



Maria Eduarda Moreno da Silveira

**TOLERÂNCIA A BIOCIDAS E DISPERSÃO DA RESISTÊNCIA A
ANTIBIÓTICOS: UMA ABORDAGEM FENOTÍPICA E MOLECULAR
EM *Enterococcus* spp DE DIFERENTES ORIGENS**

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

Trabalho realizado sob orientação da Professora Doutora Carla Novais (Faculdade de Farmácia, Universidade de Porto, Portugal) e co-orientação da Professora Doutora Luísa Vieira Peixe (Faculdade de Farmácia, Universidade de Porto, Portugal) e Doutora Teresa M. Coque (Servicio de Microbiología, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Espanha).

Janeiro 2014

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

TOLERÂNCIA A BIOCIDAS E DISPERSÃO DA RESISTÊNCIA A ANTIBIÓTICOS: UMA ABORDAGEM FENOTÍPICA E MOLECULAR EM *Enterococcus* spp DE DIFERENTES ORIGENS

Este trabalho foi financiado por:

Fundação para a Ciência e Tecnologia, Ministério da Ciência, Tecnologia e Ensino Superior em Portugal (Bolsa SFRH / BD / 63955 / 2009, QREN - POPH - Tipologia 4.1 - Formação Avançada, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MEC); Projetos PTDC/AAC-AMB/103386/2008) e PEst-C/EQB/LA0006/2011.



Ao meu filho

AGRADECIMENTOS

Agradeço à Professora Doutora Carla Novais, Professora Doutora Luísa Peixe e Doutora Teresa Coque, minhas orientadoras, a oportunidade que me concederam para desenvolver o meu conhecimento científico e, implicitamente, o pessoal. Serei reconhecida o bastante para que jamais menospreze estes quatro anos de aquisição, amadurecimento e sedimentação de saber. Expresso o meu reconhecimento à orientação, dedicação, disponibilidade e entrega em todos os momentos mais prementes ocorridos durante este percurso. Quero ainda deixar, indelevelmente, expresso que sem a dedicação e entrega da Professora Doutora Carla Novais não seria possível concretizar a entrega desta tese. Agradeço à Professora Doutora Luísa Peixe por me ter concedido o privilégio de integrar a equipa que coordena. Muito obrigada.

Agradeço à Professora Doutora São José Nascimento por me ter permitido desenvolver e realizar este trabalho no Laboratório de Microbiologia da Faculdade de Farmácia da Universidade do Porto.

Ao REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto agradeço o ter-me proporcionado as condições necessárias para a realização e desenvolvimento deste trabalho.

À Fundação para a Ciência e Tecnologia, agradeço o apoio financeiro concedido ao longo destes quatro anos.

À Professora Doutora Patrícia Antunes agradeço a colaboração, ajuda e incentivo em alguns momentos menos fáceis, assim como a sua disponibilidade e dedicação nesta fase final e decisiva.

À Doutora Ana Freitas, companheira de laboratório no mundo dos *Enterococcus*, nesta minha estreia passagem no mundo da ciência, agradeço o seu contributo na transmissão de conhecimento, a sua disponibilidade e ajuda durante este percurso e, em particular, nesta etapa final e decisiva.

Agradeço à Cristina Pinto da Costa e ao Nuno Oliveira toda a colaboração, disponibilidade e carinho demonstrados ao longo deste período, sem esquecer nunca as suas palavras de afago.

À Doutora Filipa Grosso e Doutora Ângela Novais o meu muito obrigado pela vossa ajuda, partilha de conhecimento, dedicação e palavras de ânimo, que tanto me incentivaram nos momentos mais difíceis.

À Joana Mourão, Teresa Gonçalves, Carla Rodrigues e João Pires expresso a minha gratidão pelo apoio nesta fase crucial da minha vida, não podendo deixar de recordar a vossa alegria contagiante, risos cúmplices, carinho e predisposição de entre ajuda.

À Mestre Raquel Branquinho, amiga e colega de laboratório, companheira deste percurso académico, que comigo partilhou gargalhadas e angústias, ilusões e certezas e que velou, como se, presencialmente, ao meu lado estivesse, nestes últimos dias que antecederam a entrega desta tese. Um imenso bem-haja para ti.

À Joana Campos, Liliana Silva, João Botelho um muito obrigada pela companhia e carinho.

À Mariana Barros e Patrice Marques agradeço todo o apoio laboratorial, dedicação e afecto.

A todos os amigos, que renunciei, provisoriamente, por força das circunstâncias e que compreenderam o meu silêncio, o meu muito obrigada.

Por fim, agradeço à minha mãe e irmã todo o amor, preocupação, palavras de ânimo e acima de tudo o terem acreditado em mim há cerca de dez anos atrás – jamais o apagarei da minha memória.

Ao António, meu filho e grande amigo, a quem dedico este trabalho, a minha gratidão profunda pelo seu altruísmo, apoio incondicional, pela sua forma doce de me olhar, mesmo quando espelhava solidão – isto é, simplesmente, amor.

Qualquer desempenho, mesmo em ciência, desde que realizado com humildade, dedicação e probidade, permite-nos, a cada dia que passa, apreender um pouco mais do imenso que desconhecemos – é apenas uma filosofia de vida.

“Transeuntes eternos por nós mesmos, não há paisagem senão o que somos. Nada possuímos, porque nem a nós possuímos. Nada temos porque nada somos. Que mão estenderei para que universo? O universo não é meu: sou eu.”

Fernando Pessoa, *in* Livro do Desassossego.

ÍNDICE

CAPÍTULO 1 – INTRODUÇÃO GERAL	1
1. Género <i>Enterococcus</i>	2
1.1. Taxonomia e perspetiva	2
1.2. Características fisiológicas	3
1.3. Estrutura populacional	4
1.3.1. Estrutura populacional de <i>Enterococcus faecium</i>	5
1.3.2. Estrutura populacional de <i>Enterococcus faecalis</i>	7
1.4. Resistência a antibióticos: mecanismos e epidemiologia	7
1.4.1. Resistência intrínseca	8
1.4.2. Resistência adquirida	9
1.4.3. Epidemiologia da resistência aos antibióticos: contexto global e de Portugal	12
1.5. Elementos genéticos móveis em <i>Enterococcus</i> spp	16
1.5.1. Elementos de transposição	17
1.5.1.1. Sequências de Inserção	17
1.5.1.2. Transposões compostos	18
1.5.1.3. Derivados do Tn3	18
1.5.1.4. Transposões conjugativos ou elementos de integração cromossomal	19

1.5.2 Plasmídeos	20
1.5.2.1. Plasmídeos RCR	21
1.5.2.2. Plasmídeos Teta	22
a) Plasmídeos Teta de pequeno tamanho	22
b) Família Inc18	23
c) Família RepA_N	23
d) Plasmídeos da família pHT β /pMG1	25
e) Megaplasmídeos	25
1.6. Biocidas	26
1.6.1. Perspetiva histórica	26
1.6.2. Mecanismos de ação	28
1.6.3. Tolerância bacteriana aos biocidas e sua relação com a resistência a antibióticos	31
1.6.4. Cobre, mercúrio, e compostos de amónio quaternário	33
1.6.4.1. Metais pesados: cobre e mercúrio	33
1.6.4.1.1. Cobre	33
1.6.4.1.2. Mercúrio	38
1.6.4.1.3. Compostos de amónio quaternário	40
1.7. Referências Bibliográficas	43

CAPÍTULO 2 – OBJETIVOS E LISTA DE TRABALHOS	89
CAPÍTULO 3 – RESULTADOS E DISCUSSÃO	97
3.1. Resistência a antibióticos no ambiente de produção animal: determinantes genéticos e linhagens clonais	99
3.1.1. Spread of multidrug-resistant <i>Enterococcus</i> to animals and humans: an underestimated role for the pig farm environment	99
3.2. Dispersão de genes associados a tolerância a metais em <i>Enterococcus</i> spp. resistentes a antibióticos de diferentes comunidades ecológicas e linhagens clonais	113
3.2.1. Co-transfer of resistance to high concentrations of copper and first-line antibiotics among <i>Enterococcus</i> from different origins (humans, animals, the environment and foods) and clonal lineages	113
3.2.2. <i>Enterococcus</i> spp. from different origins and clonal lineages carry two <i>merA</i> genes found in several <i>Firmicutes</i> members and are co-transferred with antibiotic resistance and copper tolerance	113
3.3. Modularização de plataformas genéticas móveis portadoras de genes que codificam para tolerância a metais, compostos de amônio quaternário e/ou resistência a antibióticos	147
3.3.1. Antibiotic resistance, copper and mercury tolerance genes are carried by RepA_N pLG1-like megaplasms among <i>Enterococcus</i> from several origins and clones	147
3.3.2. First description of a multidrug-resistant ST17 <i>Enterococcus faecium</i> carrying a transferable Inc18-like::qacZ plasmid	147
3.3.3. Different genetic supports for the <i>tet(S)</i> gene in Enterococci	147
CAPÍTULO 4 – CONCLUSÕES GERAIS	189
ANEXOS	197

ÍNDICE DE FIGURAS

CAPÍTULO 1 – INTRODUÇÃO GERAL	1
1.1. Figura 1. Posicionamento filogenético do género <i>Enterococcus</i> baseado na sequenciação do 16S rRNA aplicada a diferentes géneros de bactérias de Gram positivo	3
1.4.3. Figura 2. Percentagem de <i>E. faecium</i> invasivos resistentes à vancomicina nos países Europeus, dados referentes ao ano de 2012	14
1.4.3. Figura 3. Vias de disseminação de bactérias resistentes a antibióticos e respetivos elementos genéticos entre diferentes nichos	16
1.6.2. Figura 4. Esquematização dos mecanismos de toxicidade dos metais	30
1.6.4.1.1. Figura 5. Mecanismo de homeostase do cobre em <i>Enterococcus hirae</i>	37
CAPÍTULO 3 – RESULTADOS E DISCUSSÃO	97
3.2.1. Figura 1. Distribution of <i>tcrB</i> and <i>cueO</i> genes among the <i>Enterococcus</i> spp. from different sources	118
3.2.1. Figura 2. Distribution of antibiotic resistance among <i>Enterococcus</i> with (n¼164) and without (n¼758) <i>tcrB</i> and/or <i>cueO</i>	119
3.2.2. Figura 1. Distribution of antibiotic resistance among <i>Enterococcus</i> with <i>merA2</i> (n=29), <i>merA3</i> (n=19) and without <i>merA</i> genes (n=871)	145
3.3.1. Figura 1. Schematic representation of <i>tcrB</i> , <i>cueO</i> and <i>merA2</i> genetic environment	168
3.3.1. Figura 2. Schematic representation of <i>merA3</i> genetic environment	169

3.3.2. Figura 1. Primers used to search diverse genes coding for quaternary ammonium compounds tolerance and for the characterization of <i>qacZ</i> genetic environment	179
3.3.2. Figura 2. Hybridization assays of S1 and <i>ICeu-I</i> digested DNA of the <i>qacZ</i> carrying <i>E. faecium</i>	180
3.3.3. Figura 1. Diversity of complete and partial CTn6000 backbones and other genetic platforms carrying <i>tet(S)</i>	186

ÍNDICE DE TABELAS

CAPÍTULO 1 – INTRODUÇÃO GERAL	1
1.6.2. Tabela 1. Local de ação de diferentes grupos de biocidas	28
CAPÍTULO 3 – RESULTADOS E DISCUSSÃO	97
3.1.1. Tabela 1. Resistance to 12 antibiotics of <i>E. faecium</i> and <i>E. faecalis</i> recovered from different piggery samples	105
3.1.1. Tabela 2. Epidemiological features and clonal relationship of representative <i>E. faecalis</i> and <i>E. faecium</i> from the piggeries studied	106
3.1.1. Tabela 3. Transfer of antibiotic resistance from wild <i>E. faecium</i> and <i>E. faecalis</i> using media supplemented with different antibiotics for transconjugant selection	108
3.1.1. Tabela S1. Distribution of antibiotic-resistant <i>Enterococcus</i> by the samples studied	111
3.2.1. Tabela 1. MICs of CuSO₄ for different <i>Enterococcus</i> species	118
3.2.1. Tabela 2. Epidemiological features of <i>Enterococcus</i> carrying Cu tolerance genes representative of different species and sources	120
3.2.1. Tabela S1. Primers used to search copper resistance genes described in different Gram positive genus	124
3.2.1. Tabela S2. Co-transfer of copper and antibiotic resistance genes and transconjugants recovered from plates supplemented with different compounds	125
3.2.2. Tabela 1. Primers used for <i>merA</i> genes amplification	142

3.2.2. Tabela 2. Epidemiological background, antibiotic resistance and copper tolerance genes of <i>Enterococcus</i> carrying <i>merA</i> genes	142
3.3.1. Tabela 1. Epidemiological background and plasmid content of <i>Enterococcus</i> carrying copper and mercury tolerance genes.....	166
3.3.1. Tabela 2. Data of Long-PCR obtained with different primer combination	167
3.3.3. Tabela 1. Epidemiological and genetic background of enterococci carrying <i>tet(S)</i>	184
3.3.3. Tabela 2. Strategies and conditions used for characterizing CTn6000	185

LISTA DE ABREVIATURAS

AFLP - Amplified Fragment Length Polymorphism
Ag - Símbolo do elemento químico prata
Al - Símbolo do elemento químico alumínio
As - Símbolo do elemento químico arsénio
BAPs - Bayesian Analysis of Population Structure
CC - Clonal Complex
CMI – Concentração Mínima Inibitória
Co - Símbolo do elemento químico cobalto
Cr - Símbolo do elemento químico crómio
CTns - Conjugative Transposons
Cu - Símbolo do elemento químico cobre
CueO - Multi-copper oxidase
D-Ala:D-Ala - D-Alanina-D-Alanina
D-Ala:D-Lac - D-Alanina-D-Lactato
D-Ala:D-Ser - D-Alanina-D-Serina
DDAC - Didecyldimethylammonium chloride
DNA - Deoxyribonucleic acid
ECDC - European Centre for Disease Prevention and Control
EDTA - Ethylenediaminetetraacetic acid
EFSA - European Food Safety Authority
EMA - European Medicines Agency
Fe - Símbolo do elemento químico ferro
Ga - Símbolo do elemento químico gálio
Hg - Símbolo do elemento químico mercúrio
HgCl₂ - Mercury(II) chloride
ICE - Integrative and Conjugative Element
IR - Inverted Repeat
IS - Insertion Sequence
Kb - Kilobase
LAB - Lactic acid bacteria
Mb - Megabase

MCO - Multicopper oxidase
MerA - Mercuric reductase
MLS_B - Macrolide-Lincosamide-Streptogramin B
MLST - Multilocus Sequence Typing
mM - Milimolar
Mn - Símbolo do elemento químico manganês
NADPH – Reduced form of nicotinamide adenine dinucleotide phosphate (NADP)
oriT - Origin of transfer
Pb - Símbolo do elemento químico chumbo
PBPs - Penicillin Binding Proteins
PFGE - Pulsed-field Gel Electrophoresis
ppm - Partes por milhão
PSK - Postsegregational killing systems
QACs – Quaternary Ammonium Compounds
RAPD - Random Amplified Polymorphic DNA
RCR - Rolling-circle replicating
Rep - Plasmid-specific replication initiation protein
rep-PCR - Repetitive element-Polymerase Chain Reaction
RFLP - Restriction Fragment Length Polymorphism
RND - Resistance-nodulation-cell division
rRNA - Ribosomal ribonucleic acid
SCENIHR - Scientific Committee on Emerging and Newly Identified Health Risks
SDS - Sodium Dodecyl Sulfate
ST - Sequence Type
Te - Símbolo do elemento químico telúrio
TMP/SMX - Trimetoprim-Sulfametoxazol
Zn - Símbolo do elemento químico zinco
µg/ml - Microgram per milliliter

RESUMO

Enterococcus spp. são bactérias ubíquas na natureza e capazes de sobreviver a diversos agentes químicos e físicos. Esta capacidade está, largamente, associada à aquisição de genes, que codificam para diversas características, que permitem a sua adaptação a diferentes ambientes e hospedeiros. Nas duas últimas décadas, algumas linhagens clonais de *Enterococcus faecium* e *Enterococcus faecalis*, com resistência múltipla aos antibióticos, emergiram, mundialmente, em unidades hospitalares sendo, atualmente, estas espécies reconhecidas como importantes agentes nosocomiais. A seleção e manutenção de bactérias com resistência múltipla a antimicrobianos em vários ambientes é, reconhecidamente, um problema complexo e multi-fatorial. Tem sido sugerido que, juntamente com os antibióticos, outros compostos designados de biocidas, incluídos em produtos utilizados quotidianamente (v.g., desinfetantes, conservantes, aditivos alimentares), podem contribuir para a seleção e manutenção de bactérias resistentes aos antibióticos. Esta preocupação está associada à identificação de mecanismos, que conferem, simultaneamente, resistência/tolerância a antibióticos e biocidas, assim como à possibilidade de co-aquisição e co-seleção de diferentes genes associados a estas resistências/tolerância num mesmo elemento genético.

Entre os biocidas, largamente, utilizados em diversas atividades antropogénicas e dispersos em diferentes ambientes destacam-se o cobre (Cu) (v.g., usado como aditivo alimentar animal em rações e na inibição de crescimento microbiano em superfícies), o mercúrio (Hg) (v.g., envolvido em diferentes processos industriais) e compostos de amónio quaternário (QACs) (v.g., em cosméticos e desinfetantes). Assim, o objetivo geral desta tese foi o de caracterizar o contexto ecológico e genético da tolerância a biocidas em *Enterococcus* spp. resistentes a antibióticos. Foram incluídos 920 *Enterococcus* spp. de diferentes origens (humana, animal, ambiental, alimentos), obtidos durante um período de 16 anos (1997-2012) e com características fenotípicas e genotípicas variadas. Para além da avaliação da resistência a antibióticos, estudou-se a tolerância a compostos de Cu, Hg e QACs através de métodos fenotípicos e genotípicos. Genes que codificam para as bombas de efluxo (tolerância ao Cu: *tcrB*, *copB*; tolerância a QACs: genes *smr*; *qacA/B*, *qacC/D*, *qacG*, *qacH*, *qacZ*, *qacEA*), redutases do Hg (4 tipos de *merA*, designados de 1, 2, 3, 4) e oxidases do Cu (*cueO*, *mco_pBMB171/P0245*, *mco_p5578*), descritos em bactérias de diferentes géneros, foram pesquisados por PCR. Entre os genes que codificam para a tolerância a metais foram detetados *tcrB*, *cueO* e dois tipos de *merA* (*merA2* e *merA3*). Os genes *tcrB* e *cueO* encontraram-se, frequentemente, nos mesmos isolados e, ocasionalmente, com *merA2*. Estes genes associados a uma maior tolerância ao Cu e, potencialmente, ao Hg, estavam distribuídos por diferentes

comunidades ecológicas (nicho humano, animal, ambiental e alimentos), sugerindo um fluxo destes genes entre ambientes e hospedeiros distintos. A prevalência de *tcrB* e *cueO* foi superior no ambiente de suiniculturas do que na maioria dos outros nichos ecológicos ($p < 0.05$), apontando a existência de uma maior pressão seletiva, possivelmente, associada ao consumo de Cu como aditivo alimentar. Estes genes foram observados em diferentes espécies de *Enterococcus*, designadamente *E. faecium*, *E. faecalis*, *E. hirae*, *E. gallinarum* e *E. casseliflavus*. Também foram observados em diversas linhagens clonais de *E. faecium* (destacam-se linhagens de elevado risco de infecção humana ST18 e ST78, assim como CC5, CC9, CC22, CC94 e vários singletons) e de *E. faecalis* (CC2, CC21 e vários singletons). Entre os genes que codificam para QACs, apenas *qacZ* foi detetado num *E. faecium* isolado de uma amostra de esgoto hospitalar e identificado na linhagem clonal ST17, frequentemente, associada a infeções nosocomiais. *Enterococcus* portadores dos genes *tcrB*, *cueO* (principalmente *E. faecium*), ou de *qacZ* mostraram ser, respetivamente, mais tolerantes a CuSO_4 (CMI=16-36mM), ou ao cloreto de benzalcónio (CMI=10-11mg/L), uma observação aplicável quer aos isolados selvagens, quer aos transconjugantes. A expressão dos genes detetados sugere que estes estão envolvidos no processo adaptativo de *Enterococcus* spp. a diferentes desafios ambientais e não constituem resíduos genéticos de eventos recombinacionais durante a evolução genómica destas bactérias. A deteção de CMI>12mM para o CuSO_4 , na ausência dos genes *tcrB* e/ou *cueO*, foi, fundamentalmente, observada em espécies diferentes de *E. faecium* e indica que mecanismos de tolerância ao cobre diferentes dos pesquisados, podem também contribuir para a tolerância a este biocida.

