG AAT TG	End of resolvase and *vanR* of Tn*1546* in *E. faecium* BM4147	M97297	3569-3585	1225 bp		
42	P10-R	TCC AAT CCC CAA GTT TC			4793-4777			
43	P11-F	AAA CGA CTA TTC CAA AC	*vanS* and begin of *vanH* of Tn*1546* in *E. faecium* BM4147	M97297	4675-4691	1679 bp		
44	P12-R	CAT AGT ATA ATC GGC AA			6353-6337			
45	P13-F	GTG TGA AAT ATA TTT CT	*vanH, vanA*and begin of *vanX* of Tn*1546* in *E. faecium* BM4147	M97297	6229-6245	1793 bp		
46	P14-R	TTC CAT GCT TAT CAC CC			8021-8005			
47	P15-F	ATG AAT AGA ATA AAA GT	*vanA* and *vanX* of Tn*1546* in *E. faecium* BM4147	M97297	6979-6995	1942 bp		
48	P16-R	GGA TTT ACT ATT ATC AC			8920-8904			
49	P17-F	AAT TCA TCT ACA TTG GT	*vanY* and teicoplanin resistance protein (*vanZ*) of Tn*1546* in *E. faecium* BM4147	M97297	8889-8905	1585 bp		
50	P18-R	TCA GTC CAA GAA AGC CT			10473-10457			
51	P19-F	TAT CTT CGC TAT TGG AG	Teicoplanin resistance protein (*vanZ*) of Tn*1546* in *E. faecium*	M97297	10403-10419	428 bp	PCR pair with P1-F	
52	IS*Ef1*-F	GGTGTTACGATGTCTGAAATTGC	Transposase of insertion sequence:ISEf1 in *E. faecalis* 229710	EF064799	574-596	variable	PCR pair with P18-R	(306)
53	IS*1216*-F	ACC TTC ACG ATA GCT AAG GTT	TnpA (transposase) in *E. faecalis* MMH594	AY032999	3981-3901	439 bp (variable)	1 cycle 10'-94ºC; 30 cycles 30''-94ºC, 30''-52ºC, 30''-72ºC; 1 cycle 10'-72ºC	(181)
54	IS*1216*-R	AGG ATT ATA TAA GAA AAC CCG			4419-4399			
55	IS*1251*-F	GCA TCC ACT GTA AAC ACC AG	Putative transposase: mobile_element="insertion sequence:IS*1251*" in *E. faecium*	L34675	273-292	427 bp	1 cycle 10'-94ºC; 30 cycles 30''-94ºC, 30''-58ºC, 30''-72ºC; 1 cycle 10'-72ºC	(190)
56	IS*1251*-R	CGC TGT GTT TGA CCA TCC AT			699-680			

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Reference
Characterization of Tn1549/Tn5382 (vanB-transposons)								
57	vanB_long-F	GTT TGA TGC AGA GGC AGA CGA CT	*vanRB, vanSB, vanYB, vanW, vanHB, vanB* and *vanXB* in *E. faecalis* V583	U35369	450-472	5,959 bp	1 cycle 10'-94ºC; 35 cycles 30''-96ºC, 1'-59ºC, 7'-72ºC; 1 cycle 10'-72ºC	(158)
58	vanB_long-R	ACA AGT TCC CCT GTA TCC AAG TGG			6408-6385			
59	vanB_consens-F	CAA AGC TCC GCA GCT TGC ATG	D-Ala:D-Lac ligase (*vanB*) in *E. faecalis* V583	U35369	5340-5360	484 bp	1 cycle 10'-94ºC; 30 cycles 30''-94ºC, 30''-64ºC, 30''-72ºC; 1 cycle 10'-72ºC	(85)
60	vanB_consens-R	TGC ATC CAA GCA CCC GAT ATA C			5823-5802			
61	$vanS_B$-F	ATA TGC GCT GGA AAA CAC CTC	Intergenic region between *vanSB* and *vanYB* in *E. faecalis* V583	U35369	2114-2134	309 bp	1 cycle 10'-94ºC; 30 cycles 30''-94ºC, 30''-58ºC, 30''-72ºC; 1 cycle 10'-72ºC	(85)
62	$vanY_B$-B	CCC CAG ATT GTT TCA TAT GCC			2422-2402			
63	Tn*5382*_like-F	GTT CTT ATT CCG CAG GTG GTG ATT	Non-integrase left region of Tn*5382* in *E. faecium* C68	AF175739	1-24	311 bp	1 cycle 10'-94ºC; 30 cycles 30''-94ºC, 30''-68ºC, 30''-72ºC; 1 cycle 10'-72ºC	(50)
64	Tn*5382*_like-R	ACG CCA TGC TAT TTA CTT CCG GC			311-289			
65	pbp5_Tn*5382*-F	TCA GCC GAT TTG CGA CAG GTT ATG	Low affinity penicillin-binding protein PBP5 and transposon Tn*5382* in *E. faecium* C68	AF117609	3947-3960	1069 bp	1 cycle 10'-94ºC; 30 cycles 30''-94ºC, 30''-68ºC, 1'-72ºC; 1 cycle 10'-72ºC	(84)
66	pbp5_Tn*5382*-R	TGG GGT GGC GGG TAT TAG CAG TAT			5015-4992			
Characterization of Conjugative Transposons (CTns) and Tn917								
67	Int_Tn*916/1545*-F	GCG TGA TTG TAT CTC ACT	"Int-Tn" (Tn*1545*-related conjugative transposon integrases) in Tn*916* from *E. faecalis* DS16	U09422	16802-16819	1046 bp	Multiplex PCR	(106)
68	Int_Tn916/1545-R	GAC GCT CCT GTT GCT TCT			17847-17830		1 cycle 10'-95ºC; 25 cycles 30''-94ºC, 30''-52ºC, 30''-72ºC; 1 cycle 10'-72ºC	
69	Xis_Tn*916/1545*-F	AAG CAG ACT GAC ATT CCT A	"Xis-Tn" in Tn*916* from *E. faecalis* DS16	U09422	16359-16377	194 bp		
70	Xis_Tn*916/1545*-R	GCG TCC AAT GTA TCT ATA A			16552-16534			
71	tnpA_Tn*917*-F	ATC GAT TCG GTA GTG CTA AT	Transposase (tnpA) in Tn*917* from *E. faecalis* DS16	M11180	2704-2723	854 bp		(307)
72	tnpA_Tn*917*-R	CAA TAA CAT TGG CGT GTA TC			3557-3538			
73	tnpR_Tn*917*-F	GTG AGT ACG GAT GAT CAA AA	Resolvase (tnpR) in Tn*917* from *E. faecalis* DS16	M11180	1827-1846	517 bp		
74	tnpR_Tn*917*-R	TGA GGT AAC GGT AAA AGG TT			2343-2324			

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Characterization of CTns (cont.)								
75	tndX_Tn*5397*-F	ATG ATG GGT TGG ACA AAG A	TndX (responsible for insertion and excision) in Tn*5397* from *Clostridium difficile*	AF333235	19106-19124	611 bp	Multiplex PCR	(6)
76	tndX_Tn*5397*-R	CTT TGC TCG ATA GGC TCT A			19716-19698		1 cycle 10'-95ºC; 25 cycles 30''-94ºC, 30''-55ºC, 45''-72ºC; 1 cycle 10'-72ºC	
77	CW459/Tn*5801*-F	CTG TTT CCG ATA TTG AGC	Int459; putative integrase in transposon: CW459-*tet(M)* from *Clostridium perfringens*	AF329848	7589-7606	857 bp		(307)
78	CW459/Tn*5801*-R	GTT TCG CAA GTA GTC TAC AG			8445-8426			
79	Tn*4451/4453*-F	GGA TAT GAG TTG TGC AAG GG	*tnpZ* of Tn*4451* from *Clostridium perfringens* CP590	U15027	5048-5067	260 bp		M.V. Francia
80	Tn*4451/4453*-R	GTT CAC ATC AAT CAC AAT CTC			5306-5286			
81	Int_Tn*6000*-F	CAT CGA GTC TAA CCG ATT GT	Int (tyrosine integrase) in Tn*6000* (fomer CTn*1*) from *Enterococcus faecium* 664.1H1	DQ370176	817-836	869 bp	Multiplex PCR	(307)
82	Int_Tn*6000*-R	GAC CCA AAC GAA CTT GTA CT			1685-1666		1 cycle 10'-95ºC; 25 cycles 30''-94ºC, 30''-55ºC, 30''-72ºC; 1 cycle 10'-72ºC	
83	Xis_Tn*6000*-F	CGA AGT ATT AAC CGA ACA GA	Xis (excisionase) in Tn*6000* (former CTn*1*) from *Enterococcus faecium* 664.1H1	DQ370176	304-323	203 bp		
84	Xis_Tn*6000*-R	TAT CAT CGC GAT CAA AAC GA			506-487			
85	Int_Tn*5386*-F	CTT GTT CCT ACG GAC AGA GT	Integrase (Int-Tn*5386*) in Tn*5386* from *E. faecium* D344R	DQ321786	28140-28159	1021 bp		
86	Int_Tn*5386*-R	AGC CGT GAG CGT AAT AAT TC			29160-29141			
87	Xis_Tn*5386*-F	ATA CTG ATG TGC CTG TAT GG	Excisionase (Xis-Tn5386) in Tn*5386* from *E. faecium* D344R	DQ321786	27731-27750	155 bp		
88	Xis_Tn*5386*-R	TCG CTT TAT CTG AAT ACG G			27885-27867			
Identification of virulence genes								
89	agg-F	GCA CGC TAT TAC GAA CTA TGA	Aggregation substance (*prgB*) in *E. faecalis* pMG2200	AB374546	96511-96531/96888-96868	378 bp	Multiplex PCR	(443)
90	agg-R	TAA GAA AGA ACA TCA CCA CGA	Aggregation substance (*asp1*) in *E. faecalis* pPD1	X62656	2636-2656/3013-2993		1 cycle 10'-94ºC; 30 cycles 1'-94ºC, 1'-56ºC, 1'-72ºC; 1 cycle 10'-72ºC	
91	gel-F	TAT GAC AAT GCT TTT TGG GAT	"Coccolysin", known as gelatinase, in *E. faecalis* V583	AE016830	1763396-1763416/1763608-1763588	213 bp		
92	gel-R	AGA TGC ACC CGA AAT AAT ATA	Gelatinase (*gelE*) in *E. hirae* S9 from cattle feces	EU423274	2-22 / 214-194			
93	cyl-F	ACT CGG GGA TTG ATA GGC	EF0048; cytolysin activator (*cylA*) in *E. faecalis* PAI	AF454824	48382-48399 / 49069-49052	688 bp		