Verificou-se uma maior associação ($p < 0.05$) entre isolados portadores de *tcrB*, *cueO* e/ou *merA* e resistência a antibióticos como tetraciclina (frequentemente observada com *tcrB*⁺, *cueO*⁺ e/ou *merA2*⁺), eritromicina (*tcrB*⁺, *cueO*⁺; *merA3*⁺), ampicilina (*merA2*⁺), ciprofloxacina (*merA3*⁺), cloranfenicol (*merA3*⁺), estreptomicina (*tcrB*⁺, *cueO*⁺; *merA2*⁺), gentamicina (*merA3*⁺), ou nitrofurantoína (*tcrB*⁺, *cueO*⁺; *merA2*⁺). Transconjugantes portadores de genes de tolerância a biocidas e recuperados de meios suplementados com antibióticos (tetraciclina, eritromicina, ampicilina, vancomicina, ou gentamicina), ou Cu apresentavam resistência a um ou dois dos antibióticos testados acima mencionados, excetuando ciprofloxacina e nitrofurantoína. Este estudo mostrou, pela primeira vez, que a transferência por conjugação da resistência à ampicilina (*pbp5*) ocorre, juntamente com um mecanismo de tolerância ao cobre, diferente dos que foram pesquisados, em linhagens clonais clinicamente relevantes ST18 e ST78 de *E. faecium*. Estudos posteriores são necessários para a identificação do mecanismo envolvido e sua importância na manutenção da resistência à ampicilina nestas linhagens, ou para a patogenicidade de *E. faecium*.

Através de ensaios de hibridação de DNA digerido com S1, ou I-Ceul e separado por PFGE, os genes *tcrB*, *cueO*, *merA2* ou *qacZ* foram detetados em plasmídeos mobilizáveis e de diferentes tamanhos (40-300kb). O *merA3* foi identificado num plasmídeo e também no cromossoma de isolados clínicos. Os genes *tcrB* e *cueO* e, ocasionalmente, *merA2* estavam, frequentemente, co-localizados nos mesmos plasmídeos tipo pLG1 em *E. faecium*, espécie com maior prevalência destes três genes. Esta prevalência pode ser explicada pelo facto dos plasmídeos do tipo pLG1 possuírem um espectro reduzido de hospedeiros. O gene *qacZ* foi localizado num plasmídeo do tipo Inc18 (largo espectro de hospedeiros), reconhecidos, previamente, por estarem envolvidos na plasticidade genética de *Enterococcus* spp. Para além dos genes de tolerância a biocidas, os plasmídeos estudados também possuíam, frequentemente, genes de resistência a antibióticos, nomeadamente à tetraciclina [*tet*(M), *tet*(L)], à eritromicina [*erm*(B)], ou à vancomicina (*vanA*), sugerindo que diversos compostos podem seleccionar estirpes de *Enterococcus* spp. resistentes aos antibióticos. A análise do ambiente genético dos genes de tolerância ao Cu e Hg (nossos dados de sequenciação e análise de genomas disponíveis no Genbank) revelou que se encontram em regiões genéticas adjacentes e fazem parte de módulos de genes, frequentemente, flanqueados por sequências de inserção (IS), nomeadamente pela IS1216. Estas regiões podem também incluir genes, que codificam para a tolerância a outros metais, como cádmio e arsénio. A IS1216 e outras IS parecem fazer parte de transposões compostos, que incluem o gene *qacZ*, ou o gene *tet*(S) associado à resistência à tetraciclina (em CTn6000 parciais). Estruturas completas do transposão conjugativo CTn6000, outro exemplo de estrutura modular, foram detetadas em *Enterococcus* spp. de vários nichos. Os resultados obtidos permitiram concluir que *Enterococcus* spp. possuem uma diversidade de genes, que codificam para a tolerância a biocidas (metais e, ocasionalmente, QACs) embora sem especificidade de nicho, de espécie ou clonal. A presença/uso de Cu, Hg e QACs pode facilitar a persistência e disseminação de *Enterococcus* spp. com resistência múltipla a antibióticos em diferentes comunidades ecológicas e, indiretamente, a evolução de elementos genéticos aliada a fenómenos de recombinação de módulos de genes, potencialmente, abundantes nos metagenomas locais.

Palavras chave: *Enterococcus*, biocidas, antibióticos, transferência genética horizontal, cobre, mercúrio, compostos de amónio quaternário.

ABSTRACT

Enterococcus spp. are ubiquitous bacteria capable of surviving to different chemical and physical challenges. This ability is associated with the acquisition of diverse genes coding for features allowing their adaptation to different environments and hosts. In the last two decades, some multidrug resistant clonal lineages of *Enterococcus faecium* and *Enterococcus faecalis* emerged in hospitals throughout the world, with these species being currently recognized as important nosocomial agents.

The selection and maintenance of multidrug resistant bacteria in different environments is a recognized complex multi-factorial problem. It has been suggested that, together with antibiotics, other compounds designated as biocides and included in commonly used products (e.g. disinfectants, preservatives, food additives), may contribute for the selection and maintenance of antibiotic resistant bacteria. This concern associates with the identification of mechanisms simultaneous diminishing the activity of antibiotic and biocides or of biocide tolerance mechanisms which genes are co-located in common mobile genetic elements along with antibiotic resistant genetic determinants. Within the biocides largely used in diverse anthropogenic activities and spread in different environments, copper (Cu) stands out (e.g., used as animal food additive, and to inhibit microbial growth in surfaces), as does mercury (Hg; e.g., involved in different industrial processes), and quaternary ammonium compounds (QACs; e.g., in cosmetics and disinfectants). Thus, the main goal of this thesis was to characterize the ecological and genetic context of biocide tolerance in antibiotic-resistant *Enterococcus* spp.

This characterization included 920 *Enterococcus* spp. from different ecological niches (human, animal, environmental, food) sampled during a 16-year period (1997-2012), which presented diverse phenotypes and genotypes. Beyond antibiotic-resistance, the study addressed tolerance to Cu, Hg and QACs, through phenotypic and genotypic methods. Genes encoding for efflux pumps (tolerance to Cu: *tcrB*, *copB*; tolerance to QACs: *smr*, *qacA/B*, *qacC/D*, *qacG*, *qacH*, *qacZ*, *qacEΔ*), Hg reductases (4 types of *merA*, designated as 1, 2, 3, 4) and Cu oxidases (*cueO*, *mco_pBMB171/P0245*, *mco_p5578*), described in bacteria from different genera, were searched by PCR. Among genes encoding for metal tolerance, we detected *tcrB*, *cueO* and two types of *merA* (*merA2* and *merA3*). The *tcrB* and *cueO* genes were frequently found in the same isolates and occasionally with *merA2*. These genes associated with higher tolerance to Cu, and potentially Hg, were distributed in different ecological communities (human, animal, environmental and food niches), suggesting that they can flow between distinct hosts and environments. The prevalence of *tcrB* and *cueO* was higher in piggeries environment than in most other ecological niches ($p < 0.05$), suggesting a higher selective pressure due to Cu

consumption as food additive. These genes were found in different *Enterococcus* species, namely *E. faecium*, *E. faecalis*, *E. hirae*, *E. gallinarum*, *E. casseliflavus*. They were also observed in several clonal lineages of *E. faecium* (highlighting high-human-risk infection clonal lineages ST18 and ST78 as well as CC5, CC9, CC22, CC94, and several singletons) and of *E. faecalis* (CC2, CC21, and several singletons).

The only QACs tolerance gene detected in this study was *qacZ*, isolated from a hospital sewage *E. faecium* identified as ST17, a clonal lineage frequently associated with nosocomial infections. *Enterococcus* spp. harboring *tcrB*, *cueO* (mostly *E. faecium*) or *qacZ* genes were, respectively, more tolerant to CuSO₄ (CMI=16->36mM) or benzalkonium chloride (MIC=10-11mg/L), an observation applicable to either wild-type isolates or transconjugants. Expression of the detected genes suggests their involvement in *Enterococcus* spp. adaptation to different environmental challenges, and not merely silent genetic remnants left by recombination events during genomic evolution of these bacteria. The detection of CMI>12mM for CuSO₄, in the absence of *tcrB* and/or *cueO* genes, was essentially observed in non-*E. faecium* species, indicating that Cu tolerance mechanisms other than those searched may also contribute for tolerance to this biocide.

There was a significant association ($p<0.05$) between isolates harboring *tcrB*, *cueO* and/or *merA* and the resistance to antibiotics such as tetracycline (frequently observed with *tcrB*⁺, *cueO*⁺ and/or *merA2*⁺), erythromycin (*tcrB*⁺, *cueO*⁺; *merA3*⁺), ampicillin (*merA2*⁺), ciprofloxacin (*merA3*⁺), chloramphenicol (*merA3*⁺), streptomycin (*tcrB*⁺, *cueO*⁺; *merA2*⁺), gentamicin (*merA3*⁺) or nitrofurantoin (*tcrB*⁺, *cueO*⁺; *merA2*⁺). Transconjugants carrying genes encoding for biocide tolerance and recovered from antibiotic (tetracycline, erythromycin, ampicillin, vancomycin or gentamicin) or Cu-supplemented media were resistant to one or more of the tested antibiotics mentioned above, except to ciprofloxacin and nitrofurantoin. This study was the first to show that conjugative transfer of ampicillin resistance (*pbp5*) occurs together with a Cu tolerance mechanism different from those searched, and involving the clinically relevant ST18 and ST78 *E. faecium* clonal lineages. Further studies will be required for identifying the associated mechanism and its importance for the maintenance of ampicillin resistance in these lineages, or for *E. faecium* pathogenicity.

Using hybridization assays of DNA digested with S1 or I-CeuI and separated by PFGE, the *tcrB*, *cueO*, *merA2* or *qacZ* genes were detected in mobilizable plasmids of different sizes (40-300kb). The *merA3* was identified in a plasmid and also in the chromosome of clinical isolates. The *tcrB*, *cueO* and occasionally *merA2* were frequently co-localized in the same pLG1-type plasmids in *E. faecium* – the species with highest observed prevalence of these genes ($p<0.05$). The narrow host range of pLG1- type plasmids may explain this prevalence. The *qacZ* gene was detected in an Inc18 type plasmid (wide host

range), previously recognized by their involvement in *Enterococcus* spp. genetic plasticity. Beyond biocide tolerance genes, the studied plasmids also frequently harbored antibiotic-resistance genes, namely to tetracycline [*tet*(M), *tet*(L)], erythromycin [*erm*(B)], or vancomycin (*vanA*), suggesting that diverse compounds may select antibiotic-resistant *Enterococcus* spp. strains. Genetic environment analysis of Cu and Hg tolerance genes (our sequencing data and Genbank available genomes) revealed that they are in adjacent genetic regions, and are part of gene modules frequently flanked by insertion sequences (IS), namely IS1216. These regions may also include tolerance genes for other metals such as cadmium and arsenic. IS1216 and other IS may also integrate composite transposons containing *qacZ* or the *tet*(S) gene associated with tetracycline resistance (in partial CTn6000).

Complete structures of the conjugative transposon CTn6000 (another example of modular structure) were detected in *Enterococcus* spp. of different niches. Results in this thesis allow to conclude that *Enterococcus* spp. possess diverse genes encoding tolerance to biocides (metals and occasionally QACs), albeit without niche, species or clone specificity. The presence/use of Cu, Hg and QACs may facilitate the persistence and dissemination of *Enterococcus* spp. with multiple antibiotic-resistance in different ecological communities and, indirectly, the evolution of genetic elements associated with recombination of gene-modules potentially abundant in local metagenomes.

Keywords: *Enterococcus*, biocides, antibiotics, horizontal gene transference, copper, mercury, quaternary ammonium compounds.

Estrutura da tese

Esta tese está dividida em quatro capítulos:

- **CAPÍTULO 1- Introdução geral**

Neste capítulo são apresentados os conhecimentos e conceitos atuais sobre a relevância, fisiologia, ecologia, estrutura genómica (populacional e de elementos genéticos móveis), resistência a antibióticos e tolerância a biocidas de *Enterococcus* spp., de forma a permitir a compreensão da importância, oportunidade e originalidade do estudo efectuado.

- **CAPÍTULO 2- Objetivos e lista de publicações**

Neste capítulo são indicados os principais objetivos do estudo e a organização dos trabalhos realizados e apresentados no capítulo 3.

- **CAPÍTULO 3- Resultados e discussão**

Neste capítulo são apresentados os principais resultados encontrados neste estudo assim como a sua discussão face ao estado da arte. Estas informações estão integradas em 3 publicações, 1 artigo submetido para publicação e 2 artigos em preparação.

- **CAPÍTULO 4- Conclusões gerais**

Neste capítulo procedeu-se a uma integração dos principais resultados encontrados, destacando a sua relevância para o avanço do conhecimento no tópico “*biocidas e resistência a antibióticos*” em *Enterococcus* spp. São listadas e sumariadas as principais conclusões suportadas pelos dados resultantes desta tese.

CAPÍTULO 1

Introdução geral

Este capítulo tem como objetivo facultar uma perspetiva sobre os aspetos mais atuais e relevantes relacionados com a resistência a antibióticos e tolerância a biocidas em *Enterococcus* spp. e a importância deste género bacteriano para a saúde pública. Nos pontos 1.1 a 1.5 abordaram-se tópicos relacionados com as suas características gerais, impacto na saúde humana e dispersão na natureza, resistência a antibióticos e plasticidade genética. A partir do ponto 1.6 pretendeu-se fornecer informação sobre a importância dos biocidas e o seu possível impacto na seleção de bactérias resistentes aos antibióticos. Abordaram-se temas como mecanismos de ação e mecanismos de tolerância bacteriana aos biocidas, assim como o potencial impacto do uso destes compostos na co-seleção de bactérias resistentes a antibióticos, nomeadamente *Enterococcus* spp. Deu-se particular relevo aos compostos biocidas, alvo de estudo desta tese, designadamente cobre, mercúrio e compostos de amónio quaternário.

1. GÉNERO *Enterococcus*

1.1. TAXONOMIA E PERSPETIVA HISTÓRICA

Enterococcus spp. são cocos de Gram positivo incluídos no filo dos *Firmicutes*, classe dos *Bacilli*, ordem dos *Lactobacillales* e família das *Enterococcaceae* (Deák, 2011).

A designação “Entèrocoque” surge em 1899 quando Thiercellin descreve, pela primeira vez, um novo grupo de diplococos de Gram-positivo de origem intestinal, agrupados em pares e pequenas cadeias. No mesmo ano, MacCallum e Hastings descreveram um microrganismo isolado de uma endocardite como *Micrococcus zymogenes* que, atualmente, é reconhecido como fazendo parte do género *Enterococcus* (Wood & Holzapfel, 1995). Em 1906, Andrewes and Horder designaram estes cocos como *Streptococcus faecalis*, sendo posteriormente descobertas novas espécies como *Streptococcus faecium*, *Streptococcus avium* e *Streptococcus durans* que possuíam características bioquímicas distintas de *Streptococcus faecalis* (Wood & Holzapfel, 1995). Em 1919, Sherman divide o género *Streptococcus* em dois grupos: o dos não entéricos (piogénicos, do grupo *viridans* e lácticos) e o dos entéricos, sendo esta divisão baseada na capacidade de crescimento destes microrganismos a diferentes temperaturas e pH (Sherman *et al.*, 1937). Na década de 1930, o grupo dos *Streptococcus* entéricos, incluindo aqueles que hoje sabemos pertencerem ao género *Enterococcus*, foi suportado pela classificação serológica de Lancefield (baseada na presença e diferença química do carboidrato C da parede celular) que os juntou no grupo D (Lancefield, 1933).

Foi apenas em 1984, com a introdução das técnicas de biologia molecular, que Schleifer e Kilpper-Bälz verificaram que *S. faecalis* e *S. faecium* tinham características, suficientemente distintas de outros *Streptococcus*, justificando a criação de um novo género: *Enterococcus* (Wood & Holzapfel, 1995). Esta separação é fundamentada em técnicas de hibridação de DNA-DNA e DNA-rRNA (Kilpper-Bälz *et al.*, 1981, 1982, 1984; Schleifer *et al.*, 1985), obtendo-se a confirmação das diferenças entre *Enterococcus* spp., *Streptococcus* spp., *Lactococcus* spp. e outros cocos de Gram positivo, através da análise de sequências do gene 16S rRNA (Klein, 2003; Collins *et al.*, 1989; Ludwig *et al.*, 1985). O posicionamento do género *Enterococcus* face a outros géneros de bactérias de Gram positivo está esquematizado na Figura 1. Atualmente encontram-se identificadas mais de 50 espécies de *Enterococcus* (<http://www.bacterio.net/enterococcus.html>).

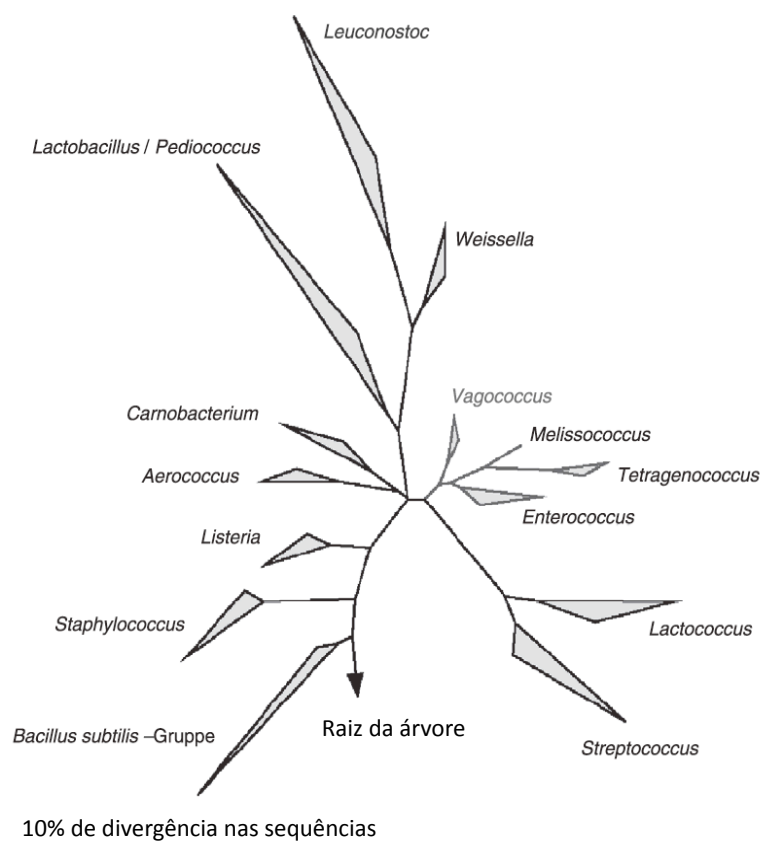


Figura 1. Posicionamento filogenético do género *Enterococcus* baseado na sequenciação do gene 16S rRNA aplicada a diferentes géneros de bactérias de Gram positivo (retirado de Klein 2003; Reprodução com permissão da *American Society for Microbiology* - Anexo1).

1.2. CARACTERÍSTICAS FISIOLÓGICAS

Enterococcus caracterizam-se por serem cocos de Gram positivo, aerotolerantes, produtores de ácido láctico a partir da glucose, sem capacidade de esporular e não produtores de catalase. Estas bactérias têm a capacidade de sobreviver em condições adversas, que incluem largos intervalos de temperatura (10-45°C) e pH (4.8-9.6), concentrações elevadas de cloreto de sódio (v.g. 6,5%) e sais biliares (v.g. 40%) (Teixeira *et al.*, 2003; Fisher & Phillips, 2009). Além disso, são tolerantes ao stress oxidativo, azida, detergentes, metais pesados e etanol e à dissecação prolongada (Huycke, 2002). Ainda, têm a capacidade de utilizar diversas fontes de energia como carboidratos complexos, glicerol, lactato, malato, citrato e vários α -ceto ácidos (Huycke, 2002; Bøhle *et al.*, 2011). *Enterococcus* spp. também produzem bacteriocinas, que impedem o crescimento de outros microrganismos competidores, tendo um papel

importante em alguns setores da indústria alimentar, nomeadamente no desenvolvimento de características organoléticas de queijos artesanais (Franz *et al.*, 2001; Moreno *et al.*, 2006; Nes *et al.*, 2007).

A sobrevivência de *Enterococcus* spp. a várias condições físico-químicas permite-lhes ter uma distribuição ubiqüitária na natureza, uma boa competitividade face a outras bactérias, que partilham os mesmos nichos ecológicos, assim como colonizar vários hospedeiros (Huycke, 2002; Leblanc, 2006). Entre outros, destaca-se o trato gastrointestinal dos humanos, animais vertebrados e invertebrados e plantas (Flahaut *et al.*, 1996; Santagati *et al.*, 2012). Esta disseminação de *Enterococcus* spp. por vários ambientes está, implicitamente, associada à contaminação de diversos alimentos frescos e processados, águas doces, marinhas e de consumo, assim como de superfícies inanimadas, entre outros (Guardabassi *et al.*, 2002; Iversen *et al.*, 2002; Simjee *et al.*, 2002; Wilson & McAfee 2002; Johnston & Jaykus 2004; Novais *et al.*, 2005b; Macedo *et al.*, 2011; Santagati *et al.*, 2012). Pelo facto de serem habitantes do trato gastrointestinal são usados como um parâmetro indicador de contaminação fecal na análise microbiológica de água e alimentos (Castillo-Rojas *et al.*, 2013).