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Identification of virulence genes (cont.)								
94	cyl-R	GCT GCT AAA GCT GCG CTT	*cylA* in pAD1 from *E. faecalis*	L37110	6657-6674 / 7344-7327	688 bp	Multiplex PCR (cont.)	(443)
95	hyl_{Efm}-F	ACA GAA GAG CTG CAG GAA ATG	Putative hyaluronidase (Hyl) in *E. faecium* CV133	AF544400	1026-1046	276 bp	1 cycle 10'-94ºC; 30 cycles 1'-94ºC, 1'-56ºC, 1'-72ºC; 1 cycle 10'-72ºC	
96	hyl_{Efm}-R	GAC TGA CGT CCA AGT TTC CAA			1301-1281			
97	esp-F	AGA TTT CAT CTT TGA TTC TTG G	Enterococcal surface protein (Esp) in *E. faecalis* PAI	AF454824	56102-56123/56612-56592	511 bp		
98	esp-R	AAT TGA TTC TTT AGC ATC TGG	Surface protein Esp variant (Esp) in *E. faecium* E0300	AF443999	92-113/602-582			
99	acm-F2	CAG GCA GAG ATA TCA GCA G	Collagen adhesin precursor in *E. faecium* TX2555	AY135217	489-507	1496 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-56ºC, 1'-72ºC; 1 cycle 10'-72ºC	(299)
100	acm-R1	ATT CTC ATT TGT AAC GAC TAG C			1984-1963			
Multilocus sequence typing of Enterococcus faecium								
101	ddl-F	GAG ACA TTG AAT ATG CCT TAT G	D-alanine:D-alanine ligase in *E. faecium* BM4147	AF294727	376-397	560 bp	1 cycle 15'-95ºC; 35 cycles 30''-94ºC, 30''-50ºC, 30''-72ºC; 1 cycle 5'-72ºC	(177)
102	ddl-R	AAA AAG AAA TCG CAC CG			935-919			
103	gdh-F	CCA AGA TTG GGC AACTTCGTCCCA	Glucose-6-phosphate 1-dehydrogenase in *E. faecalis* V583	AE016830	963342-963365	660 bp	(identical to all seven genes)	
104	gdh-R	GGC GCA CTA AAA GAT ATG GT			964001- 963982			
105	gyd-F	CAT TTC GTT GTC ATA CCA AGC	Glyceraldehyde 3-phosphate dehydrogenases in *E. faecalis* V583	AE016830	1901561-1901581	494 bp		
106	gyd-R	CAA ACT GCT TAG CTC CAA TGG C			1902054-1902033			
107	adk-F	TAT GAA CCT CAT TTT AAT GGG	Adenylate kinase (Adk) in *E. faecalis* V583	AE016830	209233-209253	529 bp		
108	adk-R	GTT GAC TGC CAA ACG ATT TT			209761-209742			
109	purK-F	GCA GAT TGG CAC ATT GAA AGT	Phoshoribosylaminoimiazol carboxylase ATPase subunit (purK) in *E. faecium* E0158	AF443355	Not available	492 bp for sequencing		
110	purK-R	TAC ATA AAT CCC GCC TGT TTC T						
111	pstS-F	TTG AGC CAA GTC GAA GCT GGA G	Phosphate ABC transporter (pstS) in *E. faecium* E0158	AF443368	Not available	583 bp for sequencing		
112	pstS-R	CGT GAT CAC GTT CTA CTT CC						

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Reference
Multilocus sequence typing of Enterococcus faecium (cont.)								
113	atpA-F	AAG TTC ACG ATA AGC CAC GG	ATP synthase F1, alpha subunit (AtpA) in *E. faecalis* V583	AE016830	2527525-2527544	649 bp	(cont.)	(177)
114	atpA-R	CGG TTC ATA CGG AAT GGC ACA			2528173-2528153			
Multilocus sequence typing of Enterococcus faecalis (cont.)								
115	gdh-F	CCA AGA TTG GGC AACTTCGTC CCA	Glucose-6-phosphate 1-dehydrogenase in *E. faecalis* V583	AE016830	963342-963365	660 bp	1 cycle 5'-94ºC; 30 cycles 30''-94ºC, 30''-52ºC, 1'-72ºC; 1 cycle 7'-72ºC	(354)
116	gdh-R	GGC GCA CTA AAA GAT ATG GT			964001-963982			
117	gyd-F	CAT TTC GTT GTC ATA CCA AGC	Glyceraldehyde 3-phosphate dehydrogenases in *E. faecalis* V583	AE016830	1901561-1901581	494 bp	(identical to all seven genes)	
118	gyd-R	CAA ACT GCT TAG CTC CAA TGG C			1902054-1902033			
119	pstS-F	ATT TAC ATC ACG TTC TAC TTG C	Phosphate-binding protein in *E. faecalis* V583	AE016830	1651231-1651252	643 bp		
120	pstS-R	CGG AAC AGG ACT TTC GC			1651873-1651857			
121	gki-F	ACC ATT AAA GCA AAA TGA TCG C	Putative glucokinase (glcK) in *E. faecalis* V583	AE016830	2691781-2691802	527 bp		
122	gki-R	GAT TTT GTG GGA ATT GGT ATG G			2692307-2692286			
123	aroE-F	TGG AAA ACT TTA CGG AGA CAG C	Shikimate 5-dehydrogenase (aroE) in *E. faecalis* V583	AE016830	1513712-1513733	524 bp		
124	aroE-R	GTC CTG TCC ATT GTT CAA AAG C			1514235-1514214			
125	xpt-F	AAC GTC ACC GTT CCT TCA CTT A	Xanthine phosphoribosyltransferase (xpt) in *E. faecalis* V583	AE016830	2287612-2287633	538 bp		
126	xpt-R	AAA ATG ATG GCC GTG TAT TAG G			2288149-2288128			
127	yiqL-F	CAG CTT AAG TCA AGT AAG TGC CG	Acetyl-CoA acetyltransferase / hydroxymethylglutaryl-CoA reductase in *E. faecalis* V583	AE016830	1338944-1338966	523 bp		
128	yiqL-R	GAA TAT CCC TTC TGC TTG TGC T			1339466-1339445			
Amplification of 23S rDNA								
129	23SrDNA-F	CGC ATG TAC AGG ATA GGT AGG	23S ribosomal RNA in *E. faecium* ATCC 27273	EU547788	2105-2125	669 bp	1 cycle 10'-94ºC; 30 cycles 30''-94ºC, 30''-56ºC, 30''-72ºC; 1 cycle 10'-72ºC	(86)
130	23SrDNA-R	AGG TGG GCT TCA ACA TTA GAT			2774-2754			