Apesar da presença de características metabólicas e fisiológicas comuns entre os membros do género *Enterococcus*, existem diferenças fisiológicas entre as espécies, que podem contribuir para a sua adaptação a diferentes ambientes e hospedeiros, nomeadamente de *Enterococcus faecalis* e *Enterococcus faecium* associadas, frequentemente, à infeção em humanos (Huycke, 2002; Leblanc, 2006). Entre os genes de patogenicidade, que podem estar presentes nestas duas espécies, encontram-se aqueles que codificam para proteínas de superfície, que permitem uma melhor adesão (*v.g.*, codificadas pelos genes *acm* e *scm*), produção de biofilme (*v.g.*, *esp*) e para reguladores da transcrição (*v.g.*, *fsr*), que modulam a expressão genética em resposta aos desafios ambientais (Coque *et al.*, 2002a; Coque *et al.*, 2002b; Willems *et al.*, 2005; Willems *et al.*, 2010). Para informação mais detalhada sobre os fatores de patogenicidade de *E. faecalis* e *E. faecium* recomenda-se a consulta das seguintes referências bibliográficas: Willems *et al.*, 2001; Coque *et al.*, 2002b; Leavis *et al.*, 2004; Willems *et al.*, 2005; Leavis *et al.*, 2007; Abriouel *et al.*, 2008; Heikens *et al.*, 2008.

1.3. ESTRUTURA POPULACIONAL

Apesar do género *Enterococcus* incluir várias espécies, a sua distribuição na natureza não é homogénea. Apenas algumas espécies têm sido associadas a infeções no Homem (*E. faecium*, *E. faecalis*, *E. gallinarum*, *E. raffinosus*, *E. durans*, entre outras), nomeadamente de tecidos moles, do trato urinário e endocardites (Samuel *et al.* 2008;

Dombrádi *et al.*, 2012; Vijayakrishnan & Rapose, 2012). Embora infecções por *Enterococcus* spp. possam ser descritas em pacientes da comunidade, é no ambiente hospitalar que estas bactérias se destacam, principalmente *E. faecalis* e *E. faecium* (Hidron *et al.*, 2008), espécies que estão, frequentemente, envolvidas em situações epidêmicas e endêmicas em diferentes continentes (Bonten *et al.*, 2001; Novais *et al.*, 2004; Novais *et al.*, 2005; Leavis *et al.*, 2006; Hidron *et al.*, 2008; Top *et al.*, 2008; Liu *et al.*, 2011; Palazzo *et al.*, 2011; Ruiz-Garbajosa *et al.*, 2011; Matsushima *et al.*, 2012). Para a avaliação destes contextos epidemiológicos e para tentar compreender a contribuição de vários nichos ecológicos na estrutura populacional dos isolados envolvidos na infecção humana, têm sido aplicadas diversas metodologias, que permitem determinar a relação clonal entre isolados da mesma espécie (Kaufmann, 1998). Entre elas destacam-se *Pulsed-Field-Gel-Electrophoresis* (PFGE) e, mais recentemente, *MultiLocus Sequence Typing* (MLST) (Homan *et al.*, 2002).

A identificação e distribuição ecológica de diversas linhagens clonais de *E. faecalis* e *E. faecium* têm sido descritas em vários estudos (Mcbride *et al.*, 2007; Agersø *et al.*, 2008; Burgos *et al.*, 2009; Freitas *et al.*, 2009a; Freitas *et al.*, 2009b). A emergência, persistência e a evolução dessas diversas linhagens clonais é, largamente, influenciada por recombinação homóloga relacionada com a aquisição de diferentes genes e características fenotípicas associadas, determinando o seu maior ou menor sucesso em contextos epidemiológicos específicos (Smith *et al.*, 1993; Smith *et al.*, 2000; Feil *et al.*, 2001; Gupta *et al.*, 2001; Urwin *et al.*, 2003; Willems *et al.*, 2012; Freitas *et al.*, 2013).

1.3.1. Estrutura populacional de *Enterococcus faecium*

A partir dos anos 90, *E. faecium* emergiu como um agente responsável por infecções nosocomiais importantes (Hidron *et al.*, 2008). O aumento da incidência de infecções enterocócicas por esta espécie, comparativamente a *E. faecalis* (predominante nos anos 80), esteve relacionado com a sua capacidade de sobreviver a distintos desafios ambientais, nomeadamente a diversos antibióticos usados na terapêutica humana, como a ampicilina e a vancomicina (Jureen *et al.*, 2003; Coque *et al.*, 2005; Lester *et al.*, 2008; Top *et al.*, 2008). A aplicação de MLST associado à utilização do algoritmo eBURST [agrupa *Sequence Types* (STs) que diferem em um ou dois alelos], permitiu detetar a existência de uma subpopulação de *E. faecium* geneticamente distinta e frequentemente associada a infecções nosocomiais de diversas regiões geográficas (Willems *et al.*, 2005; Leavis *et al.*, 2007). Esta subpopulação, altamente disseminada, foi designada de linhagem C1, que mais tarde assumiu a designação de complexo clonal 17 (CC17) (Feil

et al., 2004; Willems *et al.*, 2005; Willems *et al.*, 2011). Este sucesso deveu-se a uma aquisição sucessiva e cumulativa de genes e/ou elementos genéticos, que conferem resistência aos antibióticos, virulência e melhoria da capacidade de colonização (Willems *et al.*, 2011). De facto, *E. faecium*-CC17 são, frequentemente, resistentes à ampicilina, vancomicina e quinolonas (Klare *et al.*, 2005; Willems *et al.*, 2005; Bonora *et al.*, 2007; Khan *et al.*, 2008; Freitas *et al.*, 2009a; Lopez *et al.*, 2009; Valdezate *et al.*, 2009). Outros marcadores com ocorrência variável são os genes de virulência *hyl* e *esp* (Coque *et al.*, 2002b; Rice *et al.*, 2003; Willems *et al.*, 2005; Arias *et al.*, 2009; Rice *et al.*, 2009; Freitas *et al.*, 2010; Willems *et al.*, 2011), ilhas genómicas relacionadas com o metabolismo de carboidratos (Heikens *et al.*, 2008) e sequências de inserção (IS), importantes na modulação genómica (Leavis *et al.*, 2007).

Recentemente, detetou-se que a análise da estrutura populacional baseada no algoritmo de eBURST parece não ser a mais adequada para a análise filogenética de espécies com elevada taxa de recombinação associada a uma baixa taxa de mutação, como *E. faecium* (Willems *et al.*, 2012; de Been *et al.*, 2013). Uma alternativa ao eBURST é a análise da estrutura genética populacional combinando a identificação de linhagens que ramificaram há mais tempo e a recombinação genética entre elas. Isto pode ser determinado usando o software de *Bayesian Analysis of Population Structure* (BAPs), que separou os diferentes STs em grupos BAPs (Willems *et al.*, 2012). Este tipo de análise revelou que CC17 não é uma única população, mas sim constituído por três linhagens clonais designadas de ST17, ST18 (pertencentes ao grupo BAPs 3-3) e ST78 (BAPs 2-1), que evoluíram a partir de diferentes ancestrais (Willems *et al.*, 2012). A análise de 1720 *E. faecium* sugeriu que o grupo BAPs 3-3 tem uma forte associação ao ambiente hospitalar e o BAPs 2-1 ao de produção animal. No entanto, este último co-agrupa cerca de 30% dos isolados de origem hospitalar pertencentes ao ST78 juntamente com isolados de origem animal. Os autores sugerem que o ST78 evoluiu, separadamente, dos isolados hospitalares de BAPS 3-3, tendo em comum a resistência a ampicilina e o fator de virulência *esp*, que se encontra implicado na formação de biofilmes associados a infeções do trato urinário e endocardite (Heikens *et al.*, 2007; Leendertse *et al.*, 2009; Heikens *et al.*, 2011). Assim, foi sugerido que a emergência de infeções hospitalares associadas a determinadas linhagens não é o resultado da disseminação de um clone ou complexo clonal, CC17, mas sim de uma evolução constante de diferentes linhagens clonais no ambiente hospitalar, através da transferência horizontal de genes e de um confinamento ecológico, que diminuiu a sua recombinação com outras linhagens genéticas dispersas em diferentes nichos ecológicos (Willems *et al.*, 2012). Apesar dos STs de CC17 evoluírem de ancestrais diferentes, a maioria dos isolados relacionados com surtos e infeções hospitalares (pertencentes a ST17, ST18 e ST78) são, claramente,

distintos da maioria das estirpes comensais de humanos saudáveis e animais (Willems & van Schaik, 2009; Lebreton *et al.*, 2013), implicando origens evolutivas distintas, que podem ter ocorrido há milhares de anos (Willems & van Schaik, 2009; Galloway-Pena *et al.*, 2012; Lebreton *et al.*, 2013).

Outras linhagens clonais de *E. faecium* predominantes em nichos ecológicos particulares são CC5 em suínos, CC9 em aves, CC1 em bovinos e CC22 e CC94 em humanos saudáveis (Homan *et al.*, 2002; Freitas *et al.*, 2009b; De Leener *et al.*, 2004; Willems *et al.*, 2005; Donabedian *et al.*, 2010), apenas causando infecção humana ocasionalmente (CC5, CC9) (Freitas *et al.*, 2011a; Lopez *et al.*, 2013).

1.3.2. Estrutura populacional de *Enterococcus faecalis*

Numerosos métodos moleculares foram aplicados para a análise epidemiológica, ou filogenética da espécie *E. faecalis* (PFGE, AFLP-*Amplified fragment length polymorphism*, ribotipagem, tipagem da cápsula, RFLP- *Restriction fragment length polymorphism*, rep-PCR e RAPD- *Random Amplified Polymorphic DNA*) (Gordillo *et al.*, 1993; Tomayko *et al.*, 1995; Seettulsingh *et al.*, 1996; Malathum *et al.*, 1998; Hufnagel *et al.*, 2006). A sua estrutura populacional atual inclui uma grande diversidade de STs e de complexos clonais (CC2, CC9, CC21, CC40, CC87) e um *singleton* predominante – o ST16 (Feil *et al.*, 2001; Leavis *et al.*, 2006; Ruiz-Garbajosa *et al.*, 2006; McBride *et al.*, 2007; Willems *et al.*, 2011).

ST6/CC2 é uma das linhagens clonais mais disseminadas, enquanto o CC40 e CC87 são endêmicos em determinadas regiões geográficas (Kawalec *et al.*, 2007; McBride *et al.*, 2007; Willems *et al.*, 2011). ST21/CC21 e o ST1 foram identificados em isolados oriundos de ambiente hospitalar, assim como em humanos saudáveis, animais e produtos alimentares (Mcbride *et al.*, 2007; Agersø *et al.*, 2008; Burgos *et al.*, 2009; Chowdhury *et al.*, 2013). Outros *singletons* menos prevalentes como o ST166 foram encontrados quer em humanos, quer em animais, mais uma vez sugerindo que linhagens clonais de *E. faecalis* são, frequentemente, partilhados por diferentes reservatórios (Mcbride *et al.*, 2007; Agersø *et al.*, 2008; Burgos *et al.*, 2009).

1.4. RESISTÊNCIA A ANTIBIÓTICOS: MECANISMOS E EPIDEMIOLOGIA

A crescente importância de *Enterococcus* spp. ao longo das últimas décadas esteve, estreitamente, relacionada com a emergência da resistência aos antibióticos verificada entre os membros deste género, nomeadamente a aqueles utilizados na terapêutica

humana e na produção animal (Aarestrup *et al.*, 2002). A resistência aos antibióticos em *Enterococcus* pode ser intrínseca ou adquirida, e ambas as formas tiveram um papel relevante na emergência das infecções por este género bacteriano (Rice, 2012). A facilidade de *Enterococcus* spp. adquirirem genes por transferência horizontal, aliada à sua abundância na natureza, foram fatores condicionantes para estas bactérias serem consideradas bons indicadores para estudar a resistência aos antibióticos em diversos nichos ecológicos (EFSA, 2012a). Entre os antibióticos mais relevantes para o tratamentos de infecções enterocócicas e aos quais este género bacteriano tem desenvolvido resistências, destacam-se os glicopéptidos, β -lactâmicos (ampicilina), aminoglicosídeos (gentamicina e estreptomicina), oxazolidinonas (linezolid) e lipopéptidos (daptomicina) (Werner *et al.*, 2001; Werner *et al.*, 2008b; Arias *et al.*, 2010; Arias *et al.*, 2011).

1.4.1. Resistência intrínseca

A resistência intrínseca aos antibióticos é aquela que faz parte das características naturais do microrganismo, transmitida apenas verticalmente à descendência (Murray, 1990). *Enterococcus* spp. são, intrinsecamente, resistentes a diferentes classes de antibióticos, limitando as alternativas terapêuticas disponíveis e facilitando a sua seleção em ambientes onde esses antibióticos são largamente usados. Entre os antibióticos aos quais *Enterococcus* spp. são, intrinsecamente, resistentes destacam-se os β -lactâmicos, nomeadamente cefalosporinas e monobactâmicos, cuja ligação aos PBPs (*Penicillin Binding Proteins*) de *Enterococcus* spp. é de baixa afinidade (Sousa, 2006; Hollenbeck & Rice, 2012). O uso intenso de cefalosporinas de terceira geração, para o tratamento de infecções por bactérias de Gram negativo (*v.g.*, *Enterobacteriaceae*), associado ao uso de metronidazol para o tratamento de infecções por anaeróbios e ao qual *Enterococcus* spp. também são intrinsecamente resistentes foram fatores relevantes para uma seleção intensa destas bactérias nas unidades hospitalares durante os anos 1990 (Cetinkaya *et al.*, 2002).

Enterococcus spp. também possuem resistência intrínseca a baixas concentrações de aminoglicosídeos (CMI=8-64 $\mu\text{g/mL}$), associada a uma reduzida permeabilidade membranar, que impede a entrada destes antibióticos para o citoplasma bacteriano. Esta resistência intrínseca condiciona o uso de aminoglicosídeos na terapêutica, embora possam ser usados quando associados a um antibiótico anti-parietal, como ampicilina ou vancomicina (Sousa, 2006). Em *E. faecium* a resistência aos aminoglicosídeos é ainda mediada pelos genes cromossomais *efmM*, que codificam para uma metiltransferase e o

gene *aac(6')-li* que codifica para uma acetiltransferase, que afetam terapias sinérgicas com tobramicina (Murray, 1990; Galimand *et al.*, 2011; Arias *et al.*, 2012).

As espécies *E. gallinarum*, *E. casseliflavus* e *E. flavescens* são, intrinsecamente, resistentes aos glicopéptidos devido à presença dos genes cromossomais *vanC1*, *vanC2* e *vanC3*, respetivamente. Os genes do operão onde *vanC* está incluído originam a formação de D-Ala-D-Ser em vez de D-Ala-D-Ala no local de ligação da vancomicina, diminuindo, assim, a afinidade do antibiótico para o seu local alvo (Centinkaya *et al.*, 2002). Esta resistência é, geralmente, constitutiva e traduz-se fenotipicamente por uma baixa resistência à vancomicina, com uma concentração mínima inibitória (CMI) variável entre 2-32µg/mL, e suscetibilidade à teicoplanina (CMI=0,5-1µg/mL) (Murray, 1990; Courvalin, 2006).

Embora *Enterococcus* spp. sejam suscetíveis ao trimetoprim-sulfametoxazol (TMP/SMX) *in vitro* (quando testados em meio isento de timina que reverte a ação do TMP/SMX), esta combinação pode não ter atividade *in vivo*. Isto acontece quando estas bactérias têm disponíveis no meio circundante os folatos necessários escapando assim à inibição da síntese destes compostos por ação do antibiótico, especialmente *E. faecium* (Murray, 1990; Leclercq *et al.*, 2005).

As espécies *E. faecalis*, *E. avium*, *E. gallinarum* e *E. casseliflavus* exibem baixo nível de resistência às lincosamidas (clindamicina e lincomicina; CMI=12,5-100µg/mL) e estreptograminas do grupo A, associada a um mecanismo de efluxo destes antibióticos. Todos os *E. faecalis* são, naturalmente, resistentes a compostos derivados da estreptogramina A por possuírem o gene *lsa*, que confere resistência quer à clindamicina, quer à quinupristina-dalfopristina (Murray, 1990; Singh *et al.*, 2002; Hollenbeck & Rice, 2012).

Outros antibióticos, aos quais *Enterococcus* spp. têm resistência natural, são as polimixinas e o metronidazol (Sousa, 2006). A resistência intrínseca de *Enterococcus* spp. a esta diversidade de antibióticos contribuiu para a seleção deste género bacteriano em diversos nichos ecológicos (v.g., humano e animal), onde estes compostos são largamente utilizados.

1.4.2. Resistência adquirida

A resistência adquirida está associada à ocorrência de genes que sofreram mutações, ou à aquisição de novos genes (DNA externo), que não existem na população selvagem e que codificam para novas características, que conduzem a fenótipos de diminuição de suscetibilidade a um determinado antibiótico (Murray, 1990).

Este fenómeno tem sido observado em *Enterococcus* spp. de diversas origens, que demonstram resistência a classes de antibióticos como os fenicóis, tetraciclina, macrólidos, lincosamidas, estreptograminas, aminoglicosídeos, β -lactâmicos, glicopéptidos ou fluoroquinolonas, entre outros (Murray, 1990; Kak *et al.*, 2002; Leclercq *et al.*, 2005). Embora a resistência adquirida em *Enterococcus* spp. possa ser mediada pela ocorrência de mutações (*v.g.*, nos genes *gyrA* e *parC* associadas à resistência às quinolonas; no gene *pbp5* associado à resistência à ampicilina) (Rice *et al.*, 2004; Werner *et al.*, 2010), geralmente está associada à aquisição de diversos elementos genéticos envolvidos em eventos de transmissão horizontal, nomeadamente por conjugação, transposição e/ou transdução (Coque *et al.*, 2011; Werner *et al.*, 2013). Diversos elementos genéticos (*v.g.*, plasmídeos, transposões e integrões) (Coque *et al.*, 2011; Werner *et al.*, 2013) têm sido associados à aquisição e transferência de genes de resistência em *Enterococcus* spp. Alguns destes elementos serão abordados mais detalhadamente no ponto 1.5.

Das resistências adquiridas mais disseminadas e/ou relevantes destacam-se:

Glicopéptidos - O mecanismo de resistência aos glicopéptidos envolve a alteração do local alvo através de uma substituição aminoacídica no dipeptídeo D-Ala:D-Ala por D-Ala:D-Lac ou Ala:D-Ser, obtendo-se um precursor do peptidoglicano com baixa afinidade para estes agentes antimicrobianos e impedindo a transferência das unidades recém-sintetizadas para o peptidoglicano em crescimento (Murray, 1990; Sousa, 2006). Para que se observe a formação deste precursor com baixa afinidade e a expressão do fenótipo de resistência aos glicopéptidos, são necessários um conjunto de genes que se agrupam em transposões (*v.g.*, Tn1546 e Tn1547) e que codificam para proteínas com funções específicas na expressão da resistência (Courvalin, 2008). Os diferentes conjuntos de genes, que definem o tipo de resistência aos glicopéptidos são designados em função do nome do gene que codifica para uma ligase. Já estão descritos em *Enterococcus* spp. os tipos *vanA* a *vanN*, sendo os mais, frequentemente, identificados os genes *vanA* e *vanB* (Courvalin, 2008; Novais *et al.*, 2008; Werner *et al.*, 2008a; Werner *et al.*, 2012). Os diferentes agrupamentos de genes não se traduzem na resistência a todas as moléculas de glicopéptidos. Por exemplo, o tipo *vanA* está relacionado com um elevado nível de resistência à vancomicina e teicoplanina, enquanto que o tipo *vanB* a níveis variáveis de resistência à vancomicina e suscetibilidade à teicoplanina (Werner *et al.*, 2008a).

β -lactâmicos - A resistência adquirida aos β -lactâmicos pode estar associada à produção de β -lactamases, que conferem resistência à penicilina, aminopenicilinas (*v.g.*,

ampicilina, amoxicilina) e ureídopenicilinas (v.g., piperacilina) e baixo nível de resistência ao imipenemo. Estas enzimas são inibidas pelo ácido clavulânico, sulbactam e tazobactam (Murray, 1992). Embora a presença de β -lactamases tenha sido ocasionalmente descrita em *E. faecium* e *E. faecalis* (Patterson *et al.*, 1988; Sarti *et al.*, 2012), o mecanismo mais comum associado à resistência aos β -lactâmicos em *Enterococcus* spp. é a produção de PBPs modificadas, com diminuição de afinidade para esta classes de antibióticos devido a mutações nos genes *pbp* (v.g., *pbp5* em *E. faecium* e *pbp4* em *E. faecalis*), ou sobreexpressão destas (Rice *et al.*, 2004). Outros genes como *ddcP*, *ldt_{fm}*, *pgt* também têm sido apontados como possivelmente envolvidos na resistência à ampicilina (Zhang *et al.*, 2012). A transferência horizontal de genes *pbp* associada à mobilização de transposões ou plasmídeos foi também já descrita (Carias *et al.*, 1998; Raze *et al.*, 1998; Rice *et al.*, 2005).