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Detection of replicase genes for plasmid typing								
131	Rep(1)-F	TCG CTC AAT CAC TAC CAA GC	Unnamed protein product; repR gene product (AA 1-496) in pIP501	X17655	695-714 / 1318-1299	624 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-52ºC, 30''-72ºC; 1 cycle 10'-72ºC	(191)
			RepR in pGB354 from *Streptococcus agalactiae*	U83488	79-98 / 702-698			
			RepE (replication protein) in pAMβ1 from *E. faecalis*	AF007787	3188-3207 / 3811-3792			
			Putative RepS protein in pRE25 (orf6) from *E. faecalis* RE25	X92945	4740-4759 / 5363-5344			
			RepE protein in pTEF1 from E. *faecalis* V583	AE016833	45996-46015/46619-46600			
			Putative replication protein (repE) in pIP816	AM932524	17694-17713/18317-18298			
			RepS (initiation of plasmid replication) in pSM19035	AY357120	712-731 / 1335-1316			
			RepS in pMD101 from *Streptococcus pyogenes*	X64695	577-596 / 1200-1181			
			RepS in *Staphylococcus aureus* CM05	EF450709	312-331 / 935-916			
132	Rep(1)-R	CTT GAA CGA GTA AAG CC CTT	Putative repE protein in pKL0018 from L*actococcus garvieae*	AB290882	75-94 / 698-679			
133	Rep(2)-F	GAG AAC CAT CAA GGC GAA AT	RepR in pEF1 from *E. faecium* 6T1a; putative Rep protein of pRE25	DQ198088	16626-16645/17255-17230	630 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-52ºC, 30''-72ºC; 1 cycle 10'-72ºC	(191)
			Putative Rep protein in pRE25 (orf1) from *E. faecalis* RE25	X92945	363-382 / 992-967			
			Putative replication protein (repE) in pIP816 in *E. faecium* BM4147	AM932524	29062-29081/29691-29666			
			Replication protein (repR) in pVEF1 in *E. faecium* 399/F99/H8	AM296544	23818-23837/24447-24422			
134	Rep(2)-R	ACC AGA ATA AGC ACT ACG TAC AAT CT	Putative replication protein (repR) in pVEF2 in *E. faecium* 399/F99/H8	AM410096	23906-23925/24535-24510			
			Putative replication protein (repR) in pVEF3 in *E. faecium* 399/S99/A7	AM931300	30095-30114/30724-30699			
135	Rep(3)-F	ACA TTT TCC TCA AAG AAC AT	Replication protein Rep63A in pAW63 from *Bacillus thuringiensis*	NC_010599	42922-42941/43325-43298	404 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-49ºC, 30''-72ºC; 1 cycle 10'-72ºC	(191)
			Replication protein Rep165 in pBMB165 from *Bacillus thuringiensis*	DQ242517	15751-15770/16154-16128			
136	Rep(3)-R	CCTAATGTATATAATTTTGGT ACA TAT	Replication protein in pBT9727 from *Bacillus thuringiensis*	CP000047	44281-44300/44684-44658			
			Hypothetical protein in pX02 from *Bacillus tanthracis*	NC_002146	33445-33464/33848-33822			

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Detection of replicase genes for plasmid typing (cont.)								
137	Rep(6)-F	ACG AAT GAA AGA TAAAGGAGTAG	Rep protein in pS86 from *E. faecalis* S-86	AJ223161	607-629/1157-1134	551 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-52ºC, 30''-72ºC; 1 cycle 10'-72ºC	(191)
			RepE replication protein in pAMα1 from *E. faecalis*	AF503772	4533-4556/5083-5061			
138	Rep(6)-R	TAA ATT CTA GTTTGGCAATCTTAT	Replication protein (repE) in pEF47 from *E. faecalis* 47/3	AY842500	27-49 / 577-554			
139	Rep(18)-F	ACA CCA GTC GAA ATG AAT TT	Putative replication protein (repA) in pEF418 from *E. faecalis* 418	AF408195	258-277	462 bp	Identical	(191)
140	Rep(18)-R	AGGAATATCAAGTAATTCATGAAAGT						
141	Rep(18b)-F	GTG TCT AGA ATG CGTGAAAAAGG	Replication protein (repA) in pEF1071 from *E. faecalis* BFE 1071	AF164559	7933-7952 / 8353-8332	421 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-54ºC, 30''-72ºC; 1 cycle 10'-72ºC	M.V. Fran-cia, P.C.
			Replication protein (repA) in pB82 from *E. faecium*	AB178871	4250-4272 / 4670-4649			
			Replication initiation protein (repA) in pJS33 from *E. faecium* JH95	EU370687	1-19 / 421-400			
142	Rep(18b)-R	CTA ATA ATT CAA GAA AGT CTTC	RepA protein in plasmid encoded-bacteriocin locus from *E. faecalis*	AY063485	2786-2805 / 3206-3185			
143	Rep(18c)-F	TGT TCT AAAATGAAA AGAAAAGG	Putative plasmid replication protein in pCIZ2 from *E. faecium* L50	DQ832184	5783-5805	427 bp	Identical to the former, except annealing at 56ºC	M.V. Fran-cia, P.C.
144	Rep(18c)-R	CTA ATA GAG CTC TAA AAT CTT C			6209-6188			
145	Rep(5)-F	ATG TGT AAT AAATTAAAAGAGCA	Replication protein in pSAS from *S. aureus*	BX571858	17667-17689/18303-18281	637 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-52ºC, 30''-72ºC; 1 cycle 10'-72ºC	(191)
			Replication protein (Rep) in pMW2 from *S. aureus*	AP004832	2218-2240 / 2854-2832			
146	Rep(5)-R	ATT GTC TTG ATT TAT CTATCTTG	Replication protein (Rep) in pN315 from *S. aureus*	AP003139	21660-21682/22296-22274			
147	Rep(14a)-F	GAT ATT GCT TGC GAT ATT	Replication protein in pEFNP1 from *E. faecium*	AB038522	661-678 / 1128-1110	468 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-50ºC, 30''-72ºC; 1 cycle 10'-72ºC	M.V. Fran-cia, P.C..
			Replication initiation protein (repA) in pJS42 from *E. faecium*	EU370688	25-42 / 492-474			
148	Rep(14a)-R	CTC TCG RAA ATG ATT CGT C	Replication initiation protein (rep) in pRI1 from *E. faecium*	EU327398	3248-3266 / 3715-3698			
149	Rep(7)-F	AGA CGT AAT ATG CGT RTT GA	Hypothetical protein, repC protein in pT181 from *S. aureus*	NC_001393	162-181 / 388-369	227 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-49ºC, 30''-72ºC; 1 cycle 10'-72ºC	(191)
			Replication initiation protein in pUSA02/pUSA03 from *S. aureus*	NC_007791	3681-3700 / 3907-3888			
150	Rep(7)-R	CCA AAA TAY TTY GTT TCT GG	RepD in pK214 from *Lactococcus lactis*	X92946	19322-19341/19548-19529			

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Detection of replicase genes for plasmid typing (cont.)								
151	Rep(10)-F	TAT AAA GGC TCT CAG AGG CT	Replication protein in pIM13 from *Bacillus subtilis*	M13761	413-432 / 795-776	383 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-54ºC, 30''-72ºC; 1 cycle 10'-72ºC	(191)
			Replication and maintenance protein in pUSA03 from *S. aureus*	CP000258	6705-6724/7087-7068			
152	Rep(10)-R	CCA AAT TCG AGT AAG AGG TA	Replication protein (RepL) in pWBG738 from *S. aureus*	DQ088624	71-90 / 453-434			
153	Rep(10b)-F	TAAATAAAGACTCAGGAGAAGTA	Replication protein in pSK3 from *S. aureus*	NC 001994	922-944 / 1122-1103	201 bp	Identical	(191)
154	Rep(10b)-R	TAG CAA GTT CTC GAA CTG TT	ORF 154 in pOX2000 from *S. aureus*	X55798	633-655 / 833-814			
155	Rep(12)-F	GAG CCT ATA ACA GAG TAC ACA	Replication protein ori43 in pBMB67 from *Bacillus thuringienesis*	DQ363750	21249-21269/21819-21799	571 bp	Identical, except annealing at 52ºC	(191)
156	Rep(12)-R	CAA ATA TAG GCT TTG TAG TTC	Replication protein in *B. thurigiensis* HD263	M60513	853-873 / 1423-1403			
157	Rep(4)-F	ACTATGTCGTTGAGTCTAATGACT	Putative replication protein RepB in pMBB1 from *E. faecium*	U26268	1121-1144 / 1544-1516	424 bp	Identical, except annealing at 56ºC	(191)
158	Rep(4)-R	AGCAAGATAGAATATTTACTTTTAAGTTT						
159	Rep(17)-F	TACTAACTGTTGGTAATTCGTTAAAT	Putative RepA in pRUM; similar to protein PrgW from pCF10	AF507977	20562-20587/21063-21043	502 bp	Identical, except annealing at 52ºC	(191)
160	Rep(17)-R	ATC AAG GAC TCA ACC GTA ATT	Putative RepA in plasmid = "pRUM" from *E. faecium* VRE3	EU376117	1470-1495 / 1971-1951			
161	Rep(8)-F	TAGATACGACAAAAG AAGAATTACA	Replication-associated protein (EP0002) in pAM373 from *E. faecalis*	AE002565	1743-1764 / 2137-2113	395 bp	Identical, except annealing at 52ºC	(191)
162	Rep(8)-R	CCA ATC ATG TAA TGT TAC AAC C	Replication-associated protein A (repA) in pEJ97-1 from *E. faecalis*	AJ490170	7448-7472 / 7842-7821			
163	Rep(9)-F	GCT CGA TCA RTT TTC AGA AG	RepA in pPD1; pheromone related transfer gene from *E. faecalis*	D78016	1618-1637 / 1818-1797	201 bp	Identical, except annealing at 52ºC	(191)
			PrgW ("replication") in pCF10 from *E. faecalis*	AY855841	3664-3683 / 3864-3843			
			Replication-associated protein (repA) in pAD1 from *E. faecalis*	L01794	333-354 / 533-514			
			Replication-associated protein RepA (repA-1) in pTEF1 and pTEF2	AE016833	733/5-752/4 / 933/5-912/4			
164	Rep(9)-R	CGC AAA CAT TTG TCW ATT TCT T	Plasmid replication protein (repA) in pMG2200 from *E. faecalis*	AB374546	83638-83657/83838-83817			
165	Rep(13)-F	ATG ATG CAA TAT ATT AAG CA	Replication protein in pSK89 from *S. aureus*	M37889	513-534 / 915-896	403 bp	Identical, except annealing at 46ºC	(191)
166	Rep(13)-R	TAC CAG AAT AYT TAG CCA TTT C	Putative replication initiation protein in pSSP1	AP008935	34138-34159/34540-34521			