Aminoglicosídeos - *Enterococcus* spp. podem adquirir um nível elevado de resistência aos aminoglicosídeos, fundamentalmente, por dois mecanismos: i) modificação do local alvo; e ii) alteração enzimática (Murray, 1990; Kak *et al.*, 2002; Chow, 2000). O primeiro está associado a mutações cromossômicas, enquanto que o último é mediado por elementos genéticos móveis, que transportam genes, que codificam para diferentes enzimas como fosfotransferases [por exemplo, *aph(2'')-Ib*], ou nucleotidiltransferases [por exemplo, *ant(4')-Ia*], dependendo se o tipo de inativação do antibiótico é realizada por O-fosforilação ou O-nucleotidilação, respetivamente (Leclercq *et al.*, 2005). De acordo com a enzima produzida nem todos os aminoglicosídeos são afetados. Um dos genes mais disseminados é *aac6'-Ia-aph2''-Ie*, que codifica para uma enzima bifuncional com funções de acetiltransferase e fosfotransferase, conferindo elevada resistência a todos os aminoglicosídeos comercializados (v.g., CMI $\geq$ 500 μ g/mL à gentamicina, CMI $\geq$ 2000 μ g/mL à canamicina) com exceção da estreptomicina (Chow, 2000). Genes como *aadE* ou *aadK* estão associados à elevada resistência à estreptomicina (CMI $\geq$ 2000 μ g/mL) (Chow, 2000). O elevado nível de resistência adquirida aos aminoglicosídeos impede o sinergismo entre qualquer aminoglicosídeo e antibióticos anti-parietais comprometendo, deste modo, o tratamento de infeções graves (Leclercq *et al.*, 2005).

Tetraciclinas - Atualmente, a resistência às tetraciclinas tem uma incidência elevada entre várias espécies de *Enterococcus* (Novais *et al.*, 2005c; Ahmad *et al.*, 2011; Seputiene *et al.*, 2012; Doud *et al.*, 2013). Dois grandes mecanismos condicionam a resistência às tetraciclinas nestas bactérias: i) bombas de efluxo, que expulsam o antibiótico da célula, sendo frequentemente codificadas em *Enterococcus* spp. pelos genes *tet(K)* ou *tet(L)*; e ii) proteção ribossomal, que dificulta a ligação do antibiótico ao

seu local alvo. Os genes que codificam este tipo de mecanismo mais, frequentemente, descritos em *Enterococcus* spp. são *tet*(M), *tet*(O) ou *tet*(S) (Aarestrup *et al.*, 2000; Brouwer *et al.*, 2010; Thaker *et al.*, 2010; Ahmad *et al.*, 2011; DiCesare *et al.*, 2013; Radhouani *et al.*, 2013).

Macrólidos, lincosamidas e estreptograminas do grupo B - A resistência adquirida a macrólidos, lincosamidas e estreptograminas do grupo B, cujo fenótipo é designado de MLS_B, está, largamente, distribuída em *Enterococcus* spp. (Aarestrup *et al.*, 2000; Novais *et al.*, 2005c; de Graef *et al.*, 2007; Thumu *et al.*, 2013). Os mecanismos mais relevantes estão relacionados com a modificação do local alvo por metilases do gene rRNA [genes *erm*(A), *erm*(B), *erm*(C), *erm*(F) e *erm*(T)], enzimas inativadoras como liases (*vgaA*) ou transferases (genes *lnuB*, *vatB*, *vatD*, *vatE*), ou bombas de efluxo (*msrA*, *msrC*, *msrD*, *lsaA*, *vgaB*, *mefA*) (Roberts *et al.*, 1999) (<http://faculty.washington.edu/marilynr/>).

Oxazolidinonas, glicilciclinas e lipopéptidos - Alguns estudos já têm descrito resistência às moléculas mais recentemente introduzidas no mercado. A resistência ao grupos das oxazolidinonas, representadas pelo linezolid, tem sido associada a mutações no *loop* central do domínio V do RNA ribossomal 23S. Recentemente, foi também associada a um gene adquirido por transferência horizontal, *cfr*, que codifica para a metilação do A2503 do gene rRNA 23S (Long *et al.*, 2006). A resistência às glicilciclinas, representadas pela tigeciclina, tem sido raramente descrita em *Enterococcus* spp., sendo o mecanismo ainda desconhecido neste género bacteriano (Werner *et al.*, 2008b; Freitas *et al.*, 2011b; Cordina *et al.*, 2012). A resistência aos lipopéptidos, representados pela daptomicina, tem sido associada a mutações nos genes que codificam as proteínas LiaF e a família da GdpD (Arias *et al.*, 2011).

1.4.3. Epidemiologia da resistência aos antibióticos: contexto global e de Portugal

A emergência de *Enterococcus* spp. com resistência múltipla aos antibióticos encontra-se, fortemente, associada ao consumo excessivo destas moléculas no tratamento de infeções humanas (hospital e comunidade), em veterinária (animais de estimação e de produção para consumo humano) e na agricultura (ECDC, 2013a; EMA, 2012). Dados recentes demonstraram que Portugal está entre os 10 países Europeus, que mais utiliza antibióticos no tratamento das infeções humanas da comunidade (DGS, 2013), havendo também registo do seu elevado uso no ambiente de produção animal (DGV, 2010).

Atualmente, *E. faecium* e *E. faecalis* fazem parte do grupo dos microrganismos designados de “ESKAPE” (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* e *Enterobacter* spp.), definidos como aqueles capazes de escapar à ação dos antibióticos, representando novos paradigmas na patogénese, transmissão e resistência (Rice *et al.*, 2009; Pendleton *et al.*, 2013). Para além de *Enterococcus* spp. serem reconhecidos a nível mundial como importantes agentes de infeções nosocomiais, nos Estados Unidos da América e Europa são a segunda e terceira causas de infeção em ambiente hospitalar, respetivamente (Arias *et al.*, 2008; Hidron *et al.*, 2008; ECDC, 2013b). Em Portugal, encontram-se classificados como o quarto agente etiológico de infeções nosocomiais (ECDC, 2013c).

Infeções por *Enterococcus* spp. são sobretudo associadas a estirpes de *E. faecium* com resistência a múltiplos antibióticos, nomeadamente a antibióticos críticos na terapêutica como ampicilina (através do gene *pbp5* mutado), glicopéptidos (do tipo *vanA* e *vanB*) e aminoglicosídeos (sobretudo associada aos gene *aac6'-Ie-aph2''-Ia*). A resistência à ampicilina é considerada um marcador de linhagens clonais de elevado risco (ST17, ST18 e ST78) emergentes em diferentes partes do mundo, incluindo Portugal (Novais *et al.*, 2005a; Freitas *et al.*, 2009a; Willems *et al.*, 2012). Estas linhagens podem apresentar também resistência a outros antibióticos como à vancomicina, apresentando Portugal uma das maiores taxas de resistência a este antibiótico entre *E. faecium* isolados de infeções invasivas (Figura 2). A resistência a aminoglicosídeos a nível hospitalar tem sido sobretudo associada à espécie *E. faecalis* e aos complexos clonais CC2, CC9 e CC87 (Ruiz Garbajosa *et al.*, 2006; Kuch *et al.*, 2012; http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/map_reports.aspx.)

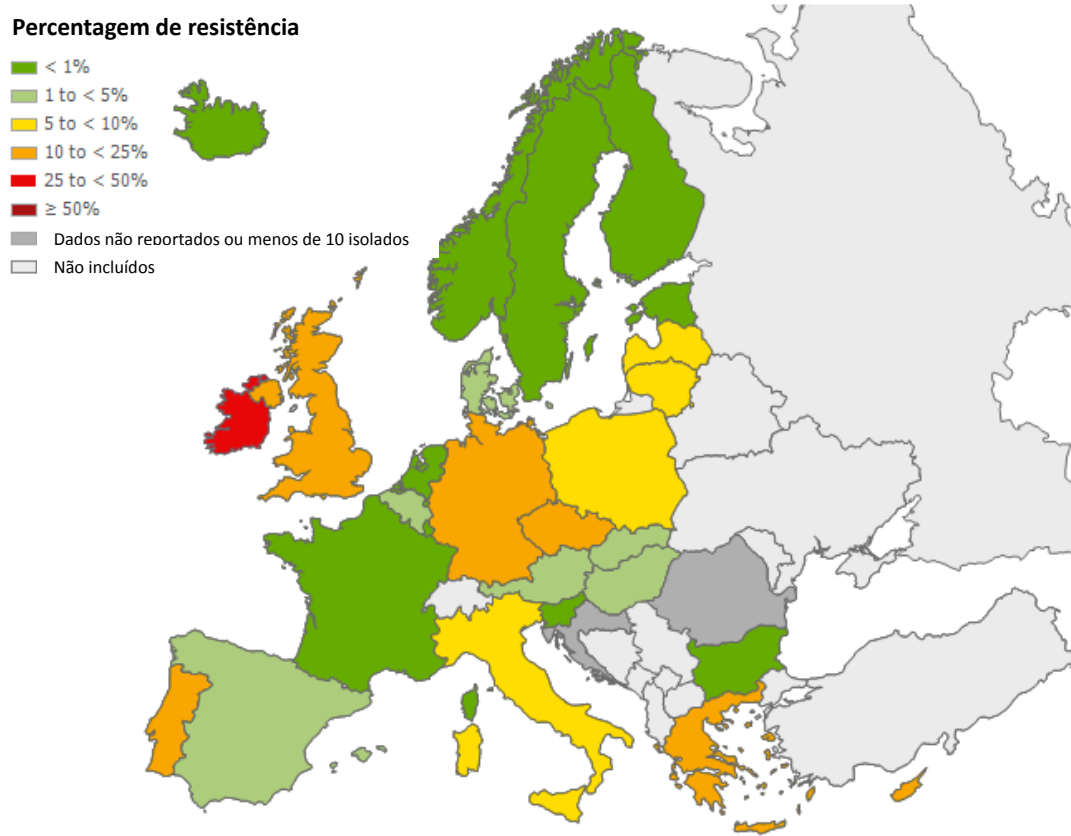


Figura 2. Percentagem de *E. faecium* invasivos resistentes à vancomicina nos países Europeus, dados referentes ao ano de 2012. Figurrada de http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/map_reports.aspx.

Para além de serem um problema nas unidades hospitalares, *Enterococcus* spp. com resistência a múltiplos antibióticos encontram-se, largamente, disseminados na comunidade de diferentes países (Aarestrup *et al.*, 2000; Larsen *et al.*, 2010; DiCesare *et al.*, 2013; Hammad *et al.*, 2014). Em Portugal, destaca-se a sua presença no ambiente de produção animal (Novais *et al.*, 2005a; Braga *et al.*, 2012), animais de estimação e selvagens (Poeta *et al.*, 2006; Gonçalves *et al.*, 2013; Santos *et al.*, 2013), no ambiente aquático (Novais *et al.*, 2005b; Marinho *et al.*, 2013), alimentos e água para consumo humano (Macedo *et al.*, 2011; Novais *et al.*, 2005c; Campos *et al.*, 2013) e fezes de humanos saudáveis (Novais *et al.*, 2006). Entre as resistências mais comuns descritas na literatura e detetadas em nichos extrahospitais de diversos países destaca-se a resistência às tetraciclina [por exemplo, associada a genes *tet(M)*, *tet(L)*, *tet(S)*], a macrólidos [por exemplo, genes *erm(B)*] e aminoglicosídeos como a estreptomicina (v.g., gene *aadA* e *aad9*) (Mahbub *et al.*, 2005; Barreto *et al.*, 2009; Ahmad *et al.*, 2011). O elevado consumo destes antibióticos na comunidade, nomeadamente no tratamento de infeções humanas e animais (ECDC, 2013a; EMA, 2012), contribui para seleção de

Enterococcus spp. resistentes aos antibióticos mencionados. Também têm sido descritos em animais de produção e de estimação, humanos saudáveis e ambiente aquático, *Enterococcus* spp. com resistência aos glicopéptidos (vancomicina e teicoplanina), ampicilina ou gentamicina, importantes no tratamento das infeções enterocócicas. Estes pertencem a várias espécies e linhagens clonais, nomeadamente às de elevado risco (*E. faecium* ST18 e ST78; *E. faecalis*-CC2), ou outras, ocasionalmente, associadas a infeções humanas (*E. faecium*-CC5; *E. faecalis*-CC21) (Freitas *et al.*, 2011a; Freitas *et al.*, 2009a). É de destacar que isolados de *E. faecium*-CC5 resistentes à vancomicina, tetraciclina e outros antibióticos, está disseminado em suínos oriundos de vários países Europeus e Estados Unidos, em humanos saudáveis e humanos hospitalizados, nomeadamente em Portugal (Freitas *et al.*, 2011a).

A dispersão da resistência aos antibióticos entre nichos e hospedeiros está associada não só à dispersão clonal, mas sobretudo à transferência horizontal de elementos genéticos móveis. De facto, transposões que codificam para a resistência à vancomicina (v.g., Tn1546 e Tn5382), ou tetraciclina (v.g., Tn916 e Tn5397) foram detetados entre isolados de humanos, animais e/ou ambiente (Torres *et al.*, 2003; Novais *et al.*, 2008; Valdezate *et al.*, 2009; Ahmad *et al.*, 2011; Radhouani *et al.*, 2013; Song *et al.*, 2013). Adicionalmente, o mesmo plasmídeo portador de Tn1546 foi detectado em humanos e suínos (Freitas *et al.*, 2011a) e o gene *cfr* emergente em diversas unidades hospitalares parece ter uma origem animal (Liu *et al.*, 2012; Liu *et al.*, 2013; Patel *et al.*, 2013).

As vias de contaminação para a dispersão de estirpes e elementos genéticos entre diferentes hospedeiros e ambientes são diversas, destacando-se a cadeia alimentar e o contato direto com ambientes contaminados, ou hospedeiros colonizados por estas bactérias (animais de produção, manipulação de alimentos contaminados, atividades recreativas em águas contaminadas) (Figura 3) (Witte, 2000; Aarestrup *et al.*, 2008; Byappanahalli *et al.*, 2012).

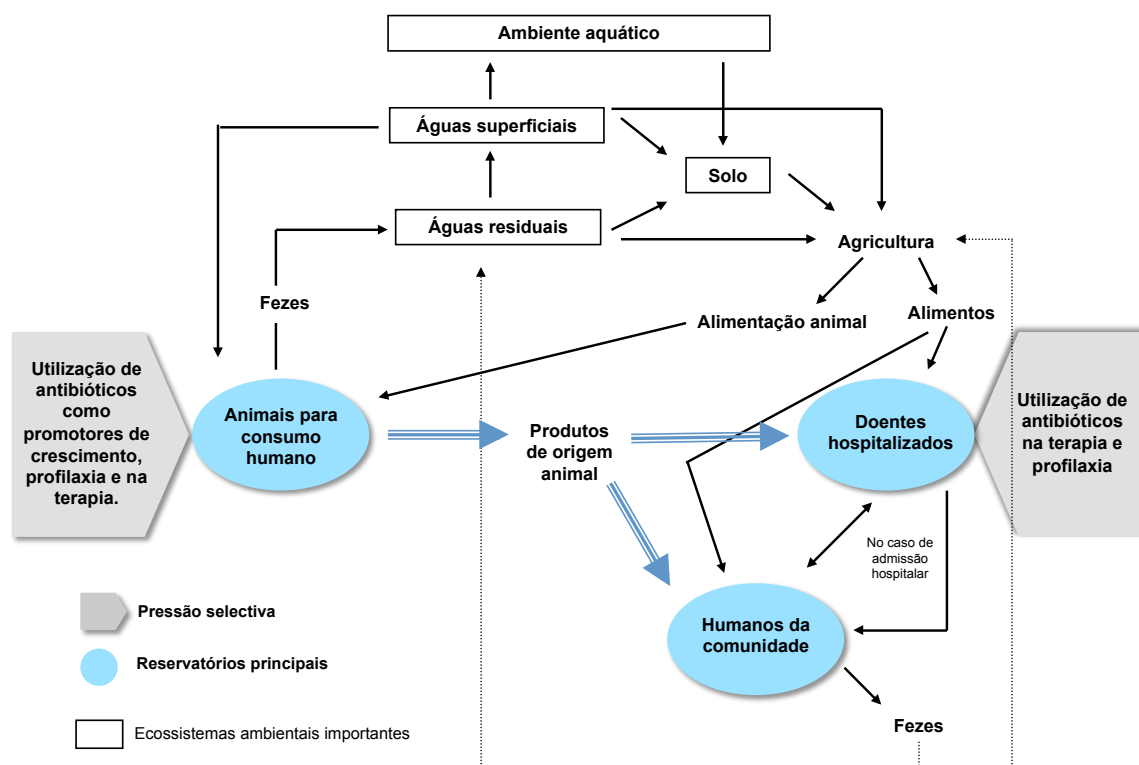


Figura 3. Vias de disseminação de bactérias resistentes a antibióticos e respetivos elementos genéticos entre diferentes nichos (Figura adaptada de Witte *et al.*, 2000).

1.5. ELEMENTOS GENÉTICOS MÓVEIS EM *Enterococcus* spp.

A emergência de estirpes de *Enterococcus* spp. com novas características adaptativas conferidas pela resistência a antibióticos, capacidade de colonização, ou fenótipos/genótipos de virulência, ilustram a importância da transferência horizontal na evolução destes microrganismos (Coque *et al.*, 2011). Esta parece ser importante na adaptação de *E. faecium* e *E. faecalis* a ambientes específicos conferindo-lhes propriedades que favorecem a colonização, virulência, ou persistência em diferentes hospedeiros e ambientes (Palmer *et al.*, 2010; Werner *et al.*, 2013). Além de genes que codificam para resistência para antibióticos, os elementos genéticos móveis podem albergar genes que codificam para adesinas, biocidas, bacteriocinas, ou proteínas que participam em diferentes vias metabólicas (de la Cruz *et al.*, 2000; Osborn *et al.*, 2002; Toussaint *et al.*, 2002; Frost *et al.*, 2005).

A transferência horizontal ocorre não só entre membros de *Enterococcus* spp., mas também com outros microrganismos, que partilham as mesmas comunidades ecológicas.

Esta partilha de genes parece ser mais influenciada pela proximidade física entre os microrganismos que partilhem propriedades genómicas similares (*v.g.*, tamanho do genoma, composição em guaninas e citosinas), ou pelo uso de compostos orgânicos do que pela proximidade filogenética (Jain *et al.*, 2003; Skippington *et al.*, 2011).

Dentro do mobiloma (repertório de elementos genéticos móveis) de *Enterococcus* spp. serão apenas abordados os elementos genéticos envolvidos em eventos de transposição e plasmídeos.

1.5.1. Elementos de transposição

Os elementos de transposição estão largamente distribuídos no género *Enterococcus* e incluem sequências de inserção (IS), transposões compostos, transposões tipo Tn3 e transposões conjugativos. Os transposões compostos e a família de transposões tipo Tn3 encontram-se disseminados nas bactérias de Gram positivo e de Gram negativo, enquanto que os transposões conjugativos são, na maior parte dos casos, encontrados em bactérias de Gram positivo (Weaver *et al.*, 2002; Clewell *et al.*, 2004).

1.5.1.1. Sequências de Inserção (ISs)

As ISs são elementos de transposição muito simples, constituídos por pequenas sequências de DNA, que variam entre 700 e 2500 pares de bases (<http://www-is.biotoul.fr/> and Mahillon *et al.*, 1999; Siguier *et al.*, 2006; Depardieu *et al.*, 2007). Estes elementos contêm um gene, que codifica para uma transposase e sequências denominadas de repetições invertidas (IR) nas extremidades do elemento, ambas necessárias para a transposição (Mahillon & Chandler, 1998).

As ISs podem existir como elementos de transposição autónomos, mobilizando-se para diferentes partes do genoma (cromossoma, ou plasmídeos), participando na eliminação da atividade, ou na expressão de genes circundantes (Mahillon & Chandler, 1998). Além disso, por eventos de recombinação homóloga podem participar na criação de plataformas genéticas modulares (*v.g.*, Tn5385), ou na variabilidade de elementos genéticos como Tn1546, ou plasmídeos, contribuindo assim para a plasticidade genética dos genomas bacterianos (Mahillon & Chandler, 1998; Rice *et al.*, 1998b; Novais *et al.*, 2008; Freitas *et al.*, 2013, Werner *et al.*, 2013). Quando as ISs estão a flanquear um ou mais genes, fazem parte de transposões compostos (ver seção 1.5.1.2). Para uma revisão detalhada sobre a contribuição das ISs na plasticidade genómica dos microrganismos recomenda-se a leitura de Mahillon & Chandler, 1998.

Encontram-se descritas doze famílias de ISs disseminadas entre *Firmicutes* e identificadas em *Enterococcus* spp. de diferentes nichos ecológicos (Coque, 2008), tanto em *E. faecium* como em *E. faecalis*. São exemplos as IS16 e IS256 (família IS256), IS1216 (família IS6), IS1476 e IS1251 (família ISL3) e ISEf/IS6770 (família IS30) (Rice *et al.*, 1994a, 1995; MacKinnon *et al.*, 1997; Huh *et al.*, 2004; Camargo *et al.*, 2005; Novais *et al.*, 2008; Werner *et al.*, 2011).