Table 3 *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Detection of replicase genes for plasmid typing (cont.)								
167	Rep(15)-F	CAG TAG AAG AAA ATT ATA AAG AAC	Putative replication initiation protein Rep in pSK41 from *S. aureus*	AF051917	13224-13247/13551-13531	328 bp	Identical, except annealing at 52ºC	(191)
168	Rep(15)-R	GTT ATG GCT GGT TTT AAT AAA	Replication initiation protein in pUSA03 from *S. aureus*	NC_007792	120-143 / 447-427			
169	Rep(16)-F	CAG GAA AAC ACT TCG TTT AT	Replication protein in pSAS, similar to *S. aureus* plasmid pSK1	BX571858	4737-4761 / 5328-5309	592 bp	Identical, except annealing at 52ºC	(191)
170	Rep(16)-R	CTT CTA TAT CAC TAT CAT TGTCATT	Protein of unknown function in pSJH101 from *S. aureus* JH1	CP000737	6071-6090 / 6662-6630			
171	Rep(19)-F	GWG ATC GCTTARAYTTATCTAT	Replication initiation protein Rep in pSA1379 from *S. aureus*	NC_007931	131-152 / 671-653	540 bp	Identical, except annealing at 46ºC	(191)
172	Rep(19)-R	YMT TGT TST GGM AAT TCT T	Hypothetical protein (repA) in pUB101 from *S.aureus*	NC_005127	3639-3657 / 4194-4173	555 bp		
173	Rep(21)-F	TCG TTT CAC TCA TTGGACACC	Plasmid replication protein in pVEF1 from *E. faecium*	AM296544	207-227 / 406-388	200 bp	Identical, except annealing at 52ºC	M.V. Fran-cia, P.C.
174	Rep(21)-R	TCA TTG TCG GCA TAA AGC C	Plasmid replication protein in pVEF2 from *E. faecium*	AM410096	207-227 / 406-388			
175	Rep(22)-F	GTA TTA ACA CAC TGG ACT C	Replication protein in pHTβ from *E. faecium*	AB183714	49977-49995/50175-50157	199 bp	Identical, except annealing at 52ºC	M.V. Fran-cia, P.C.
176	Rep(22)-R	TCA GTG TAG GCA ATA ACC C	Rep protein in pMG1 from *E. faecium*	AB206333	7713-7731/ 7911-7893			
177	Rep(20)-F	AGTCGAAAATATCCAGAACCTG	Replication-associated protein RepA in pLG1 from *E. faecium* 64/3xUW2774	HM565183	40776-40797	135 bp	Identical, except annealing at 60ºC	G. Wer-ner, P.C.
178	Rep(20)R	AAC CAGTCTTTCGTAATAGTGC			40910-40889			
Detection of relaxase genes for plasmid typing								
179	traA(pHTβ)-F	TAAA CTG TTC GCC GGAACTG	TraA in pHTβ from *E. faecium*	AB183714	47971-47990/48508-48490	538 bp	Identical, except annealing at 56ºC	This study
180	traA(pHTβ)-R	TGC TCC TGT GTC GTA CAGG	Hypothetical protein (traA) in pMG1 from *E. faecium*	AB206333	1048-1067 / 1585-1567			
181	Rel(pHTβ)-F	TAA AGA TTT GCG TGA AATGG	Putative relaxase/nickase (traE) in pHTβ from *E. faecium*	AB183714	31777-31796/31968-31949	192 bp	Identical, except annealing at 52ºC	(149)
182	Rel(pHTβ)-R	TCT TGG AAC ATC ACC GATCC	Hypothetical protein (orf46) in pMG1 from *E. faecium*	AB206333	42358-42377/42549-42530			
183	Rel(pRE25)-F	AAT GGC TTC TTA TCGAAGTGG	Nickase in pRE25 (orf24) from *E. faecalis* RE25	X92945	19681-19701/20005-19986	325 bp	Identical, except annealing at 52ºC	(149)
184	Rel(pRE25)-R	TTC ATC ATC TCT ATG AAT GG	ORF1 (nickase) in pIP501 from *Streptococcus* pVA1702	L39769	1459-1479 / 1783-1764			

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Detection of relaxase genes for plasmid typing (cont.)								
185	Rel(pEF1)-F	AAG CAA ATC TTC CAA AGA GG	Hypothetical protein in pEF1; orf34; ef1887	DQ198088	20948-20967	217 bp	Identical to the former	(149)
186	Rel(pEF1)-R	GGTGAATCGTTGCCATCCAGTAGCC			21164-21140			
187	Rel(pRUM)-F	AAT GTA TGG CAA AAC AGA TGG	Rlx protein (orf27) in pRUM in *E. faecium* U37	AF507977	18940-18960/19077-19058	138 bp	Identical with annealing at 56ºC	(149)
188	Rel(pRUM)-R	ACG GCA ACT TGA TGA TTA GG	MobA (relaxase) in pC223 from *S. aureus*	AY355285	2476-2496 / 2613-2594			
189	Rel(pCIZ2)-F	ATG GCA ACA GTT AAA GTA AG	Putative mobilization protein in pCIZ2 from *E. faecium* L50	DQ832184	2994-3013 / 3201-3181	208 bp	Identical with annealing at 52ºC	(149)
			Mobilization protein (mobA) in pB82 from *E. faecium*	AB178871	1465-1484 / 1672-1652			
			Relaxase domain protein in *E. hirae* operon hiracin JM79	DQ664500	1607-1626 / 1814-1794			
190	Rel(pCIZ2)-R	CAG CGG TAA TTT CTC CTG GTC	Mobilization Protein A (mobA) in p200B from *E. faecium*	AB158402	4868-4887 / 5075-5055			
191	Rel(pAD1)-F	TAA TTT ATG TRA CAC CTA GT	EF0025 in *E. faecalis* pathogenicity island	AF454824	28849-28868/28981-28955	133 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-49ºC, 30''-72ºC; 1 cycle 10'-72ºC	(149)
			Unknown product (orf57) in pAD1 from *E. faecalis* DS16	AF343837	9143-9162 / 9275-9249			
			Hypothetical protein (EP0019) in pAM373 from *E. faecalis*	AE002565	13059-13085/13191-13172			
192	Rel(pAD1)-R	TCAAATTCTTCRGCTGAAAAAAYATGC	Hypothetical protein (EFA0025) in pTEF1 from *E. faecalis* V583	AE016833	18166-18192/18298-18279			
193	Rel(pCF10)-F	TAA TCC AAT CMT TTT CTC C	PcfG (relaxase) in pCF10 from *E. faecalis*	AY855841	33634-33652/33951-33931	318 bp	Identical to the former	(149)
194	Rel(pCF10)-R	TGG ACT SWG TTT GCC ATT TTG	Relaxase in pTEF2 from *E. faecalis* V583	AE016831	30711-30729/31028-31008			
195	Rel(pAMα1)-F	GAA AGA CAC AAA TTT AAG TGG	MobE mobilization protein (mobE) in pAMα1 from *E. faecalis*	AF503772	7983-8003 / 8357-8337	375 bp	Identical with annealing at 54ºC	(149)
196	Rel(pAMα1)-R	ACA ATT CCT AAG TGC ATA TGG	Mobilization protein (mob) in pS86 from *E. faecalis* S-86	AJ223161	2462-2482 / 2836-2816			
197	Rel(pEF1071)-F	CCA TGG CCG AAC GAA AAC TC	MobA (mobilization protein) in pEF1071 from *E. faecalis* BFE-1071	AF164559	6301-6320 / 6552-6533	252 bp	Identical to the former	(149)
198	Rel(pEF1071)-R	ACT CAT GTT TAA TCA CGT CC						
199	Rel(pMV158)-F	AAC TAY GAK TTG AYG GAT CG	Hypothetical protein (pMV158_p4) in pMV158 from *S. agalactiae*	NC_010096	3859-3878 / 4145-4126	287 bp	Identical with annealing at 52ºC	(149)
200	Rel(pMV158)-R	ACB CCY AWR TGC ATR TGR GG	Mob (mobilization protein) in pER13 from *S. thermophilus*	NC_002776	2126-2145 / 2412-2393			

Table 3. *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Reference
Detection of relaxase genes for plasmid typing (cont.)								
201	Rel(pRI1)-F	TTG TTC TAA ATA GAT ATTTTCATC	MobA (mobilization protein) in pJS42 from *E. faecium* JH95	EU370688	1941-1964 / 2045-2022	105 bp	Identical with annealing at 48ºC	M.V. Francia, P.C.
202	Rel(pRI1)-R	AGC ATT TCT CAT AAT AATCGTGAG	Mobilization protein (mob) in pRI1 from *E. faecium* AHA15	EU327398	1695-1728 / 1799-1776			
203	Rel(Tn*1549*)-F	GTA AGG GCA AGA GCT ATG CC	Rlx-like protein in Tn1549 (orf28) from *E. faecalis* BM4382	AF192329	21041-21061/21394-21375	354 bp	Identical with annealing at 58ºC	(149)
204	Rel(Tn*1549*)-R	CAT AGC CTG CCG ACT GCT GGG	Hypothetical protein in pMG2200 from *E. faecalis*	AB374546	29619-29639/29972-29953			
205	Rel(BM4518)-F	GCA AGA AAA AGC CCT ATG CC	Rlx-like protein in BM4518 (ORFG11) from *E. faecalis* BM4518	AY271782	4890-4909	208 bp	Identical with annealing at 58ºC	(149)
206	Rel(BM4518)-R	GGA ATT TTC GCT GAT ACG CTC			5097-5078			
207	Rel(pLG1)-F	CGC CTG AAG ACA AAG TGA CA	Putative relaxase in pLG1 from *E. faecium* 64/3xUW2774	HM565192	2341-2360	332 bp	Identical with annealing at 56ºC	This study
208	Rel(pLG1)-R	GCG AAT TTC AGA ACG AAA GC			2672-2653			
Detection of toxin-antitoxin genes for plasmid typing								
209	Fst-F	AGCAGCTTACTT TCGGCTATCGTCT	*par* toxin and antidote in pAD1 from *E. faecalis*	L01794	4174-4198	146 bp	Identical with annealing at 70ºC	K. Weaver, P.C.
210	Fst-R	TAA TGC GGC AGC TCG CCT CGATT			4319-4297			
211	ε-F	TTA ACG AAT TAT CGG CAA GC	Antidote of epsilon-zeta PSK system in pSM19035	AY357120	6784-6803	1063 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-52ºC, 1'-72ºC; 1 cycle 10'-72ºC	(193)
212	ζ-R	GTG GTT TAG GTG GCT GCA AG	Toxin of epsilon-zeta PSK system in pSM19035		7846- 7827			
213	omega-F	GGG AAA TTT AGG CGC ACA	Transcriptional repressor (omega) in pSM19035	AY357120	6520-6537	150 bp		This study
214	omega-R	AAT GGC GGA AAC GTA AAA GA			6669-6650			
215	Axe-Txe-F	CTG ACC CTT TCC TTA CTT CCG	Toxin and antitoxin of Axe-Txe in pRUM from *E. faecium*	NC_005000	4937-4957	557 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-52ºC, 30''-72ºC; 1 cycle 10'-72ºC	(286)
216	Axe-Txe-R	GGG TGA AAG GAA TGG AAG CAG			5493-5473			
217	mazF-F	CAACTGTTCCTTTCTTCGTTGCTCC	Toxin of the ChpA-ChpR TA system in *Escherichia coli* K-12	NC_000913	2908831-2908855	444 bp	Identical with annealing at 54ºC	(286)
218	mazE-R	GATGATGAAGTGAAGATTGACCTGG	Antitoxin of the ChpA-ChpR TA system in *Escherichia coli* K-12		2909274-2909250			

Table 3 *Cont.*

N.º	Primers	Oligonucleotide sequence (5' to 3')	Description (examples)	GenBank accession number	Position of amplicon (F/R)	Amplicon size	PCR conditions	Refe-rence
Detection of toxin-antitoxin genes for plasmid typing (cont.)								
219	relE-F	CAG AGA ATG CGT TTG ACC G	Toxin of the RelE-RelB TA system in *Escherichia coli* K-12	NC_000913	1643371-1643389	457 bp	Identical to the former	(286)
220	relB-R	GGT GTA ACT CCT TCT GAA GCG	Antitoxin of the RelE-RelB TA system in *Escherichia coli* K-12		1643827-1643807			
Detection of bacteriocins								
221	EntA-F	GTA CCA CTC ATA GTG GA	Enterocin A in *E. faecium* CTC492	X94181	160-176	451 bp	1 cycle 10'-94ºC; 35 cycles 30''-94ºC, 30''-50ºC, 30''-72ºC; 1 cycle 10'-72ºC	(398)
222	EntA-R	GAG ATT TAT CTC CAT AAT CT	Enterocin A immunity in *E. faecium* CTC492		610-591			
223	EntB-F	GAA AAT GAT CAC AGA ATGCCTA	Enterocin B in *E. faecium* T136	EFU87997	146-167	159 bp	Identical with annealing at 56ºC	(398)
224	EntB-R	GTT GCA TTT AGA GTA TACATTTG			304-282			
225	EntP-F	ATG AGA AAA AAA TTA TTTAGTTT	Enterocin P precursor in *E. faecium* P13	AF005726	173-195	216 bp	Identical with annealing at 50ºC	(398)
226	EntP-R	TTA ATG TCC CAT ACC TGC CAA ACC			388-365			
227	EntQ-F	AGG GCT ACT TGG ATA GTA CAC	Enterocin Q in pCIZ2 from *E. faecium*	NC_008259	45-66	678 bp	Identical with annealing at 52ºC	(81)
228	EntQ-R	AAC AAG AAA ATT GCG GCT G	Enterocin EntQ ABC-transporter in pCIZ2 from *E. faecium*		722-704			
229	EntL50A/B-F	TCC CTA CAG TCT CCC TTCC	Enterocin L50A and L50B in pCIZ1 from *E. faecium*	AJ223633	877-895 / 1753-1735	877 bp	Identical with annealing at 52ºC	(81)
230	EntL50A/B-R	TCT AGC GTT AAG CCG AAT G	Enterocin I (entI) and Enterocin J (entJ) in pEF1 from *E. faecium*	Q198088	1331-1349 / 2206-2188			
231	Bac32-F	AAT GGT GGA GTG GTT GAA GC	Bacteriocin and immunity proteins in p200B from *E. faecium*	AB205024	162-181	462 bp	Identical with annealing at 56ºC	This study
232	Bac32-R	GAG TGA TTA TTT TCG CCC GT			623-604			
233	Bac43-F	CGA AAA GG AAA AAC AAT CATG	*bacA* and *bacB* in pB82 from *E. faecium*	AB178871	104-124	576 bp	Identical with annealing at 50ºC	(417)
234	Bac43-R	TCC CAT TTT CAT TTT ATT CC			679-660			

Abbreviations: F, forward, R, reverse; P.C., personal communication.

6.2. Reagents, Solutions and Equipment

6.2.1. Reagents

a) Box 1. PCR reagents

- T*aq* DNA polymerase - GoTaq® Flexi DNA polymerase supplying reaction buffer and $MgCl_2$ (*Promega, Madison, WI USA*)
- LA *Taq* polymerase for long PCRs (supplying dNTPs, reaction buffer and $MgCl_2$) (*Takara Bio Inc., Shiga, Japan*)
- Deoxynucleoside triphosphates (dNTPs) stock solution – equimolar amounts of dATP, dTTP, dCTP, and dGTP (*PCR Nucleotide Mix, Promega, Madison, WI USA*)
- Oligonucleotide primers – acquired from *Sigma-Aldrich, UK* or *STAB VIDA, Investigação e Serviços em Ciências Biológicas, Lda. Portugal*
- Bovine serum albumin solution (BSA) - *Sigma-Aldrich, St Louis, MO USA*
- Agarose (*SeaKem® LE Agarose, Lonza, Rockland, ME USA*) – high purified polysaccharide derived from agar used in electric continuous-field agarose gel electrophoresis

b) Box 2. Restriction enzymes

For genomic DNA digestion in situ:

- ***Sma*I** – supplied with 10X NEBuffer 4; optimal activity at 25ºC *(New England Biolabs Inc.*)
- **I-*Ceu* I** - supplied with 10X NEBuffer 4 and 100X BSA; optimal activity at 37ºC *(New England Biolabs Inc.*)
- **S1 nuclease** – supplied with 10X S1 nuclease buffer; optimal activity at 37ºC *(Takara Bio Inc., Shiga, Japan*)

For RFLP analysis of long PCR products or plasmids:

- ***Eco*RI** – supplied with 10X Buffer H and BSA; optimal activity at 37ºC (*Promega, Madison, WI, USA*)
- ***Cla*I** – supplied with 10X Buffer C and BSA; optimal activity at 37ºC (*Promega, Madison, WI, USA*)
- ***Hind*III** – supplied with 10X Buffer M; optimal activity at 37ºC (*Promega, Madison, WI, USA*)
- ***Dra*I** – supplied with 10X NEBuffer 4; optimal activity at 37ºC *(New England Biolabs Inc.*)
- ***Bsp*HI** – supplied with 10X NEBuffer 4; optimal activity at 37ºC *(New England Biolabs Inc.*)
- **FastDigest™ *Eco*RI** – supplied with 10X FastDigest™ Buffer; optimal activity at 37ºC and efficient DNA digestion in only 5 minutes (*Fermentas Life Sciences*)

c) Box 3. DNA molecular weight markers

Used in electric continuous-field agarose gel electrophoresis (visualization of PCR products or digested plasmids)