1.5.1.2. Transposições compostos

Os transposições compostos são constituídos por um número variável de genes que, habitualmente, codificam para a resistência a antibióticos, ou funções catabólicas com tamanho variável entre 4.7 e 65.0 Kilobases (Kb) e são flanqueados por ISs (Merlin *et al.*, 2000).

Encontram-se descritos diversos transposições compostos em *Enterococcus* spp. associados à resistência a antibióticos. Alguns exemplos representativos incluem o Tn5405, que codifica para a resistência aos aminoglicosídeos (*aadE*, *sat4-aphA-3*) e Tn5281/Tn5384/Tn5385, que codificam para a resistência a concentrações elevadas de gentamicina (Heaton *et al.*, 1996; Quintiliani *et al.*, 1996; Werner *et al.*, 2001). A maior parte dos transposições compostos estão disseminados quer em diferentes espécies de *Enterococcus*, quer em diferentes nichos ecológicos e encontram-se localizados em plasmídeos. É o caso de Tn5405, encontrado em *Enterococcus* spp. oriundos de animais de estimação, de produção e alimentos, e presente em plasmídeos da família Inc18 (Werner *et al.*, 2001; Teuber, *et al.*, 2003; Werner *et al.*, 2003).

A presença de vários elementos de transposição relacionados é favorável à ocorrência de recombinações homólogas entre as ISs localizadas nos transposições, plasmídeos e cromossoma, contribuindo para a transferência e disseminação de transposições particulares, ou influenciando rearranjos de DNA. Estes eventos genéticos podem resultar na aquisição de diversas características como a resistência a antibióticos, virulência, ou produção de bacteriocinas (Grady *et al.*, 2003; Paulsen *et al.*, 2003; Kozitskaya *et al.*, 2004; Sletvold *et al.*, 2007, 2010).

1.5.1.3. Derivados do Tn3

Os transposições do tipo Tn3 são constituídos por uma transposase, uma resolvase e um fragmento de DNA variável (v.g., que codifica para a resistência a antibióticos) flanqueados por duas sequências invertidas e repetidas (Trun & Trempey, 2004). O seu mecanismo de transposição é replicativo, o que significa que quer a bactéria dadora, quer

a recetora possuem uma cópia do elemento genético após a sua transposição. A família de transposições tipo Tn3 divide-se em duas subfamílias, conforme a direção da transcrição dos genes da transposase e resolvase. Se a transcrição destes é feita em direção oposta designa-se de subfamília Tn3, se na mesma direção por Tn21 (Grindley *et al.*, 2001). São exemplos de transposições do tipo Tn3 em *Enterococcus* spp. o Tn1546 e Tn917/Tn3871 (subfamílias Tn3 e Tn21, respetivamente). A disseminação de transposições da família Tn3 em *Enterococcus* spp. é facilitada por plasmídeos conjugativos (Shaw *et al.*, 1985; Arthur *et al.*, 1993). O Tn1546 (10.8Kb) é constituído por um conjunto de sete genes (*vanRSHXYZ*), que medeiam a resistência aos glicopéptidos (vancomicina e teicoplanina) (Arthur *et al.*, 1993; Werner *et al.*, 2008a) e encontra-se descrito em isolados de diferentes nichos ecológicos (Weigel *et al.*, 2003; Guardabassi *et al.*, 2005; Clark *et al.*, 2005; Guardabassi *et al.*, 2006; Malik *et al.*, 2008; Novais *et al.*, 2008; Perichon *et al.*, 2009). O Tn917 (5.3Kb) codifica para a resistência aos macrólidos, lincosamidas e estreptograminas do grupo B (gene *ermB*) (Tomich *et al.*, 1980; Shaw *et al.*, 1985). Encontra-se, frequentemente, localizado em plasmídeos de *E. faecalis* (v.g., pAD1) e a sua transposição, assim como a expressão da resistência à eritromicina, são induzidas pela presença deste antibiótico (Tomich *et al.*, 1979). O Tn3871 (5.1Kb) tem grande proximidade com o Tn917 (Banai *et al.*, 1984). Estes elementos estão, amplamente, disseminados em humanos, alimentos e animais (Rollins *et al.*, 1985; Thomas *et al.*, 2005).

1.5.1.4. Transposições conjugativas ou elementos de integração cromossomal

Os transposições conjugativas (CTns) são também denominados de elementos de integração cromossomal (ICE) (Coque, 2008). Estes têm capacidade de se auto-transferir entre uma célula dadora e uma célula recetora. A sua estrutura genética compreende diversos módulos envolvidos na integração/excisão, conjugação e regulação, para além de poderem conter genes, que codificam para outras funções como resistência a antibióticos, virulência, ou metabolismo de diferentes nutrientes. Estas características conferidas pelos CTn/ICE contribuem para a rápida adaptação das bactérias a diversas condições ambientais e a colonização de novos nichos (Burrus & Waldor, 2004).

Apesar da sintenia observada nos CTn, estes diferem nas sequências dos genes das integrases e excisionases (recombinase tipo serina ou tirosina) e na especificidade do seu local de inserção (único ou múltiplo), determinando a sua promiscuidade (Roberts *et al.*, 2009, 2011). Estes elementos têm uma extraordinária capacidade de estarem envolvidos em processos de recombinação genética numa diversidade de genomas, ou

elementos genéticos móveis, resultando, frequentemente, em plataformas genéticas mosaico contendo fragmentos de outros transposições e plasmídeos (Roberts *et al.*, 2009; Burrus & Waldor, 2004).

Os CTns contribuem, largamente, para a disseminação da resistência a antibióticos tanto em microrganismos comensais como patogênicos, nomeadamente *Enterococcus* spp. de vários nichos ecológicos (Agersø *et al.*, 2006; Novais *et al.*, 2010a). Muitos dos CTns de *Enterococcus* spp. são portadores de genes de resistência à tetraciclina [*tet*(M)] (CTn916, CTn5397; CTn5801), ou *tet*(S) (CTn6000)], podendo também conter genes de resistência à canamicina e eritromicina (CTn1545) (Garnier *et al.*, 2000; Weaver *et al.*, 2002; Roberts *et al.*, 2009; Werner *et al.*, 2013). Existem ainda estruturas modulares de transposição contendo transposições do tipo CTn916 como o CTn5385 (CTn5381-Tn5384-Tn552), ou CTn5382 (*pbp5*-CTn1549), que codificam para a resistência a múltiplos antibióticos (Carias *et al.*, 1998; Rice, , 1998a).

Elementos da família CTn916/Tn1545 [*tet*(M)] são dos mais frequentes em *Enterococcus* spp. (Rice *et al.*, 1998b; Roberts *et al.*, 2009, 2011) e têm uma baixa especificidade de integração, o que permite a sua ampla disseminação (Rice, 1998a; Roberts *et al.*, 2009; Wozniak *et al.*, 2010). CTn6000 [*tet*(S)], CTn5801 [*tet*(M)] e CTn5397 [*tet*(M)] foram descritos em vários nichos ecológicos, sendo que alguns são encontrados mais, frequentemente, em algumas espécies (Brouwer *et al.*, 2010; Novais *et al.*, 2010). Os CTns têm sido descritos em bactérias de Gram positivo colonizadoras do trato gastrointestinal e cavidade oral de humanos e em alimentos de origem animal, com ou sem exposição à tetraciclina, o que sugere a elevada frequência de transferência destes elementos em condições naturais (Lancaster *et al.*, 2005).

1.5.2. Plasmídeos

Os plasmídeos são elementos genéticos móveis extra-cromossomais, capazes de se replicarem e de se manterem independentemente do tipo de hospedeiro. A sua estrutura é modular e inclui regiões estáveis, que contêm genes que codificam para funções essenciais (replicação, conjugação e manutenção) e uma região variável, que contém um conjunto de genes, que codificam para funções adaptativas (resistência a antibióticos, metais pesados, biocidas, virulência, produção de bacteriocinas, funções metabólicas e simbióticas, ou resistência à radiação) (Espinosa *et al.*, 2000; Taylor *et al.*, 2004; Thomas *et al.*, 2004; Bennett, 2008). O tamanho dos plasmídeos pode variar entre 1Kb e mais de 2Mb (<http://www.genomics.ceh.ac.uk/plasmiddb/>) (Molbak *et al.*, 2003). Especula-se que

ambientes complexos possam selecionar plasmídeos maiores se estes codificarem para características que confirmem vantagens competitivas/adaptativas (Slater *et al.*, 2008).

A classificação dos plasmídeos tem sido realizada com base em diferentes critérios, como seja o número de cópias características do hospedeiro, replicação e mobilidade, não existindo ainda um critério universal e definitivo dada a plasticidade destes elementos (Francia *et al.*, 2004; Taylor *et al.*, 2004; Kim *et al.*, 2006; Garcillan-Barcia *et al.*, 2008; Jensen *et al.*, 2010). Em *Enterococcus* spp., a replicação dos plasmídeos ocorre por uma de duas vias: a de círculo rolante ("*Rolling-circle*"), ou a replicação Teta de deslocamento de cadeia ("*strand displacement*"), sendo a última mais comum e associada a plasmídeos de maior tamanho (del Solar *et al.*, 1998). De acordo com a mobilidade, os plasmídeos podem ser classificados em conjugativos, mobilizáveis e não-mobilizáveis. A maquinaria da mobilidade inclui um grupo de genes de mobilidade (MOB), que permitem a conjugação de DNA: um local original de transferência (*oriT*) e uma relaxase, que funciona como enzima reguladora do início e final da conjugação. Estes componentes são comuns tanto em plasmídeos mobilizáveis, como em plasmídeos conjugativos, de forma a codificarem as funções necessárias à sua auto-transferência e, assim, tendendo a ser maiores (>30Kb) e com um baixo número de cópias. Os plasmídeos não mobilizáveis não são auto-transferíveis e disseminam-se, naturalmente, por transformação ou transdução, não havendo descrições em *Enterococcus* spp. (Waar *et al.*, 2002; Smillie *et al.*, 2010). Devido à distribuição universal de replicases e relaxases nos plasmídeos, estas foram utilizadas para a sua classificação (Francia *et al.*, 2004; Garcillan-Barcia *et al.*, 2009; Jensen *et al.*, 2010).

De acordo com o mecanismo de replicação, as famílias de plasmídeos em *Enterococcus* spp. dividem-se em plasmídeos RCR (rolling-circle replicating; pRI1-like), ou plasmídeos Teta (RepA_N, Inc18_Type e PHT β -like) (Weaver *et al.*, 2002; Garcia-Migura *et al.*, 2009; Rosvoll *et al.*, 2009; Weaver *et al.*, 2009; Jensen *et al.*, 2010; Sletvold *et al.*, 2010). Quatro famílias de relaxases (MobC, MobP, MobQ, MobV) têm sido associadas a diferentes tipos de plasmídeos e CTns.

1.5.2.1. Plasmídeos RCR

Plasmídeos RCR constituem uma família extensa de plasmídeos de pequenas dimensões (<10Kb), com elevado número de cópias, e capazes de se replicarem num largo espetro de bactérias de Gram positivo e de Gram negativo (del Solar *et al.*, 1993; Khan, 1997; del Solar *et al.*, 1998; Weaver *et al.*, 2002; Khan, 2004).

Atualmente, estão descritas sete famílias de plasmídeos RCR, com base na sequência das proteínas iniciadoras de replicação (Rep) e também da cadeia dupla de origem da replicação. Muitas destas famílias são crípticas (sem função conhecida na natureza), enquanto outras transportam genes de resistência a antibióticos, genes associados à mobilização e transferência, ou recombinases envolvidas na resolução de multímeros dos plasmídeos (Khan, 1997; Khan, 2005). Os plasmídeos RCR descritos em *E. faecium* e *E. faecalis* pertencem às famílias pE194/pLS1, ou pMV158 e pC194/pUB110. Recentemente, foi descrita uma nova família, que parece ser intrínseca da espécie *E. faecium* correspondendo a um novo grupo de plasmídeos RCR (pRI1-like), representado pelo pRI1 e seus derivados, sejam crípticos (pRI1, pJS42, pEFNP1), ou associados à resistência à tetraciclina [pKQ10::tet(U)]. Estes plasmídeos encontram-se, globalmente, disseminados existindo descrições, quer em animais de produção, quer a nível hospitalar (Ridenhour *et al.*, 1996; Garcia-Migura *et al.*, 2009; Jensen *et al.*, 2010; Freitas *et al.*, 2013).

1.5.2.2. Plasmídeos Teta

A replicação Teta foi descrita pela primeira vez em bactérias de Gram positivo no grupo de *Streptococcus* spp. e *Enterococcus* spp. (Bruand *et al.*, 1991; del Solar *et al.*, 1998; Espinosa *et al.*, 2000). Os plasmídeos de *Enterococcus* spp. de replicação teta incluem pequenos plasmídeos mobilizáveis (<15Kb), que são, na sua maioria, crípticos e plasmídeos conjugativos de maior tamanho (>30Kb), que podem codificar uma grande diversidade de funções adaptativas como a resistência a antibióticos, biocidas, metais, funções metabólicas e determinantes de virulência (Coque *et al.*, 2011).

a) Plasmídeos Teta de pequeno tamanho

Os plasmídeos pequenos de replicação Teta (<15Kb) têm sido cada vez mais descritos em *Enterococcus* spp.. Ainda que maioritariamente crípticos, estão, frequentemente, associados à produção de bacteriocinas, que têm atividade contra uma variedade de bactérias de Gram positivo. Podem ter sistemas de mobilização associados a plasmídeos RCR (MOBV; pAM α 1-like), ou plasmídeos Teta (MobP₇; por exemplo, pCIZ2, pB82, pEF1071, p200B) (Martinez-Bueno *et al.*, 2000; Garcillan-Barcia *et al.*, 2008; Garcillan-Barcia *et al.*, 2009) e têm sido descritos em *Enterococcus* spp. oriundos de pacientes hospitalizados, animais e alimentos (Nes *et al.*, 2007; Coque, 2008; Criado *et al.*, 2008; Freitas *et al.*, 2013).

b) Família Inc18

A família Inc18 inclui um grupo de plasmídeos com tamanho de 25-50Kb, de baixo número de cópias e que exibe um espectro de hospedeiro alargado (Schwarz *et al.*, 2001; Kurenbach *et al.*, 2003; Teuber *et al.*, 2003). Plasmídeos representativos desta família incluem o pIP501 de *Streptococcus agalactiae*, pRE25 e pAM β 1 de *E. faecalis*, e pIP186 de *E. faecium*. O tamanho e constituição dos plasmídeos da família Inc18 é muito variável devido à aquisição (ISs, transposões e ilhas genômicas) e perda de DNA, que parece ocorrer por deleção espontânea após transferência conjugativa para diferentes hospedeiros (Thompson *et al.*, 2003; Zuniga *et al.*, 2003).

Os plasmídeos Inc18 replicam por um mecanismo único, de “antisense-RNA” de atenuação transcricional, ainda não identificado em bactérias de Gram negativo (de la Hoz *et al.*, 2000; Weaver *et al.*, 2002; Brantl, 2004). A relaxase é o protótipo da subfamília MobQ3 (família MOBQ), que apenas inclui plasmídeos do filo *Firmicutes* (Garcillan-Barcia *et al.*, 2008). Também a presença de genes que codificam as Rep e sistemas de morte pós-segregacionais (PSK) são, frequentemente, encontrados em plasmídeos e cromossomas de diversas bactérias, demonstrando que os plasmídeos Inc18 estão, frequentemente, envolvidos nos processos de transferência genética horizontal (Cooper *et al.*, 2000; Moritz *et al.*, 2007; Van Melder *et al.*, 2009). Os plasmídeos da família Inc18 conferem, geralmente, resistência a macrólidos, lincosamidas, estreptograminas, alguns aminoglicosídeos (canamicina, estreptomicina), cloranfenicol e mercúrio (LeBlanc *et al.*, 1986; Schwarz *et al.*, 2001; Davies *et al.*, 2005).

c) Família RepA_N

A família de plasmídeos RepA_N compreende uma grande diversidade de subfamílias de plasmídeos de espectro de hospedeiro reduzido, que se encontram distribuídos em bactérias de Gram positivo com baixo conteúdo em guanina e citosina (Weaver *et al.*, 2009). As proteínas RepA desta família subdividem-se em cinco grupos com forte correlação a determinado género de hospedeiros (*Enterococcus*, *Streptococcus*, *Lactobacillus*, *Lactococcus* e *Staphylococcus*). Em *E. faecalis*, os membros desta família incluem plasmídeos, que respondem a feromonas e, em *E. faecium* a derivados dos plasmídeos pRUM e pLG1 (Weaver *et al.*, 2009; Laverde Gomez *et al.*, 2011). Duas famílias de relaxases filogeneticamente distantes, MobC₂ e MobP₇, têm sido descritas nestes plasmídeos (Garcillan-Barcia *et al.*, 2009).

Os plasmídeos que respondem a feromonas constituem o paradigma dos sistemas de conjugação em bactérias de Gram positivo. Estes plasmídeos possuem genes que codificam para uma resposta a feromonas produzidas por bactérias recetoras. As

feromonas são codificadas por genes cromossomais destas bactérias, enquanto as proteínas importantes para o sinal quer de detecção, quer de resposta são codificadas por genes localizados no plasmídeo, que responde à feromona sexual. Este sistema de conjugação é extremamente específico, na medida em que estes plasmídeos só respondem a feromonas específicas (Clewell *et al.*, 2004; Kozłowicz *et al.*, 2006). Assim, plasmídeos que respondem a feromonas encontram-se classificados em diferentes grupos de incompatibilidade, de acordo com a feromona sexual a que respondem (Coque *et al.*, 2011).

As famílias mais comuns correspondem a pAD1, pCF10, pPD1 e pAM373, que têm sido maioritariamente descritos em isolados de infeções humanas nosocomiais e codificando para a resistência a antibióticos [Tn1546_*vanA*, Tn1549_*vanB2*, Tn4001_*aac6'-aph2'*", Tn916-like_*tet(M)*], e/ou características de virulência (bacteriocinas e citolisinas). A produção de feromonas é restrita a *E. faecalis*, no entanto foi descrita em géneros de bactérias de Gram positivo com capacidade de produção de péptidos cAM373-like e com atividade específica para a feromona pAM373 (*E. hirae*, *S. aureus* e *Streptococcus gordonii*), o que promove a disseminação da resistência a antibióticos inter-espécies (Clewell *et al.*, 1985, 2002; Vickerman *et al.*, 2010).

Nos plasmídeos que respondem a feromonas as replicases não estão, necessariamente, relacionadas às relaxases correspondentes uma vez que pAD1 e pCF10 contêm proteínas de mobilização de diferentes famílias (Weaver *et al.*, 2009; Jensen *et al.*, 2010). A mobilização deste grupo de plasmídeos tem sido associada à transferência de resistência a antibióticos e marcadores de MLST, assim como genes que codificam para a cápsula, o que promove a diversidade cromossomal em *E. faecalis* (Manson *et al.*, 2010).

Plasmídeos derivados pRUM-like

Os plasmídeos pRUM-like (v.g., pRUM, pS177) são plasmídeos mosaico gerados por processos de recombinação entre transposões, ISs e pequenos plasmídeos. Possuem um sistema específico "PSK" designado de Axe-Txe que atua como um módulo de estabilidade segregacional numa larga diversidade de hospedeiros (Grady *et al.*, 2003; Halvorsen *et al.*, 2011). Os plasmídeos que contêm a RepA e Axe-Txe do pRUM estão disseminados em isolados de *E. faecium* de diferentes países, em sua maioria associados a linhagens clonais de origem hospitalar (Rosvoll *et al.*, 2009). Isolados de *Enterococcus* spp. oriundos de humanos e frangos e portadores de genes de resistência à vancomicina, ou tetraciclina possuem, frequentemente, plasmídeos da família pRUM (Grady *et al.*, 2003; Moritz *et al.*, 2007; Sung *et al.*, 2008; Jensen *et al.*, 2010; Halvorsen *et al.*, 2011).