- 100 bp DNA ladder (0.1-1.5kb) (*Promega, Madison, WI USA*)
- 250 bp ladder (0.25-5kb) (*Invitrogen, Carlsbad, CA USA*)
- λ DNA/Hind III (0.125-23.13kb) (*Promega, Madison, WI USA*)
- λ-EcoT14 I/Bgl II digest (0.06-22.01kb) (*Takara Bio Inc., Shiga, Japan*)

Used in Pulsed-field gel electrophoresis

- *λ-ladder PFG marker* (48.5kb to 1,018.5kb) (*New England Biolabs, Inc., Ipswich, USA*)
- *Low Range PFG marker* (0.13kb to 194kb) (*New England Biolabs, Inc., Ipswich, USA*)

6.2.2. Solutions

a) Box 4. Solutions for DNA extraction

- **Lysozyme** from chicken egg white. Lysozyme solution is prepared by dissolving the powder in Tris-HCl 10mM pH=8.0 at the final concentration required.
- **SDS**: 20% (100ml) – Dissolve 20g in 70ml of ultra-pure water, complete the volume of 100ml and sterilize with filter.
- **NaOH**: 10N (100ml) – Dissolve 40g of NaOH in ultra-pure water and make up to 100ml.
- **Tris-HCl**: 1M, pH=7.6 (1L) – Dissolve 121.1g of Tris base in 800ml of ultra-pure water adjust the pH=7.6 with concentrated HCl (~60ml), complete the volume of 1L and sterilize.
- **EDTA**: 0.5M, pH=8 (1L) – Dissolve 186.1g of $Na_2EDTA.2H_2O$ in 800ml of ultra-pure water (faster in a hot water bath), adjust the pH=8.0 with NaOH (~20g), complete the volume of 1L and sterilize.
- **NaCl**: 5M (1L) – Dissolve 292.2g of NaCl in 800ml of ultra-pure water (faster in a hot water bath), make up to 1L and autoclave.
- **PIV buffer** (1 M NaCl, 10 mM Tris-HCl, pH=7.6) – This solution is prepared from the stock solutions of NaCl 5M and Tris-HCl 1M. For 100ml add 20ml NaCl 5M, and 1ml Tris-HCl 1M to 79ml of ultra-pure water. Autoclave before use.
- **Brij58** 10% (Polyoxyethylene 20 cetyl ether) – Dissolve 10g in 100ml of ultra-pure water and autoclave.
- **Deoxycolate** 10% - Dissolve 10g of deoxycolate in 100ml of ultra-pure water.
- **Sarkosyl** 20% (10ml) - Dissolve 2g in 10ml of ultra-pure water.
- **Proteinase K** solution (25mg/ml) – A stock solution is prepared by dissolving 25mg of proteinase K in 1ml of a sterile Tris-HCl/$CaCl_2$ (50mM Tris-HCl, 100mM $CaCl_2$) solution. Store small aliquots at -20ºC.
- **Ribonuclease** (RNase) solution (10mg/ml) – A stock solution is prepared by dissolving 10mg of RNAse in 1ml of sterile ultra-pure water. This solution is then boiled for 10-30 minutes to eliminate any DNAse activity and after cooling can be stably stored at -20ºC during some months.
- **ESP** solution (0.5 M EDTA pH=9, 1% sarkosyl, 50 µg/ml proteinase K) – Mix 100ml EDTA 0.5M, 5ml sarkosyl 20% and 200µl proteinase K 25mg/ml (in ice).

Box 4 (*cont.*)

- **TE 1X** (10 mM Tris-HCl pH=7.6, 1 mM EDTA pH=8.0) – This solution is prepared from the stocks solutions of Tris-HCl 1M and EDTA 0.5M. For 1L add 10ml Tris-HCl 1M and 2ml EDTA 0.5M to 988ml of ultra-pure water. Autoclave.
- **TEG** (50 mM Tris-HCl pH=8.0, 10 mM EDTA, 2% glucose) (20ml) – Dissolve 0.4g of glucose in 1ml Tris-HCl 1M, 0.4ml EDTA 0.5M and 18.6ml of ultra-pure water.
- **Potassium acetate** solution 3M, pH=5 (100ml) – Dissolve 29.44g acetate potassium (PM=98.15g/mol) in 60ml of ultra-pure water, adjust the pH=5 with acetic acid glacial and complete the volume of 100ml with water. Autoclave for stocking.

b) Box 5. Solution for PCR, DNA separation and detection

- TAE 1X buffer (40 mM Tris-Acetate, 1mM EDTA, pH=8.0). Stock of TAE 50X buffer achieved by *Bio-Rad Laboratories GmbH, München*.
- TBE 0.5X buffer (45 mM Tris-Borate, 1mM EDTA).
  Stock of TBE 10X extended range buffer achieved by *Bio-Rad Laboratories GmbH, München*.
  For manual preparation (1L): 108g Tris base, 55 g boric acid and 40ml EDTA 0.5M pH=8.0, make up to 1L with ultra-pure water and autoclave.
- Tracking dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in distilled water.
- Ethidium bromide (EtBr) solution (stock=1% solution in water) – The ethidium bromide is prepared in TBE 0.5X or water at a final concentration of 0.5 µg/ml (for conventional agarose gels) or 1 µg/ml (for PFGE gels). Nitrile gloves must be always worn for adequate protection of this strongly mutagenic agent and the contaminated gels discard in special waste.

c) Box 6. Solutions for hybridization

- **NaOH**: 0.4N (1L) – Dissolve 16g of NaOH in 900ml of ultra-pure water and complete the volume of 1L. Do not sterilize.
- **Tris-HCl**: 0.5M pH=7 (1L) – Dissolve 60.55g of Tris base in 800ml of ultra-pure water, adjust the pH of 7.0 with HCl and complete the volume of 1L. To sterilize for stock.
- **20X SSC** [3M NaCl, 0.3M $Na_3(C_3H_5O(COO)_3)$] (1L) – Dissolve 175.31g NaCl and 88.23g $Na_3(C_3H_5O(COO)_3)$ in 800ml of ultra-pure water and make up to 1L. Sterilize as a stocking solution and dilute 1:10 for 2X SSC.
- **$NaH_2PO_4.H_2O$**: 0.5M pH=7 (100ml) – Dissolve 6.9g in 80ml of ultra-pure water, adjust the pH=7 with NaOH pellets, complete the volume of 100ml and sterilize for stock.
- **$MgCl_2$**: 1M (25ml) – Dissolve 5.08g of $MgCl_2$ in ultra-pure water, complete the volume and sterilize.
- **Hybridization buffer** [NaCl 0.5M + blocking reagent 4% (W/V)] (100ml) – 2.922g of NaCl and 4g of blocking reagent (supplied with the kit) are mixed with 100ml of hybridization buffer (supplied with the kit) and the solution is immediately placed in vigorous agitation for 2 hours at room temperature.
- **Primary wash buffer** (1L) – 120g of urea; 8.7g NaCl; 100ml of $NaH_2PO_4.H_2O$: 0.5M pH=7; 1ml $MgCl_2$: 1M; and 2g blocking reagent are dissolved in 700ml of ultra-pure water (in a boiling water bath and agitation); 1g SDS (Sodium *n*-Dodecyl Sulfate) is added and the volume of 1L is complete with water. This solution can be kept at 4ºC for one week.
- **Secondary wash buffer** (1L) – Dissolve 121g Tris base and 112g of NaCl in 800ml of ultra-pure water, adjust to the pH=10 and complete the volume of 1L. After sterilization, this stock solution can be stored at 4ºC for 4 months. The working solution must be prepared when needed by diluting the stock solution 20X in water and adding 2mL of $MgCl_2$ (for 1L).
- **SDS**: 0.1% (1L) – Dissolve 1g of SDS in 1L of distilled water and carefully warm the solution until boiling for striping.

6.2.3. Box 7. Equipment

- Termocyclers *Bio-Rad Laboratories*: Perkin Elmer Gene Amp 2400 and BioRad iCycler
- CHEF-DRIII – PFGE apparatus (*Bio-Rad Laboratories, La Jolla, CA, USA*)
- Camera system – *Gel Doc™ XR+* (*Bio-Rad Laboratories, Inc., USA*)
- Fingerprinting II Informatix software package (*Bio-Rad Laboratories, Hercules, CA, USA*)
- Wizard® SV Gel and PCR Clean-Up System (*Promega, Madison, WI, USA*)
- Gene Images™ AlkPhos Direct™ labeling and detection system kit - *Amersham GB, GE Healthcare Life Sciences UK Limited* (http://www.gehealthcare.com/lifesciences)
- CDP-Star™ reagent - *Amersham GB, GE Healthcare Life Sciences UK Limited*
- Hybridization oven (*BINDER®, Tuttlingen, Germany*)
- Autoradiography films – Hyperfilm™ ECL (*GE Healthcare Life Sciences UK*)
- Film cassettes – Hypercassette™ 18X24 cm (*GE Healthcare Life Sciences UK*)
- Nylon transfer membrane – Hybond™ N+ (*GE Healthcare Life Sciences UK*)

6.3. Protocols

Plasmid protocols *Protocol 1*

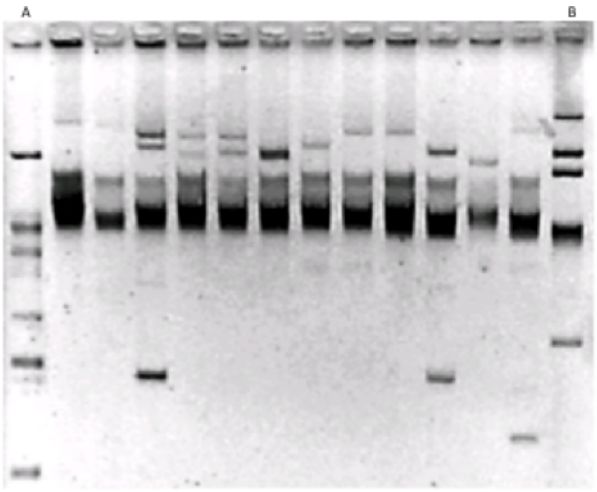
Plasmid extraction: Kado & Liu

(Kado and Liu. J Bacteriol 1981. 145:1365-1373)

***Purpose**: Obtain plasmid DNA for typing*

1. Fill an eppendorf tube with 150µl of lysozyme solution* **(**25% sacarose and 10mg/ml of lysozyme in TE 1X)
2. Pick up a colony of fresh cell culture (the size of a toothpick flat end). Apply to the side of the tube above lysozyme solution and spread out.
3. Incubate in a water bath at 37ºC for 60 minutes.
4. Add 150µl of lysis solution**. Invert carefully but thoroughly 4-5 times and incubate at 60ºC for 60 minutes.
5. Add 300µl phenol:chloroform 1:1. Invert 3 or 4 times.
6. Centrifuge for 5 minutes at maximum speed.
7. Take 35-40µl of upper layer and add 7µl tracking dye.
8. Run the samples in a 0.8% agarose gel at 120V for 3 hours with appropriate controls of plasmid size. *Escherichia coli* strains V517 (NCTC 50193; 8 plasmids of 2.1, 2.7, 3.0, 3.9, 5.1, 5.5, 7.2 and 53.4 kb) and 39R861 (NCTC 50192; 4 plasmids of 7, 36.2, 63.8 and 148.5 kb) were used as controls of plasmid size.

*Lysozyme solution (for 2ml): 0.02g lysozyme, 0.5g sucrose, 2ml TE1X
**Lysing solution (for 100ml): 3g SDS, 0.6g Tris base, 1.6ml NaOH 2N and complete the volume with water.



Notes:

This protocol has been used only to assess the plasmid content accurately since very low molecular weight plasmids are not always visible using the S1 method.

Drawbacks: frequent chromosomal contamination, obtention of different plasmid conformations, impossibility to detect plasmid size and plasmid number accurately.

Cell lysis incubation using SDS and NaOH should not be prolonged.

Do not vortex at any step of the procedure!

Figure 3. Gel electrophoresis of plasmid DNA from VRE isolates obtained by Kado & Liu (A. *E. coli* V517; B. *E. coli* NCTC).

Plasmid protocols *Protocol 2*

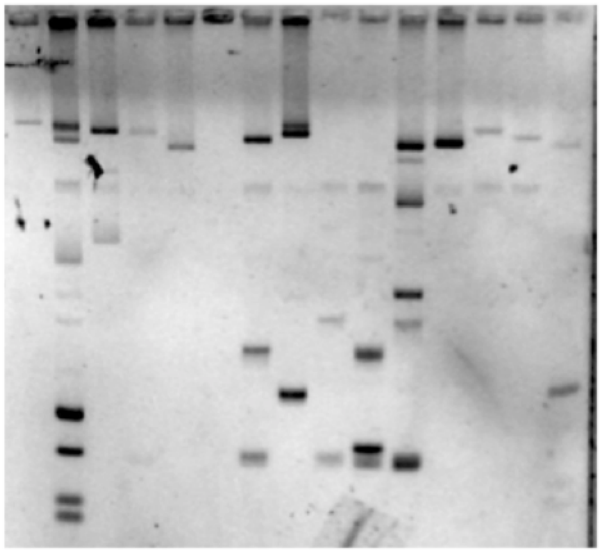
Plasmid extraction

(protocol modified from Handwerger *et al.* Antimicrob Agents Chemother 1995. 39:362-368)

***Purpose**: Obtain plasmid DNA for typing*

1. Inoculate a bacterial fresh colony in 5 ml of BHI broth supplemented with antibiotic. To incubate in a water bath at 37ºC with gentle shaking overnight.
2. Harvest the culture and wash the pellet with 100 µl of TEG*. Transfer to a 1.5ml eppendorf tube and centrifuge for 5 minutes.
3. Dissolve the pellet in 200µl of TEG containing 50 mg/ml of lysozyme. Incubate for 30 minutes at 37ºC.
4. Add 400µl of freshly prepared lysis solution** and mix carefully inverting the tube 4-6 times (no vortex!!).
5. Add 300µl of potassium acetate (3M, pH 5), invert 2-3 times (carefully to avoid genomic DNA breaking and contamination) and chill at -20ºC for at least 30 min (*it results better for 2-3 hours*).
6. Centrifuge for 15 minutes at maximum speed and extract the supernatant with the same volume of phenol-chloroform-isoamylic acid (25:24:1).
7. Centrifuge for 5 minutes and extract the supernatant again with chloroform.
8. After centrifugation, the supernatant is precipitated with 2 volumes of etanol added of 1/10 potassium acetate, for at least 30 minutes (*it results better for 1-2 hours*).
9. Centrifuge for 15 minutes and wash the pellet 3 times with etanol 70% (*the pellet is visible and white*). 10. Dry the pellet and resuspend in 30µl of water. Store at 4ºC (the pellet must dissolve overnight prior to enzyme digestion).

*TEG solution (Tris-EDTA-glucose): 50 mM Tris-HCl pH 8.0 + 10 mM EDTA + 2% glucose
**Lysis solution: 1%SDS, 0.2N NaOH



Notes:

We have extensively used different plasmid extraction protocols for enterococci including that by Neil Woodford et al. (473). Neil´s protocol works pretty well but in our hands, this from Sandra Handwerger is the best since yield less chromosomal contamination. It was slightly modified it with good results.

Optimal lysozyme concentration for efficient enterococcal cell wall lysis seems to be 10mg/ml but we obtained better results increasing up to 50mg/mL for 30 minutes.

Cell lysis incubation using SDS and NaOH should not be prolonged over 5 minutes.

Do not vortex at any step of the procedure!

Figure 4. Gel electrophoresis of plasmid DNA from VRE isolates obtained by a modification of the *Birnboim and Doly* method.

Plasmid protocols *Protocol 3*

Plasmid extraction: Qiagen

(QIAprep MidiPrep kit, QIAGEN; modified from Roberts *et al.* J Bacteriol 2006. 188:4356-4361)

***Purpose**: Obtain plasmid DNA for typing*

1. Pick a single colony from a fresh selective plate and incubate a starter culture of 10 ml of BHI broth supplemented with antibiotic. Incubate for approximately 8 hours at 37ºC with vigorous shaking.
2. Dilute 300µl of the starter culture into 100ml of pre-warmed BHI broth supplemented with antibiotics and incubate for 16h with vigorous shaking.
3. Harvest the bacterial cells by centrifugation at 1,500 x g at 4ºC for 10 minutes and resuspended in 8ml of P1 buffer – *is important to achieve a pellet homogenization, but avoid to vortex!*
4. Add 800µl of lyzozyme solution (10-20mg/ml) and incubate for one hour at 37ºC.
5. The manufacturer's instructions were followed from this point forward (available in http://www.qiagen.com/literature).
6. The final DNA pellet is difficult to dissolve in water (prewarm the tube).

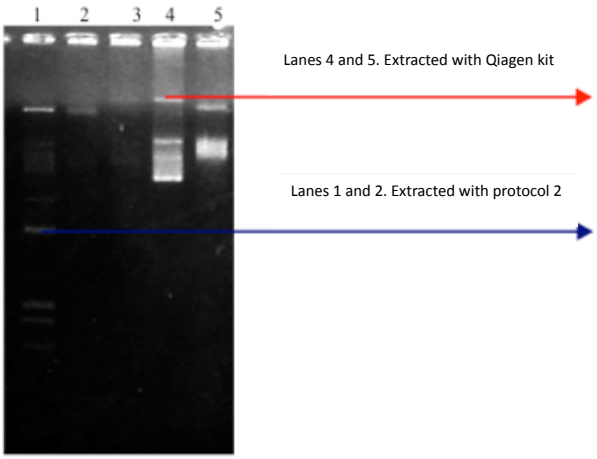


Figure 5. Gel electrophoresis of plasmid DNA from VRE isolates extracted with different methods (greater chromosomal contamination with Qiagen kit)

Notes:

We have tried different approaches using commercial kits for extraction of enterococcal plasmids and this works well with the modifications made. However, we still prefer protocol 2 because the chromosomal contamination is much lower.