Megaplasnídeos RepA_N

Os megaplasnídeos são frequentemente encontrados em isolados da espécie *E. faecium* de diferentes nichos ecológicos (Werner *et al.*, 1999; Hasman *et al.*, 2005; Biavasco *et al.*, 2007; Rosvoll, 2009; Laverde Gomez *et al.*, 2011; Zhang *et al.*, 2011). O único megaplasnídeo sequenciado na totalidade é o pLG1 (~281Kb), que foi obtido de um isolado hospitalar de *E. faecium* resistente à vancomicina na Alemanha (Laverde Gomez *et al.*, 2011). Contém um gene *rep* que codifica para uma proteína semelhante à proteína RepA-pAD1 e por isso foi incluído na família plasmídica RepA_N (Weaver *et al.*, 2009). A relaxase identificada no plasmídeo pLG1 faz parte da sub-família MOB_P que, por sua vez, é encontrada noutros plasmídeos RepA_N (Garcillan-Barcia *et al.*, 2009). Megaplasnídeos derivados do pLG1 têm sido descritos em isolados resistentes à vancomicina (tipo *vanA*) e gentamicina [*aac(6')*-*le-aph(2'')*]-*la*] oriundos de pacientes hospitalizados de Portugal (Freitas *et al.*, 2013) e da Noruega (Rosvoll *et al.*, 2012), respetivamente.

d) Plasmídeos da família pHT β /pMG1

Esta família compreende plasmídeos derivados dos pHT (pHT α , pHT β , pHT γ) e pMG1 que têm sido associados à disseminação da resistência aos glicopéptidos (Tn1546-*vanA*) e a elevados níveis de resistência aos aminoglicosídeos (elementos Tn4001-like) em isolados de *E. faecium* e *E. avium* nos Estados Unidos da América e Japão (Tomita & Ike *et al.*, 2002, 2003; Tanimoto & Ike, 2008). O seu sistema de transferência faz parte da família MobP, sendo esta classificação baseada no oriT e nas sequências das relaxases (Tomita & Ike, 2005; Garcillan-Barcia *et al.*, 2009). Até à data, este tipo de plasmídeos foi, exclusivamente, identificado em isolados de *E. faecium* oriundos de humanos (Coque *et al.*, 2011; Freitas *et al.*, 2013).

e) Megaplasnídeos

Os megaplasnídeos, enquanto componente do mobiloma bacteriano, têm sido cada vez mais reportados. Os plasmídeos de tamanho acima dos 150Kb, geralmente, associados a funções metabólicas, são, classicamente, definidos como megaplasnídeos (Maclellan *et al.*, 2004). Estes tipos de plasmídeos são comuns entre *Lactobacillus* spp., *Bifidobacterium* spp. e *Bacteroides* spp., e conferem-lhes a capacidade de utilizar complexos de carboidratos (presentes nos vegetais da dieta), de glicoproteínas e glicoconjugados (sintetizados pelo hospedeiro), que podem contribuir para o aumento da sua adaptação e persistência no cólon (Schell *et al.*, 2002; Li *et al.*, 2007). Estes plasmídeos podem codificar genes envolvidos no metabolismo dos açúcares (manitol,

glicerol, sorbitol e rafinose), variando a expressão fenotípica dos microrganismos que os possuem (Teixeira *et al.*, 1995; Zhang *et al.*, 2011). Em estudos recentes demonstrou-se que megaplasmídeos, encontrados em *E. faecium*, codificam para a resistência a vários antibióticos (macrólidos, glicopéptidos, aminoglicosídeos), metais pesados (cobre) e virulência (genes *hyl*, *pilA*) (Arias *et al.*, 2009; Freitas *et al.*, 2010; Kim *et al.*, 2010; Freitas *et al.*, 2011a; Laverde Gomez *et al.*, 2011).

A origem destes plasmídeos continua por determinar uma vez que a classificação baseada nas proteínas de replicação Rep foi designada com base nas sequências dos plasmídeos de tamanho pequeno/médio de famílias conhecidas de bactérias de Gram positivo. A diversidade destes plasmídeos em *E. faecium*, *E. durans* e *E. hirae* tem sido pouco explorada (Coque *et al.*, 2011) e não têm sido descritos em *E. faecalis*.

1.6. BIOCIDAS

1.6.1. Perspetiva histórica

Os biocidas são compostos valiosos que se traduzem em inúmeros benefícios para a sociedade devido ao controlo que exercem sobre o crescimento microbiano (bactericida, esporicida, fungicida, virucida, ou uma combinação destes mecanismos) (Russel, 2003; Cerf *et al.*, 2010). Desde a Antiguidade que os biocidas (*v.g.*, metais) são utilizados, empiricamente, devido às suas propriedades adstringentes, desinfetantes e conservantes (Alexander *et al.*, 2009; Borkow & Gabbay, 2009). Nos últimos dois séculos, os óxidos de telúrio, magnésio e arsénio, sais de cobre e mercúrio foram, amplamente, utilizados na terapêutica da lepra, tuberculose, gonorreia e sífilis (Hodges, 1889; Keyes, 1920; Frazer & Edin, 1930). A utilização destes compostos, como fungicidas, também se estendeu à agricultura. Em 1880, Pierre-Marie-Alexis-Millardet criou a “Calda Bordalesa” (sulfato de cobre, óxido de cálcio e água como solvente), que, até aos dias de hoje, tem ampla utilização, na Europa e Estados Unidos, para prevenção de fitopatologias em vinhas, batata, ou tomate (Ayres, 2004). Em 1945, assiste-se à introdução das biguanidas (*v.g.*, clorhexidina, alexidina e formas poliméricas), surfatantes anfotéricos, bifenois (triclosan), aldeídos (glutaraldeído, succinaldeído-base, orto-fetaldeído), agentes libertadores de cloreto (isocianuratos), agentes libertadores de iodo (iodóforos), isotiazolonas e ácido paracético (Russel & Russel, 1995; Ortega *et al.*, 2013).

No entanto, após a introdução dos antibióticos na terapêutica e da sua utilização em massiva, verificou-se uma franca diminuição do uso de biocidas em medicina humana

(Lemire *et al.*, 2013). Atualmente, o uso destes compostos tem sido recuperado, sendo, amplamente, aplicados em ambiente hospitalar para o controlo de infeções nosocomiais (v.g., como desinfetantes de dispositivos médicos e de superfícies, e antisépticos utilizados nas mucosas e pele), na indústria alimentar (v.g., como conservantes e desinfetantes), na indústria farmacêutica (v.g., em cosmética, ou produtos de higiene pessoal), na comunidade (v.g., em produtos de limpeza, têxteis, preservantes de madeira), na agricultura (v.g., em herbicidas ou pesticidas) e na produção animal intensiva (v.g., como desinfetantes de superfícies, ou aditivos alimentares em rações) (Maillard, 2002; EFSA/ECDC/EMEA, 2009; SCENIHR, 2009). Devido ao impacto e importância que os biocidas têm no estilo de vida atual, existem diretrizes europeias que sugerem e estimulam, que a sua utilização seja prudente, de forma a prevenir a emergência de bactérias tolerantes, ou resistentes quer a estes compostos, quer aos antibióticos (para mais detalhes neste tópico ver seções abaixo) (SCENIHR, 2010).

De acordo com o SCENIHR (2009) e o Regulamento da União Europeia 528/2012 (22 de Maio de 2012) os termos BIOCIDA e PRODUTO BIOCIDA são definidos, respetivamente, como:

- Uma molécula química ativa, presente num produto biocida, com o objetivo de controlar o crescimento bacteriano, ou matar bactérias.

- Qualquer substância, ou mistura, consistindo em, contendo, ou originando uma, ou mais substâncias ativas, com o objetivo de destruir, deter, neutralizar, prevenir a ação de, ou controlar o efeito de qualquer organismo prejudicial, através de quaisquer outros meios que não meras ações físicas, ou mecânicas.

Na Diretiva 98/8/EC, constam 23 tipos de aplicação de produtos biocidas, que se encontram divididos em quatro grandes grupos: desinfetantes e biocidas de uso geral, preservantes, usados no controlo de pestes e outros biocidas. É de referir que os compostos presentes nas formulações de produtos biocidas podem ter atividade sinérgica, levando à potenciação do efeito das substâncias ativas presentes. É o caso de combinações de compostos de amónio quaternário com o ácido etilenodiaminotetracético (EDTA), ou biguanidas com álcoois (ECDC/EFSA/EMEA, 2009).

A toxicidade de qualquer composto depende sempre da concentração utilizada. Alguns dos compostos que serão abordados nesta tese, como os de cobre, são aplicados com outros propósitos para além da sua atividade biocida (v.g., como aditivos alimentares em rações para promoção de crescimento animal) (70/524/EEC, 2004). Para facilitar a descrição da importância dos biocidas e não multiplicar terminologias, a palavra “biocida”

será empregue de uma forma genérica.

1.6.2. Mecanismos de ação

Os biocidas podem ter atividade *cida* (v.g., bactericida), ou *estática* (v.g., bacteriostática) e atuar em mais do que um local alvo nos microrganismos (Tabela 1), embora muito ainda exista por compreender acerca dos seus mecanismos de ação (Russell and Chopra, 1990; Denyer, 1995; Maillard, 2002; Poole, 2005; Cerf *et al.*, 2010). Estes compostos são classificados em vinte e duas categorias consoante os seus grupos funcionais. Existem moléculas pertencentes ao grupo dos fenóis, compostos de amónio quaternário, álcoois, biguanidas, metais, entre outros. No entanto, se nos basearmos no alvo de atuação, os biocidas podem ser classificados em três grandes grupos, os que interagem: i) com componentes celulares externos; ii) com a membrana citoplasmática, ou iii) com constituintes citoplasmáticos (Russel, 2003; Gnanadhas *et al.*, 2012; Ortega *et al.*, 2013).

Tabela 1. Local de ação de diferentes grupos de biocidas (adaptado de Gnanadhas *et al.*, 2012).

Biocidas	Local de Ação	Referências
Álcoois	Membranar Proteína Ácidos nucleicos Parede celular	Russel <i>et al.</i> , 1996; Suller <i>et al.</i> , 1999; Russel, 2003.
Biguanidas, ácidos, peróxidos	Membranar Proteína Ácidos nucleicos	Russel <i>et al.</i> , 1996; Suller <i>et al.</i> , 1999; Russel, 2003
Fenóis	Membranar Proteína Parede celular	Russel <i>et al.</i> , 1996; Suller <i>et al.</i> , 1999; Russel, 2003
Feniléteres, isotiazóis	Membranar Proteína	Russel <i>et al.</i> , 1996; Suller <i>et al.</i> , 1999; Russel, 2003
Aldeídos e derivados de metais pesados	Proteína Ácidos nucleicos Parede celular	Russel <i>et al.</i> , 1996; Moken <i>et al.</i> , 1997; Suller <i>et al.</i> , 1999; Russel, 2003.
Composto de cloreto	Proteína Ácidos nucleicos	Russel <i>et al.</i> , 1996; Suller <i>et al.</i> , 1999; Russel, 2003
QACs, terpenos, anilidas, parabenos e surfactantes aniônicos	Membranar	Russel <i>et al.</i> , 1996; McDonnell <i>et al.</i> , 1999; Suller <i>et al.</i> , 1999; Russel, 2003.
Corantes, acrilidinas, diamidinas e compostos halogenados	Ácidos nucleicos	Russel <i>et al.</i> , 1996; Suller <i>et al.</i> , 1999 Boykin <i>et al.</i> , 2002
Libertadores de cloreto	Parede Celular	Moken <i>et al.</i> , 1997

Alguns dos compostos biocidas são, altamente, reativos (aldeídos, cloretos, compostos libertadores de oxigénio), ligando-se covalentemente a proteínas e peptidoglicano

presentes na parede celular bacteriana. Exercem a sua atividade bactericida, provavelmente através da oxidação de grupos tiol, ou outros grupos químicos (Maillard, 2002; Lambert, 2004). Biocidas com atividade anti-membranar, como os compostos de amónio quaternário, causam a disrupção física da membrana celular, levando à lise e solubilização do conteúdo microbiano. Outros agentes como os álcoois e a clorohexidina, quando usados em concentrações bactericidas, provocam a desnaturação de proteínas. As biguanidas desestabilizam os catiões divalentes, que estão associados à parede celular, provocando a disrupção dos lipopolissacarídeos, o que permite a sua entrada na célula (Lambert, 2004; Gilbert & McBain, 2003).

Os metais são um grupo particular porque podem ter não só uma atividade biocida como também uma atividade fisiológica em processos vitais para as células, nomeadamente atuando como co-fatores enzimáticos, ou intervindo na manutenção da estrutura da membrana celular e DNA (Waldron & Robinson, 2009; Hood & Skaar, 2012). Por exemplo, aproximadamente metade de todas as proteínas conhecidas são dependentes de um, ou mais metais para a manutenção da sua estrutura e participação nos processos celulares, como é o caso de processos de transferência de eletrões e catálise (Andreini *et al.*, 2008; Waldron & Robinson, 2009). Os metais essenciais ao funcionamento dos sistemas biológicos nunca se encontram em estado livre dentro da célula. Uma cadeia complexa de transportadores, sensores, metaloreguladores e metalochaperonas restringem a sua especiação e transportam os metais para enzimas específicas, proteínas estruturais e de armazenamento (Finney & O'Halloran, 2003). No entanto, quando concentrações excessivas destes metais essenciais são atingidas, eles podem ser letais para as células. Por exemplo, cobre (Cu) ou zinco (Zn), que participam em funções fisiológicas, podem ser tóxicos devido à formação de radicais livres, que podem interagir com vários componentes das células quando estas não conseguem compensar estas concentrações elevadas (Porcheron *et al.*, 2013). Outros, como a prata (Ag), mercúrio (Hg) e telúrio (Te), sem qualquer função fisiológica na célula, são extremamente nocivos para a grande maioria das bactérias, tendo atividade bactericida mesmo em concentrações muito baixas (Nies, 1999; Harrison *et al.*, 2004).

São inúmeras as propriedades químicas, que medeiam a reatividade dos metais com as células e, que podem estar relacionadas com a sua toxicidade para as bactérias (Lemire *et al.*, 2013). A interação dos metais com biomoléculas está relacionada com a formação de espécies reativas de oxigénio e depleção de anti-oxidantes [ex: Cr(VI), As(III), Te(IV), Fe(II), Cu(II)], com fenómenos associados a disfunção proteica e inibição enzimática [Cr(VI)], comprometimento da função membranar [ex: Cu(II), Ag(I), Al], interferência com a assimilação de nutrientes [ex: Cr(VI), Ga(III)] e genotoxicidade [ex: Mn(II), Cr(VI), Co(II)] (Figura 4).

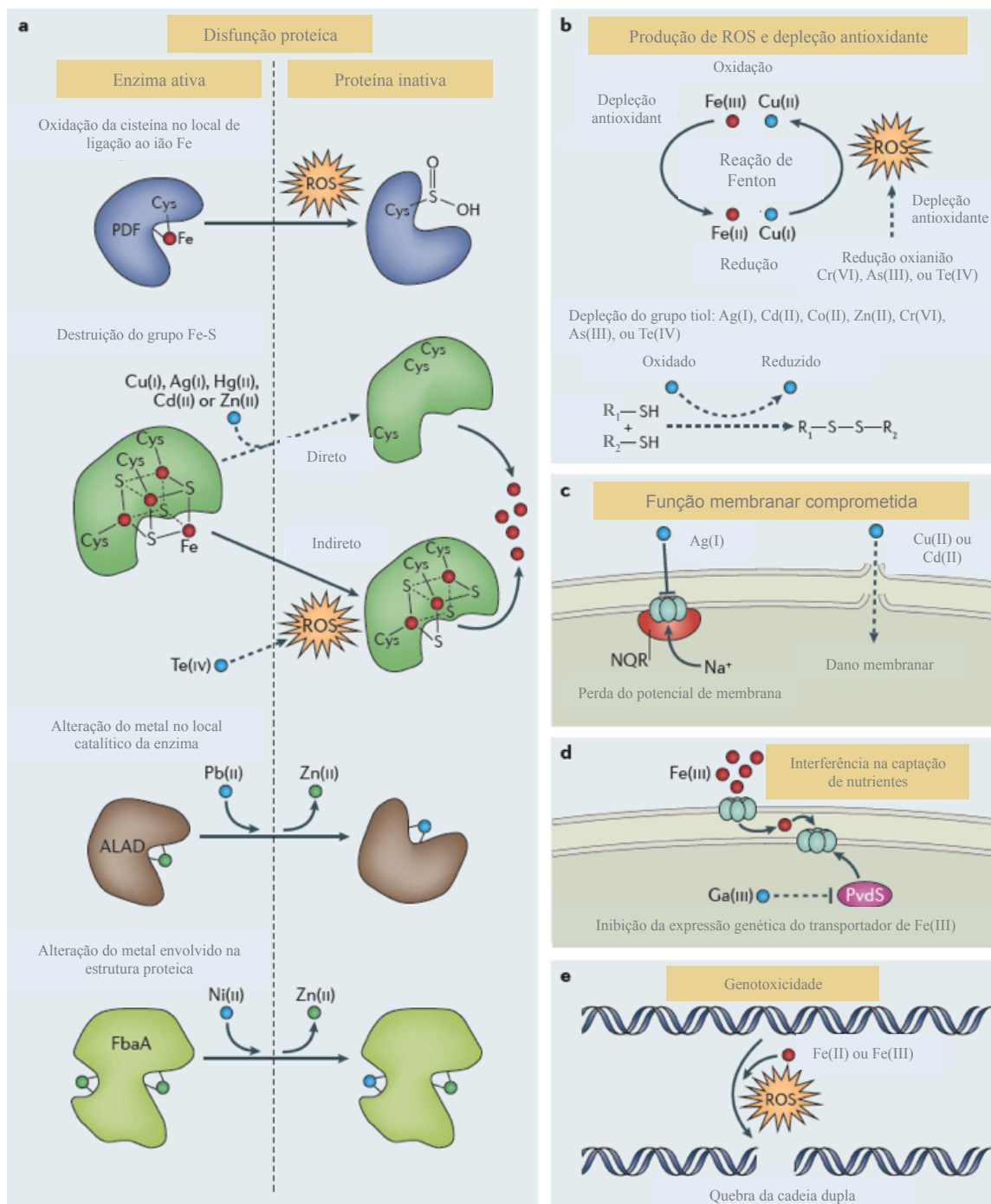


Figura 4. Esquematização dos mecanismos de toxicidade dos metais. a) Disfunção proteica, por exemplo com perda da sua atividade catalítica; b) Produção de espécies reativas de oxigênio e depleção de antioxidantes, que podem levar à degradação de DNA; c) Comprometimento da função membranar, alterando a sua integridade; d) Interferência com a assimilação de nutrientes; e) Genotoxicidade. Setas ininterruptas representam eventos bioquímicos conhecidos. Setas descontinuas representam mecanismos de toxicidade desconhecidos; ALAD – ácido δ-aminolevulínico desidratase; FbaA - frutose-1,6-bisfosfato aldase; NQR, NADH - quinona oxidoreductase; PDF - péptido deformilase; PvdS - fator σ presente em *Pseudomonas aeruginosa*. Para mais detalhe sobre estes mecanismos consultar Lemire *et al.*, 2013 (Figura retirada e traduzida de Lemire *et al.*, 2013; reprodução com permissão da Nature Publishing Group -Anexo I)

1.6.3. Tolerância bacteriana aos biocidas e sua relação com a resistência a antibióticos

De forma semelhante aos antibióticos, o termo “resistência” tem sido largamente utilizado para os biocidas. A resistência aos antibióticos é medida em concentrações inibitórias do crescimento, podendo estes ser utilizados como agentes terapêuticos quando a bactéria é suscetível a um antibiótico bacteriostático ou bactericida. No entanto, os biocidas têm a intenção de inibir a viabilidade de populações bacterianas, questionando-se, assim, se o termo “resistência” se aplica nas mesmas condições. Quando o tópico é biocidas, os termos “resistência” e “tolerância” têm sido usados, frequentemente, como sinónimos mas, recentemente, propôs-se que fossem aplicados em contextos distintos (Cerf *et al.*, 2010):

-*Resistência* - recomenda-se a sua aplicação quando o fenómeno que está a ser estudado é a morte microbiana;

-*Tolerância* - recomenda-se a sua aplicação quando o fenómeno que está a ser estudado são adaptações microbianas a concentrações inibitórias de biocidas.

Nesta tese, será adotada a palavra “tolerância” a partir deste ponto.

A resposta bacteriana aos biocidas é fundamentalmente determinada pela natureza do agente químico e o tipo de microrganismo envolvido. Outros fatores como a temperatura e tempo de contacto, pH ambiental e a presença de matéria orgânica podem exercer um efeito considerável na atividade do agente antibacteriano (Russel & Russel, 1995). Também o facto de os microrganismos se encontrarem em biofilmes, ou não, pode determinar a eficácia do biocida (Ortega *et al.*, 2013).

Tal como para os antibióticos, os microrganismos podem possuir uma tolerância intrínseca, ou adquirida aos biocidas, esta última associada ao aparecimento de mutações em genes pré-existentes, ou aquisição de novos genes. Dentro das bactérias, as micobactérias são apontadas como as menos susceptíveis, seguidas do grupo das bactérias de Gram negativo e por fim das de Gram positivo (Brown & Gilbert, 1993).

Os mecanismos de tolerância podem ser diversos e estão, diretamente, relacionados com a diminuição da concentração intracelular de biocida (EFSA, ECDC, EMEA., 2009; SCENIHR, 2009). Incluem i) alteração da permeabilidade; ii) presença de bombas de efluxo; iii) alteração/degradação enzimática; iv) destoxificação, ou v) alteração do local alvo (Maillard, 2005; Stickler *et al.*, 2004; SCENIHR, 2009). Alguns exemplos destes mecanismos de tolerância são sumariados no parágrafo seguinte.

A suscetibilidade de bactérias de Gram positivo e de Gram negativo pode variar devido às especificidades da sua parede celular. Por exemplo, já foi demonstrado que bactérias de Gram negativo são menos susceptíveis a alguns biocidas (v.g., compostos de amónio

quaternário) devido à presença da membrana externa e dos canais de porina, que impedem a difusão dos biocidas para o interior da célula (Gnanadhas *et al.*, 2012). A presença de bombas de efluxo é verificada quer em bactérias de Gram negativo, quer de Gram positivo. Os genes *qacA* e *qacJ* são responsáveis pela diminuição de susceptibilidade a compostos de amónio quaternário e biguanidas em *Staphylococcus haemolyticus* (Ye *et al.*, 2012; Correa *et al.*, 2008). A bomba de efluxo codificada pelo gene *acrAB* em *Escherichia coli* é responsável pela expulsão de compostos como brometo de etídio, acriflavina, SDS, entre outros (Gnanadhas *et al.*, 2012). Outros mecanismos são descritos em *Pseudomonas* spp., que têm a capacidade de degradar compostos como os de amónio quaternário através de processo de N-desalquilação (Nishihara *et al.*, 2000). Na alteração dos estados de oxidação do cobre, por exemplo através de enzimas *multicopper* oxidases estão envolvidos iões de cobre menos tóxicos para a célula, por exemplo em *E. coli*, ou *Staphylococcus aureus* (Singh *et al.*, 2004; Sitthisak *et al.*, 2005).

Alguns destes mecanismos de tolerância aos biocidas são comuns aos antibióticos, existindo assim fenómenos de *resistência cruzada*. Por exemplo, a bomba de efluxo codificada por *acrAB* em *E. coli* para além de expulsar os compostos mencionados, anteriormente, também confere resistência à tetraciclina, ciprofloxacina e β -lactâmicos (Ma *et al.*, 1994). A sobreexpressão da bomba de efluxo codificada pelo genes *norA*, *norB* ou *norC* de *S. aureus* contribuem para a resistência a quinolonas (Truong-Bolduc *et al.*, 2006). Também podem ocorrer fenómenos de *co-seleção*, em que genes que codificam para a tolerância a biocidas e resistência a antibióticos se encontrem nos mesmos elementos genéticos (v.g., plasmídeos, integrões e transposões) e sejam adquiridos em simultâneo, quando os microrganismos estão sujeitos a distintas pressões seletivas, quer associadas a biocidas quer a antibióticos (McIntosh *et al.*, 2008; Soge *et al.*, 2008; SCENIHR, 2009). Por exemplo, foi descrito em isolados clínicos e de alimentos de *Staphylococcus* spp., plasmídeos portadores de genes *qac* (codificam para tolerância a compostos de amónio quaternário) e genes de resistência a antibióticos (*blaZ*, β -lactâmicos; *aacA-aphD*, aminoglicosídeos; *dfrA*, trimetoprim) (Sidhu *et al.*, 2001, 2002). Adicionalmente, foi identificado em *Aeromonas salmonicida* subsp. *salmonicida*, isolada de salmão de aquaculturas, um plasmídeo tipo IncA/C, que incluía genes associados à tolerância ao mercúrio e, que codificavam para a resistência a antibióticos como aminoglicosídeos, sulfamidas, cloranfenicol, tetraciclina e β -lactâmicos (*aadA7*, *sull*, *sullI*, *floR*, *tetA*, *tetR*, *strA*, *strB*, and *bla_{CMY-2}*) (McIntosh *et al.*, 2008).

Várias bactérias de Gram negativo possuem integrões de classe 1 com diversos genes de resistência a antibióticos assim como os genes *qac* (Sandvang *et al.*, 1998; Gaze *et al.*, 2005; Buffet-Bataillon *et al.*, 2012). O elevado consumo de biocidas que se verifica,

atualmente, em diferentes setores (produção animal, ambiente clínico, vida quotidiana) tem conduzido a uma preocupação emergente de diferentes entidades (Comissão Europeia, EFSA, EMEA, ECDC) (SCENIHR, 2009), associada quer à perda da atividade destes compostos importantes para o controlo do crescimento dos microorganismos (*v.g.*, desinfecção, antisepsia, conservação), quer ao seu possível impacto na seleção de bactérias resistentes a antibióticos (SCENIHR, 2009). Esta preocupação veio acompanhada de uma análise do conhecimento disponível e de lacunas científicas, que necessitam de ser preenchidas para controlar atempadamente a emergência e extensa disseminação de microorganismos tolerantes a biocidas e resistentes a antibióticos. Assim, tem-se apelado ao desenvolvimento de estudos em tópicos relacionados com a avaliação de risco (quantificação da exposição a biocidas, disseminação de genes de tolerância, mudanças nas populações bacterianas após exposição a biocidas), com o desenvolvimento de protocolos standardizados para avaliação da susceptibilidade a biocidas, com o desenvolvimento de programas de vigilância, ou com a avaliação do impacto microbiano associado à supressão de várias substâncias ativas pela recente legislação Europeia (nº1451/2007; 4 Dezembro 2007/2008/809/CE –14 Outubro 2008).

1.6.4. Cobre, mercúrio e compostos de amónio quaternário

Nesta seção serão abordados em maior detalhe os biocidas que foram alvo de estudo desta tese.

1.6.4.1. Metais pesados: cobre e mercúrio

Os metais pesados incluem, entre outros, o cobre, mercúrio, arsénio, cádmio, chumbo e o zinco (Silver, 1996). Têm uma distribuição ubíqua na natureza e não são sujeitos a degradação, podendo por isso permanecer no ambiente durante longos períodos de tempo e exercer uma ação seletiva contínua das bactérias, que a eles são tolerantes (Bruins *et al.*, 2000).

1.6.4.1.1. Cobre

O cobre é um elemento de transição, classificado no grupo IB da tabela periódica, que apresenta quatro estados de oxidação: cobre metálico (Cu^0), ião cuproso (Cu^+), ião cúprico (Cu^{2+}) e ião trivalente (Cu^{3+}) (Linder & Hazegh-Azam, 1996). Tem uma distribuição ubiquitária na natureza e abunda na crosta terrestre em concentrações na ordem dos

68ppm, ocorrendo nas formas de sulfito, óxido ou carbonato (Tylecote, 1992). Na natureza é, frequentemente, encontrado na forma metálica, enquanto que nos sistemas biológicos o estado de oxidação predominante é o Cu^{2+} , resultado da oxidação do muito instável Cu^+ (Linder & Hazegh-Azam, 1996).

O cobre é um oligoelemento essencial para os seres vivos, desempenhando um papel fundamental em vários processos celulares. As inúmeras funções biológicas em que participa fazem com que seja, amplamente, utilizado como aditivo alimentar em rações usadas na produção animal intensiva (Diretiva 70/524/CEE, 2004; EFSA, 2012b). Tem influência no crescimento fetal e desenvolvimento pós-natal, na síntese de hemoglobina, maturação do tecido conectivo (especialmente do sistema cardiovascular e ossos), na função do sistema nervoso e em processos inflamatórios (http://ec.europa.eu/food/fs/sc/scan/out115_en.pdf). Estes benefícios estão relacionados com a participação do cobre em diferentes processos metabólicos, sendo por exemplo um componente crítico de proteínas. Os diferentes estados de oxidação do cobre (Cu^+ , Cu^{2+}) fornecem um ambiente químico enriquecido para diversos ligandos biológicos (*v.g.*, enzimas), parceiros em muitos papéis estruturais e catalíticos (Samanovic *et al.*, 2012). A deficiência em cobre pode originar nos animais fraturas espontâneas, perturbações cardíacas e vasculares, diminuição da produção de ovos, entre outros sintomas (http://ec.europa.eu/food/fs/sc/scan/out115_en.pdf). Também intervêm em processos biológicos de células procariotas, tendo a análise de 450 genomas bacterianos mostrado que 72% destes possuem genes, que codificam para pelo menos uma proteína dependente de cobre (Ridge *et al.*, 2008). Exemplos de enzimas e proteínas dos sistemas biológicos (eucariotas, ou procariotas) com os quais o cobre está envolvido são citocromo C, metano monooxidasas e superóxido dismutases, ou relacionadas com o transporte de iões. Por exemplo, a ceruloplasmina (ferroxidase I) é uma proteína associada ao transporte de cobre e necessária para a incorporação de ferro na transferrina levando-o para o citoplasma (Linder & Hazegh-Azam, 1996).

Apesar do seu papel vital em muitos processos celulares, concentrações excessivas de cobre são tóxicas, quer para células eucariotas, quer para células procariotas (Odermatt & Solioz 1995; Linder & Hazegh-Azam, 1996; Rademacher & Masepohl, 2012; Samanovic *et al.*, 2012). Vários mecanismos associados à toxicidade do cobre têm sido descritos, nomeadamente a sua capacidade de aceitar e doar eletrões, alterando o seu estado redox entre $\text{Cu}^+/\text{Cu}^{2+}$. Em condições anaeróbias, esta propriedade redox origina a produção de radicais livres, através das reações de Haber-Weiss e Fenton (Halliwell & Gutteridge 1984; Karlin, 1993; Yoshida *et al.*, 1993; Solioz & Stoyanov, 2003; Magnani & Solioz, 2007), podendo causar danos em lípidos, proteínas e ácidos nucleicos (Yoshida *et al.*, 1993; Hodgkinson & Petris, 2012). Alterações na estrutura proteica devido a

interações do cobre com cadeias polipeptídicas, associadas à exclusão de co-fatores metálicos nativos dos seus ligandos, podem também ocorrer (v.g., alterações de *clusters* de proteína ferro-enxofre, devido a este último ter maior propensão para se ligar ao Cu(I)) (Hodgkinson & Petris, 2012).

As propriedades antimicrobianas do cobre têm sido aproveitadas para a sua ampla utilização em produtos biocidas (v.g., em desinfetantes, preservantes de madeira, fungicidas, algicidas e conservantes) (EFSA/ECDC/EMEA, 2009; SCENIHR, 2009). Diversos estudos também têm sugerido a utilização de ligas de cobre em superfícies de unidades hospitalares, ou da indústria alimentar para controlar o crescimento bacteriano e transmissão horizontal de genes, nomeadamente em *Enterococcus* spp. resistentes aos glicopéptidos (Warnes *et al.*, 2010; Warnes *et al.*, 2011; Warnes *et al.*, 2012; Zhu e tal., 2012).

Os microrganismos têm desenvolvido diversos sistemas regulatórios de homeostase de modo a manter o cobre intracelular dentro de concentrações fisiológicas, evitando, desta forma, a sua toxicidade (Kim *et al.*, 2008). Entre estes sistemas de regulação encontram-se bombas de efluxo, chaperonas para a via intracelular, oxidases e redutases, que permitem alterar os estados de oxidação, e reguladores que controlam a expressão de diferentes genes (Magnani & Solioz, 2007). Podem dar-se os exemplos de sistemas de homeostase mediados por ATPases transportadoras de cobre, sistemas de bombas de efluxo pertencentes à família RND (*resistance-nodulation-cell division*), ou sistemas de destoxificação através de *multicopper* oxidases (MCO), que conferem tolerância a concentrações baixas de cobre (Rademacher & Masepohl, 2012).

As copper-ATPases, como por exemplo CopA e CopB, pertencem ao subgrupo da superfamília das P-type ATPases e são designadas de ATPases de metais pesados, ou CPx-type ATPases (Lutsenko & Kaplan, 1995; Solioz & Vulpe, 1996). Estas enzimas encontram-se disseminadas na natureza, tendo como função catalizar o transporte de metais de transição e pesados para as células (Cu^+ ; Ag^+ ; Co^{2+} ; Zn^{2+} ; Hg^{2+} e Pb^{2+}) (Axelsen & Palmgren, 1998). Além dos mecanismos de transporte do cobre são ainda de referir os de regulação que são do tipo CopY, CsoR, CutC e CueR (Stoyanov *et al.*, 2001; Latorre *et al.*, 2011; Solioz *et al.*, 2011). O tipo CopY é o principal regulador de transcrição que responde ao cobre nas bactérias ácido-lácticas (LAB), encontrando-se associado, por exemplo, à regulação dos genes do operão Cop em *E. hirae* (Strausak & Solioz, 1997), *E. faecium* (Hasman *et al.*, 2006), *Lactococcus lactis* IL1403 (Magnani *et al.*, 2008), *Streptococcus mutans* (Vats & Lee, 2001; Portmann *et al.*, 2006) e *Streptococcus gordonii* (Mitrakul *et al.*, 2004). O regulador da família de proteínas Cut é crucial para o controlo da homeostasia do cobre, havendo evidências científicas de que a expressão do gene *cutC* depende de diferentes concentrações de cobre, podendo estar

envolvido direta, ou indiretamente, na proteção da célula contra concentrações superiores às fisiologicamente suportadas (Latorre *et al.*, 2011).

O regulador CsoR-Type encontra-se mais disseminado nos procariotas que o CopY-type (Solioz *et al.*, 2011). No entanto, embora as descrições em espécies LAB sejam poucas, foi identificado em *Bacillus subtilis* (Solioz *et al.*, 2011). Outro regulador de transcrição é o CueR, encontrando-se descrito em apenas algumas espécies de *Bacillales*, que não incluem LAB, sendo comum em bactérias de Gram negativo (Solioz *et al.*, 2011). CueO é uma enzima periplasmática que cataliza a oxidação do ião cuproso a ião cúprico, protegendo a célula ao evitar formas reduzidas do metal, não permitindo a ocorrência da reação de Fenton, que levaria à formação de radicais livres (Grass *et al.*, 2004; Singh *et al.*, 2011).

Um dos mecanismos de controlo da homeostase do cobre mais conhecido e estudado é codificado pelo operão *copYAZB* de localização cromossomal em *E. hirae* e, que confere tolerância a concentrações moderadas de cobre (8mM) (Solioz & Stoyanov, 2003). O operão Cop é constituído por duas ATPases, CopA e CopB, com função de transporte do cobre intra e extracelularmente. A proteína CopA recruta o cobre para o interior da célula, mas quando são atingidas concentrações superiores às fisiológicas, CopB expulsa-o. A proteína CopY é um repressor de resposta à presença de cobre, enquanto que CopZ codifica para uma chaperona que catalisa a expulsão de cobre (Odermatt & Solioz, 1995; Solioz & Stoyanov, 2003; Solioz *et al.*, 2010) (Figura 5). O operão Cop pode também ser ativado pela presença de Cd^+ ou Ag^+ (5mM) (Odermatt & Solioz, 1995).

Em *E. faecalis* foi identificado um gene regulador da homeostasia do cobre, pertencente à família de proteínas Cut, o gene *efcutC*. Verificou-se que perante concentrações elevadas de cobre e na ausência deste gene, os níveis intracelulares de cobre aumentavam, sugerindo que *efcutC* esteja implicado nos mecanismos de homeostasia deste metal (Latorre *et al.*, 2011). Recentemente, foi também descrito, para esta espécie, que a regulação das concentrações intracelulares de cobre envolve vários módulos de genes, que podem ser ativados por diferentes concentrações deste metal. A eliminação de um dos módulos de homeostasia e a reconfiguração da expressão genética mostrou que a regulação da homeostasia do cobre nesta espécie parece estar associada a um sistema complexo (Latorre *et al.*, 2014).

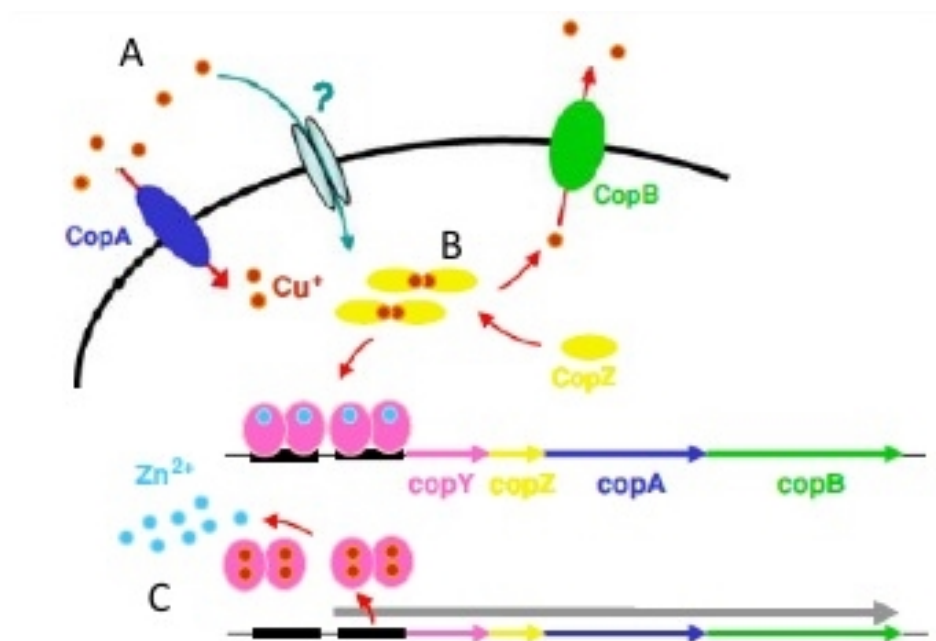


Figura 5. Mecanismo de homeostase do cobre em *Enterococcus hirae*. A - O cobre entra na célula via CopA, ou por um mecanismo não específico. B - Excesso de cobre citoplasmático liga-se a CopZ, que pode, posteriormente, fornecer Cu^+ a CopB para a sua expulsão da célula e a CopY, repressor do funcionamento do operão. C - Em concentrações baixas de cobre, dois dímeros de CopY são formados. Quando CopZ doa Cu^+ a CopY, um Zn^{2+} por cada monômero de CopY é substituído por dois Cu^+ , com a, posterior, liberação de CopY do promotor e indução da transcrição dos genes do operão a jusante. (Figura retirada de Solioz *et al.*, 2010; Reprodução com permissão da *Journal of Biological Inorganic Chemistry* - Anexo1).

Um sistema de tolerância a concentrações elevadas de cobre ($\text{CMI} > 8\text{mM}$) foi descrito por Hasman *et al.* (2005). Identificaram o operão *tcrYAZB* em *E. faecium*, sendo a funcionalidade de cada um dos genes semelhante a *copYAZB* de *E. hirae*. O operão *tcrYAZB* estava flanqueado por duas IS (IS1216 e tipo ISS1) e localizado em plasmídeos conjugativos (175-194Kb), sendo considerado o primeiro sistema de tolerância adquirida ao cobre em *Enterococcus* spp. (Hasman & Aarestrup, 2002; Amachawadi *et al.*, 2011; Amachawadi *et al.*, 2013). Este plasmídeo também era portador de genes que codificavam para a resistência a macrólidos [*erm*(B)] e glicopéptidos (*vanA*).

A pesquisa deste operão tem sido feita através do gene *tcrB* (“transferable copper resistance gene B”; codifica para uma ATPase do tipo CPx), mas apenas em isolados de algumas regiões geográficas e recuperados fundamentalmente do nicho animal, nomeadamente de suínos, que consumiam cobre como aditivo alimentar (Aarestrup *et al.*, 2002; Hasman & Aarestrup, 2002; Amachawadi *et al.*, 2010; Kim *et al.*, 2012). Só, ocasionalmente, se fez a pesquisa deste gene em outros nichos ecológicos (outros tipos de animais como aves, cabras, bovinos, ou humanos) (Hasman & Aarestrup, 2002; Kim *et al.*, 2012). A limitação de dados acerca da ecologia e estrutura populacional de

Enterococcus spp., que albergam o operão *tcrYAZB*, impede conclusões robustas acerca do seu impacto na sobrevivência deste género a concentrações elevadas de cobre e na seleção de plasmídeos portadores de genes de resistência a antibióticos.

Outro mecanismo de tolerância ao cobre, uma multicopper-oxidase (*cueO*), foi identificado em um isolado de *E. faecium* desconhecendo-se, no entanto, a sua prevalência e distribuição em *Enterococcus* spp. (van Schaik & Willems, 2010).

A identificação de genes associados à tolerância ao cobre, juntamente com genes que conferem resistência a antibióticos em *Enterococcus* spp., tem constituído uma preocupação para a Comissão Europeia, no sentido de tentar compreender o impacto deste aditivo alimentar usado em rações, na seleção e manutenção de bactérias resistentes aos antibióticos no ambiente de produção animal (SCENIHR, 2009; EFSA, 2012b).

1.6.4.1.2. Mercúrio

O mercúrio é o sexto elemento mais tóxico num universo de 6 milhões de substâncias e existe, naturalmente, no ambiente em pequenas concentrações, devido à libertação de gases a partir da crosta terrestre (v.g., erupções vulcânicas, tremores de terra). Os seus níveis na biosfera (ar, água e solo) têm vindo a aumentar através de diversas atividades antropogénicas (v.g., uso de catalisadores na indústria, uso de pesticidas, queima de carvão e produtos petrolíferos), em alguns casos em milhares de vezes comparativamente aos níveis naturais (Nascimento & Chartone-Souza, 2003; Magos & Clarkson, 2006; Skurnik *et al.*, 2010; Mathema *et al.*, 2011; EFSA, 2012c). A distribuição ubíqua deste metal na natureza expõe seres humanos, animais, plantas e seus microrganismos por diferentes vias, nomeadamente através da cadeia alimentar (Mathema *et al.*, 2011; EFSA, 2012c).