Lysozyme step is crucial and it must be added predissolved in Tris-HCl pH=8.0 solution (it doesn't work well at lower pH values). We obtained better results using 20mg/ml rather than 10mg/ml. In the kit and other references, mutanolysin besides lyzozyme is added but we have never tried it.

Do not vortex at any step of the procedure!

The final step of DNA pellet dissolution is tricky because isopropanol pellets are better dissolved using alkaline conditions. TE buffer or Tris-HCl solutions should be avoided because they might interfere with restriction enzyme digestions and/or sequencing procedures.
High quality water is the best option but also the hardest – we use prewarmed water and let the DNA dissolve for 2 days.

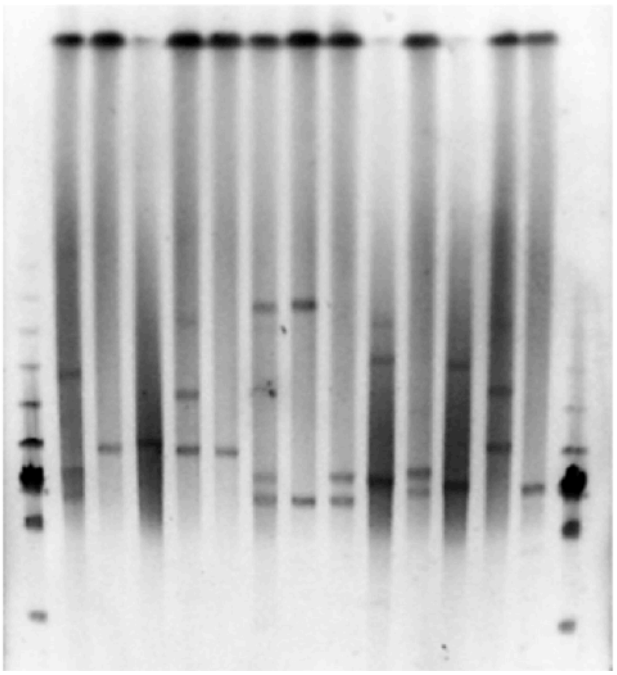
Plasmid analysis: S1-PFGE

(adapted from Barton *et al*. Anal Biochem 1995. 226:235-240)

***Purpose**: Determination of plasmid size and plasmid number*

1. DNA preparation. Any DNA standard protocol works fine. For enterococci we used a modification of the classical Cold Spring Harbor protocol.
2. To wash / equilibrate the plugs with Tris-HCl 10 mM, twice for 15 minutes.
3. DNA digestion. To digest DNA with S1 nuclease (*Takara Bio Inc., Shiga, Japan*): 10µl buffer 10X + 90µl water + S1 (14U) and incubate the reaction at 37ºC criteriously for 15 minutes.
4. To replace the solution for TE 1x for at least 1 hour.
5. PFGE run in a Chef-DR III device: Run in a 1.2% Seakem Gold agarose gel using with the following settings: switch time of 5-25 s for 6h, followed by 30-45 s for 18h.

λ-ladder PFG and *Low Range PFG* linear DNA weight markers (see section 6.2.1.) were used to estimate plasmid sizes.



Notes:

Conditions. Digestion time and amount of enzyme can slightly vary for every bacterial species or among strains of the same species. We adjust digestion time if we obtained a strong background for a particular strain.

Enzyme. This is critical for us. Takara is the best option as well as the buffer they provide.

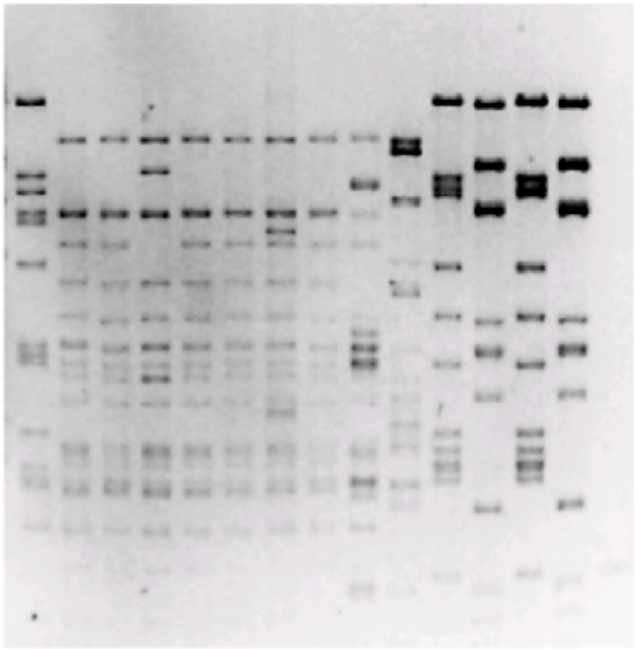
Figure 6. S1-PFGE of VRE isolates.

Plasmid fingerprinting (RFLP)

***Purpose**: To assess epidemiological relationships between different plasmids*

1. Enzymatic digestion. Make the reaction mixture (see table above) and distribute to each tube. Add the correspondent plasmid DNA (extracted with protocol 2).
2. Incubate in a water bath at 37ºC overnight (16h).
3. When possible, run the DNA in a 0.8-1% electrophoresis gel in TBE 0.5X. Otherwise store at 4ºC.

Reagent	**[Stock]**	**Volume** (20µl reaction)
DNA	____	10 µl
Enzyme	12 U/µl	2 µl
Buffer	10x	2 µl
BSA	10 mg/ml	0.2 µl
Water	____	5.8 µl



Notes:

For digestion of enterococcal plasmids we used the enzymes EcoRI, ClaI and HindIII.

ClaI revealed to be the more discriminatory and powerful enzyme to analyze vancomycin-resistant plasmids (more than 15 bands could be generated after plasmid restriction) and a good option to assess relationships between different plasmids.

Figure 7. Gel electrophoresis of *Eco*RI-digested *vanA* plasmids obtained from VRE isolates.

6.4. Sequence submitted to GenBank

6.4.1. ***Enterococcus faecium*** **strain Ef80 plasmid putative glycoside hydrolase (*hyl*$_{Efm}$) gene, complete CDS**. Authors: Freitas A.R., Tedim A.P., Novais C., Ruiz-Garbajosa P., Werner G., Laverde Gomez J.A., Canton R., Peixe L., Baquero F., and Coque T.M. Title: Global spread of the *hyl*$_{Efm}$ colonization-virulence gene in megaplasmids of the *Enterococcus faecium* CC17 polyclonal subcluster. GenBank accession number HM043740 (1673 bp).

6.4.2. ***Enterococcus faecium*** **strain E1438 plasmid putative glycoside hydrolase (*hyl*$_{Efm}$) gene, complete CDS**. Authors: Freitas A.R., Tedim A.P., Novais C., Ruiz-Garbajosa P., Werner G., Laverde Gomez J.A., Canton R., Peixe L., Baquero F., and Coque T.M. Title: Global spread of the *hyl*$_{Efm}$ colonization-virulence gene in megaplasmids of the *Enterococcus faecium* CC17 polyclonal subcluster. GenBank accession number HM043741 (1673 bp).

6.4.3. ***Enterococcus faecium*** **strain Efm42 plasmid putative glycoside hydrolase (*hyl*$_{Efm}$) gene, complete CDS**. Authors: Freitas A.R., Tedim A.P., Novais C., Ruiz-Garbajosa P., Werner G., Laverde Gomez J.A., Canton R., Peixe L., Baquero F., and Coque T.M. Title: Global spread of the *hyl*$_{Efm}$ colonization-virulence gene in megaplasmids of the *Enterococcus faecium* CC17 polyclonal subcluster. GenBank accession number HM043742 (1673 bp).

6.4.4. ***Enterococcus faecium*** **strain Efm49 plasmid putative glycoside hydrolase (*hyl*$_{Efm}$) gene, complete CDS**. Authors: Freitas A.R., Tedim A.P., Novais C., Ruiz-Garbajosa P., Werner G., Laverde Gomez J.A., Canton R., Peixe L., Baquero F., and Coque T.M. Title: Global spread of the *hyl*$_{Efm}$ colonization-virulence gene in megaplasmids of the *Enterococcus faecium* CC17 polyclonal subcluster. GenBank accession number HM043743 (1673 bp).

6.4.5. ***Enterococcus faecium*** **strain 15/02 plasmid putative glycoside hydrolase (*hyl*$_{Efm}$) gene, complete CDS**. Authors: Freitas A.R., Tedim A.P., Novais C., Ruiz-Garbajosa P., Werner G., Laverde Gomez J.A., Canton R., Peixe L., Baquero F., and Coque T.M. Title: Global spread of the *hyl*$_{Efm}$ colonization-virulence gene in megaplasmids of the *Enterococcus faecium* CC17 polyclonal subcluster. GenBank accession number HM043744 (1673 bp).

6.4.6. ***Enterococcus faecium*** **strain 64/3xUW2774 plasmid pLG1**. Authors: Laverde Gomez J.A., van Schaik W., Freitas A.R., Coque T.M., Weaver K.E., Francia, M.V., Witte W. and Werner G. Title: A multiresistance megaplasmid pLG1 bearing a *hyl*$_{Efm}$ genomic island in hospital *Enterococcus faecium* isolates. GenBank accession numbers HM565168–HM565236 (281,02 kb).