A afinidade do mercúrio para moléculas orgânicas leva, geralmente, à formação de complexos organomercuriais, que são de elevada toxicidade. Os iões de mercúrio associam-se, fundamentalmente, aos grupos sulfidrilo de resíduos de cisteína de enzimas e proteínas essenciais, tornando-as inativas e bloqueando as suas funções celulares vitais (Mathema *et al.*, 2011). Vários microrganismos desenvolveram mecanismos para combater esta toxicidade de forma a sobreviverem em ambientes onde a concentração deste metal poderia ser tóxica (Nascimento & Chartone-Souza, 2003; Mathema *et al.*, 2011). O mecanismo de tolerância bacteriano mais comum é a redução enzimática de Hg^{2+} à forma volátil Hg^0 . Esta biotransformação é mediada por uma flavina, a mercúrio redutase (MerA) codificada pelo gene *merA*, através de uma reação dependente do NADPH (Liebert *et al.*, 1997; Bogdanova *et al.*, 1998). Estes genes *merA* estão

integrados em operões, com diferentes organizações genéticas, que são encontrados quer em bactérias de Gram negativo, quer de Gram positivo (Mindlin & Petrova, 2013). Nestes operões estão envolvidos vários genes, que incluem *merD* para detecção, *merP/merT/merC* para transporte e *merA/merB* para destoxificação enzimática (Mindlin & Petrova, 2013). Os genes do operão *mer* encontram-se sob regulação de *merR*, ativado após exposição a Hg^{2+} . Existem dois grupos de operões, um que confere tolerância, unicamente, aos sais inorgânicos de mercúrio (espectro reduzido) e outro que confere tolerância a estes e compostos organomercuriais (espectro alargado) (Osborn *et al.*, 1997; Nascimento & Chartone-Souza, 2003; Mathema *et al.*, 2011). Este último grupo está presente quando o operão *mer*, para além do gene *merA*, inclui um segundo gene (*merB*, ou mais raramente *merG*), que codifica para a enzima mercúrio liase, conferindo-lhe tolerância ao mercúrio orgânico (Mathema *et al.*, 2011; Mindlin & Petrova, 2013). Variações estruturais, de organização e número de genes dentro do operão *mer* têm sido descritas em diferentes bactérias, demonstrando a sua natureza mosaico (Liebert *et al.*, 1997; Osborn *et al.*, 1997; Møller *et al.*, 2013).

Os genes *mer* podem estar localizados em plasmídeos, ou no cromossoma, e têm sido identificados em transposões de bactérias de Gram negativo e de Gram positivo (Bogdanova *et al.*, 1998; Nascimento & Chartone-Souza, 2003; Mathema *et al.*, 2011). A transferência horizontal parece contribuir para a disseminação e evolução genética dos operões *mer* (Osborn *et al.*, 1997; Mindlin *et al.*, 2002), havendo descrições da partilha das mesmas estruturas genéticas por bactérias de géneros diferentes e bactérias do mesmo género, que transportam diferentes variantes (Bogdanova *et al.*, 1998; Mindlin *et al.*, 2002; Stapleton *et al.*, 2004). Adicionalmente, a co-transferência de operões *mer* e de resistência a antibióticos tem sido detetada quer na natureza, quer em condições experimentais (Mathema *et al.*, 2011). Estudos em diferentes bactérias, demonstraram que ambientes enriquecidos em mercúrio favorecem a seleção de isolados resistentes a antibióticos (Summers *et al.*, 1993; Skurnik *et al.*, 2010).

A prevalência de genes *merA*, ou operões *mer*, em *Enterococcus* spp. tem sido objeto de escassos estudos (Ojo *et al.*, 2004; Davies *et al.*, 2005; Soge *et al.*, 2008), tendo sido descritos em isolados oriundos de primatas, de pacientes, ou amostras orais de origem humana (Zscheck & Murray 1990; Davies *et al.*, 2005; Soge *et al.*, 2008), apresentando estes isolados uma CMI a $HgCl_2$ de 9-16 $\mu g/ml$, contrastando com 2 $\mu g/ml$ obtido nos isolados sem genes *mer* (Zscheck & Murray 1990; Davies *et al.*, 2005).

A associação de genes de tolerância ao mercúrio e resistência aos antibióticos foi descrita apenas, ocasionalmente, nestas bactérias. Por exemplo, Davies *et al.* (2005) identificou um operão *mer* num plasmídeo conjugativo juntamente com *aadK* (resistência

à estreptomicina), Rice & Carias (1998b) no transposição Tn5385, portador de genes que codificam para a resistência ao β -lactâmicos, aminoglicosídeos, macrólidos e tetraciclinas e Soge *et al.* (2008) no Tn6009 (Tn916+operação *mer*) que codifica para a resistência à tetraciclina.

1.6.4.1.3. Compostos de amónio quaternário

Os compostos de amónio quaternário (QACs) são biocidas com ampla utilização em vários setores e vida quotidiana (v.g., produtos de uso doméstico, indústria, agricultura, hospital, atividade desinfetante e conservante) (Fraise, 2002; Hegstad *et al.*, 2010; Buffet-Bataillon *et al.*, 2012). O QAC mais, amplamente, utilizado é o cloreto de benzalcónio, mas outros são utilizados com o mesmo propósito como o cloretos de estearalcónio, isotiazol-benzalcónio, cetilpiridínio, cloreto ou brometo de cetrimónio e cloreto de didecildimetilamónio (DDAC) (Hegstad *et al.*, 2010; Buffet-Bataillon *et al.*, 2012).

Após a aplicação/uso dos QACs estes não sofrem qualquer metabolização, mantendo assim as suas propriedades biocidas quando libertados para o ambiente (Hegstad *et al.*, 2010; Tezel & Pavlostathis, 2011).

Os QACs são compostos orgânicos que se caracterizam por serem constituídos por quatro grupos funcionais (alquilo, metilo, benzilo ou éster) ligados, covalentemente, a um átomo de azoto (R_4-N^+). São classificados em cinco grupos mediante estes grupos funcionais: i) monoalcónio; ii) dialcónio; iii) benzalcónio; iv) diésteralcónio e v) piridalcónio (halogenetos) (Hegstad *et al.*, 2010; Tezel & Pavlostathis, 2011; Buffet-Bataillon *et al.*, 2012). O seu peso molecular varia entre 250-400g/mol. Estes compostos são constituídos por duas porções distintas, uma hidrofóbica (v.g., grupo alquilo) e outra hidrofílica (N^+), o que lhes permite manter as suas propriedades catiónicas numa escala variável de pH (Tezel & Pavlostathis, 2011; Al-Adham *et al.*, 2013). No entanto, para que os QACs tenham atividade antimicrobiana, pelo menos um dos grupos funcionais deve conter uma cadeia longa de oito a dezoito carbonos (C_8-C_{18}) (Hegstad *et al.*, 2010; Al-Adham *et al.*, 2013). QACs constituídos por 12-14C atuam melhor em bactérias de Gram positivo e aqueles com 14-16C em bactérias de Gram negativo. São capazes de eliminar 99% dos microrganismos desde que sejam utilizados em concentrações adequadas (Tezel & Pavlostathis, 2011). Compostos com menos de 4C, ou mais de 18C são, praticamente, inativos (Hegstad *et al.*, 2010).

Os QACs atuam nas bactérias interagindo com a membrana citoplasmática, nomeadamente ao nível da bicamada lipídica, levando à sua disrupção e libertação do conteúdo celular. No caso das bactérias de Gram negativo esta interação dá-se ao nível

da membrana externa. Isto acontece devido às interações iônicas entre o seu átomo de azoto (carga positiva) e fosfolípidos (carga negativa) presentes na membrana celular, o que permite a integração da sua cauda hidrofóbica no *core* hidrofóbico da membrana celular. Estas interações diminuem a fluidez da membrana, o que leva à perda de osmoregularidade e função fisiológica, conduzindo à lise celular (Gilbert & Moore 2005; Sheldon, 2005; Hegstad *et al.*, 2010; Maillard, 2002; Buffet-Bataillon *et al.* 2012). Em elevadas concentrações, os QACs solubilizam componentes da membrana celular pela formação de micelas (Hegstad *et al.*, 2010). A toxicidade, biodisponibilidade e biodegradabilidade dos QACs pode ser diminuída na presença de matéria orgânica, biofilmes e sais minerais, ou ser potenciada pelo aumento da temperatura e tempo de exposição, ou agentes quelantes como o EDTA (Hegstad *et al.*, 2010; Tezel & Pavlostathis, 2011).

A tolerância intrínseca a estes compostos pode ser mediada por alteração da permeabilidade membranar, ou expulsão destes compostos por bombas de efluxo codificadas por genes cromossomais. É devido a estes mecanismos que, na presença de baixas concentrações de QACs, as bactérias de Gram positivo apresentam uma maior suscetibilidade, que as de Gram negativo, mais protegidas devido à presença da membrana externa (McDonnell & Russell, 1999; Hegstad *et al.*, 2010). Adicionalmente, as bactérias podem adquirir tolerância a QACs pela incorporação de novos genes, ou através de mutações em genes pré-existentes conducentes a alterações fenotípicas. Estas alterações podem estar relacionadas com alteração da permeabilidade, ou composição química da membrana celular (*v.g.*, alterações de ácidos gordos, fosfolípidos, lipolissacarídeos, proteínas) (Chaplin, 1952; Guérin-Méchin *et al.*, 1999; Dubois-Brissonnet *et al.*, 2001; Ishikawa *et al.*, 2002; Loughlin *et al.*, 2002; Tabata *et al.*, 2003; Boeri *et al.*, 2007), assim como a ocorrência de bombas de efluxo (Hegstad *et al.*, 2010).

Entre os genes adquiridos, que codificam para bombas de efluxo em bactérias de Gram positivo, nomeadamente em plasmídeos que também transportam genes, que codificam para a resistência a antibióticos, destacam-se os seguintes: i) *qacA*, detetado no plasmídeo pSK1 *S. aureus* e no pSK57 também portador do gene *blaZ* (codifica para uma β -lactamase) (Gillespie *et al.*, 1986); ii) *qacA/B*, detetado em *S. aureus* no plasmídeo pMS62 juntamente com o gene *blaZ* (Anthonisen *et al.*, 2002; Sidhu *et al.*, 2002; Alam *et al.*, 2003; McGann *et al.*, 2011); iii) *smr* (*qacC* e *qacD*) descrito em *Staphylococcus pasteurii* no plasmídeo pSP187 (Bjorland *et al.*, 2007); iv) *qacG* em *Staphylococcus haemolyticus* (Ye *et al.*, 2012; Correa *et al.*, 2008); e v) *qacH* em *S. haemolyticus* (Correa *et al.*, 2008); e vi) *qacJ* em *Staphylococcus* spp. no plasmídeo pNVH01 (Bjorland *et al.*, 2003), *S. haemolyticus* (Ye *et al.*, 2012; Correa *et al.*, 2008). Diferentes genes *qac*

também foram descritos em bactérias de Gram negativo tais como *qacE*, *qacEΔ*, *qacF*, *qacH*, *qacG*, tendo sido detetados em integrões de classe 1 (Sandvang *et al.*, 1998; Gaze *et al.*, 2005; Buffet-Bataillon *et al.*, 2012).

Os genes de tolerância aos QACs, assim como a sua localização, têm sido pouco estudados em *Enterococcus* spp., havendo apenas descrições ocasionais do gene *qacA/B*, *smr* e *qacZ* em isolados de vários nichos ecológicos obtidos em Portugal e Alemanha (Braga *et al.*, 2011; Bischoff *et al.*, 2012). O *qacEΔ* foi também descrito em isolados clínicos do Japão em *Enterococcus* spp. (Kazama *et al.*, 1998). A aquisição destes genes por *Enterococcus* spp., nos estudos disponíveis, traduziu-se, em alguns casos, em alteração de suscetibilidade a diferentes QACs comparativamente à população selvagem. Por exemplo, no estudo de Bischoff *et al.*, (2012), *E. faecalis* com o gene *qacA/B* tinham uma CMI ao cloreto de didecildimetilamónio de 2,45-3,5µg/ml, contrastando com 1,05µg/ml das bactérias sem gene. No entanto, no estudo de Braga *et al.*, (2011), *Enterococcus* spp. com e sem o gene *qacZ* apresentaram uma CMI semelhante para o cloreto de benzalcónio, entre 2-8µg/ml. Nestes estudos não se avaliou a co-localização dos genes *qac* com outros importantes na sobrevivência/patogenicidade de *Enterococcus* spp., desconhecendo-se, até ao momento, o impacto do uso de QACs na epidemiologia da resistência a antibióticos nestas bactérias.

1.7. REFERÊNCIAS BIBLIOGRÁFICAS

Aarestrup, F. M., P. Butayne, and W. Witte. 2000. Nonhuman reservoirs of enterococci, p. 55-100. *In* M. S. Gilmore, Clewell, P. Couvalin, G. M. Dunny, BE Murray, and L. B. Rice (ed.), *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. ASM Press, Washington, D. C.

Aarestrup, F. M., H. Hasman, L. B. Jensen, M. Moreno, I. A. Herrero, L. Domínguez, M. Finn, and A. Franklin. 2002. Antimicrobial resistance among enterococci from pigs in three European countries. *Appl Environ Microbiol* **68**:4127–4129.

Aarestrup, F. M., H. C. Wegener, and P. Collignon. 2008. Resistance in bacteria of the food chain: epidemiology and control strategies. *Expert Rev Anti Infect Ther* **6**:733-750.

Abriouel, H., N. B. Omar, A. C. Molinos, R. L. López, M. J. Grande, P. Martínez-Viedma, E. Ortega, M. M. Cañamero, and A. Galvez. 2008. Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. *Int J Food Microbiol* **123**:38-49.

Agerso, Y., A. G. Pedersen, and F. M. Aarestrup. 2006. Identification of Tn5397-like and Tn916-like transposons and diversity of the tetracycline resistance gene *tet(M)* in enterococci from humans, pigs and poultry. *J Antimicrob Chemother* **57**:832-9.

Agersø, Y., C.H. Lester, L. J. Porsbo, I. Orsted, H. D. Emborg, K. E. Olsen, L. B. Jensen, O. E. Heuer, N. Frimodt-Møller, F. M. Aarestrup, and A. M. Hammerum. 2008. Vancomycin-resistant *Enterococcus faecalis* isolates from a Danish patient and two healthy human volunteers are possibly related to isolates from imported turkey meat. *J Antimicrob Chemother* **62**:844-845.

Ahmad, A., A. Ghosh, C. Schal, and L. Zurek. 2011. Insects in confined swine operations carry a large antibiotic resistant and potentially virulent enterococcal community. *BMC Microbiol* **11**:23.

Al-Adham, I., R. Haddadin, and P. Collier. 2013. Types of Microbicidal and Microbistatic Agents, pp. 5-55. *In* Fraise, A., J-Y Maillard, and S. Sattar (ed). *Principles and Practice of Disinfection, Preservation and Sterilization*. Fifth Edition, Blackwell Publishing Ltd.

Alam, M.M., N. Kobayashi, N. Uehara, and N. Watanabe. 2003. Analysis on distribution and genomic diversity of high-level antiseptic resistance genes *qacA* and *qacB* in human clinical isolates of *Staphylococcus aureus*. *Microbial Drug Resis* **9**:109-121.

Alexander, J. W. 2009. History of the medical use of silver. *Surg. Infect* **10**:289-292.

Amachawadi, R. G., N. W. Shelton, X. Shi, S. K. Narayanan, L. Zurek, S. S. Dritz, J. L. Nelssen, M. D. Tokach, and T. G. Nagaraja. 2010. Occurrence of *tcrB*, a transferable copper resistance gene, in fecal enterococci of swine. *Foodborne Pathog Dis* **7**:1089-97.

Amachawadi, R. G., N. W. Shelton, X. Shi, J. Vinasco, S. S. Dritz, M. D. Tokach, J. L. Nelssen, H. M. Scott, and T. G. Nagaraja. 2011. Selection of fecal enterococci exhibiting *tcrB*-mediated copper resistance in pigs fed diets supplemented with copper. *Appl Environ Microbiol* **77**:5597-5603.

Amachawadi, R. G., H. M. Scott, C. A. Alvarado, T. R. Mainini, J. Vinasco, J. S. Drouillard, and T. G. Nagaraja. 2013. Occurrence of the transferable copper resistance gene *tcrB* among fecal enterococci of U.S. feedlot cattle fed copper-supplemented diets. *Appl Environ Microbiol* **79**: 4369-75.

Andreini, C., L. Banci, I. Bertini, and A. Rosato. 2008. Occurrence of copper proteins through the three domains of life: a bioinformatic approach. *J Proteome Res* **7**:209-216.

Anthonisen, I. L., M. Sunde, T. M. Steinum, M. S. Sidhu, and H. Sørum. 2002. Organization of the antiseptic resistance gene *qacA* and Tn552-related beta-lactamase genes in multidrug- resistant *Staphylococcus haemolyticus* strains of animal and human origins. *Antimicrob Agents Chemother* **46**:3606-3612.

Arias, C. A, and B. E. Murray. 2008. Emergence and management of drug-resistant enterococcal infections. An extensive review of the treatment of enterococcal infections. *Expert Rev Anti Infect Ther* **6**:637-655.

Arias, C. A, D. Panesso, R. V. Singh, L. B. Rice, and B. E. Murray. 2009. Cotransfer of antibiotic resistance genes and a *hylEfm*-containing virulence plasmid in *Enterococcus faecium*. *Antimicrob Agents Chemother* **53**:4240-4246.

Arias, C. A., G. A Contreras, and B. E. Murray 2010. Management of multidrug-resistant enterococcal infections. *Clin Microbiol Infect* **16**:555-62.

Arias, C. A., D. Panesso, D. M. McGrath, X. Qin, M. F. Mojica, C. Miller, L. Diaz, T. T. Tran, S. Rincon, E. M. Barbu, J. Reyes, J. H. Roh, E. Lobos, E. Sodergren, R. Pasqualini, W. Arap, J. P. Quinn, Y. Shamoo, B. E. Murray, and G. M. Weinstock. 2011. Genetic basis for in vivo daptomycin resistance in enterococci. *N Engl J Med* **365**:892-900.

Arias, C. A., and B. E. Murray. 2012. The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat Rev Microbiol* **10**:266–278.

Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin. 1993. Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol* **175**:117-27.

Axelsen, K. B., and M. G. Palmgren. 1998. Evolution of substrate specificities in the P-type ATPase superfamily. *J Mol Evol* **46**:84-101.

Ayres, P.G. 2004. Alexis Millardet: France's forgotten mycologist. *Mycologist* **18**:23-26.

Banai, M., and D. J. LeBlanc. 1984. *Streptococcus faecalis* R plasmid pJH1 contains an erythromycin resistance transposon (Tn3871) similar to transposon Tn917. *J Bacteriol* **158**:1172-4.

Barreto, A., B. Guimarães, H. Radhouani, C. Araújo, A. Gonçalves, E. Gaspar, J. Rodrigues, G. Igrejas, and P. Poeta. 2009. Detection of antibiotic resistant *E. coli* and *Enterococcus* spp. in stool of healthy growing children in Portugal. *J Basic Microbiol* **49**:503-512.

Bennett, P. M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol* **153** Suppl1:S347-57.

Biavasco, F., G. Foglia, C. Paoletti, G. Zandri, G. Magi, E. Guaglianone, A. Sundsfjord, C. Pruzzo, G. Donelli, B. Facnelli. 2007. VanA-type enterococci from

humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants. *Appl Environ Microbiol* **73**:3307-3319.

Bischoff, M., J. Bauer, P. Preikschat, K. Schwaiger, G. Molle, and C. Holzel. 2012. First Detection of the Antiseptic Resistance Gene *qacA/B* in *Enterococcus faecalis*. *Microbial Drug Resist* **18**:7-12.

Bjorland, J., M. S. Bratlie, and T. Steinum. 2007. The *smr* gene resides on a novel plasmid pSP187 identified in a *Staphylococcus pasteurii* isolate recovered from unpasteurized milk. *Plasmid* **57**:145-55.

Boeri, P. S., C. E. Domenech, and G. I. Lucchesi. 2007. Modification of phospholipid composition in *Pseudomonas putida* A ATCC 12633 induced by contact with tetradecyltrimethylammonium. *J Appl Microbiol* **103**:1048-1054.

Bogdanova E. S., I. A. Bass, L. S. Minakhin, M. A. Petrova, S. Z. Mindlin, A. A. Volodin, E. S. Kalyaeva, J. M. Tiedje, J. L. Hobman, N. L. Brown, and V. G. Nikiforov. 1998. Horizontal spread of mer operons among Gram-positive bacteria in natural environments. *Microbiology*. **144**:609-620.

Bøhle, L. A., Riaz, T., Egge-Jacobsen, W., Skaugen, M., Ø L Busk, Ø. L., Vincent GH Eijsink, V. G. H., Mathiesen, G. 2011. Identification of surface proteins in *Enterococcus faecalis* V583. *BMC Genomics* **12**:35

Bonora, M. G., D. Oliosio, G. Lo Cascio, and R. Fontana. 2007. Phylogenetic analysis of vancomycin-resistant *Enterococcus faecium* genotypes associated with outbreaks or sporadic infections in Italy. *Microbiol Drug Resist* **13**:171-177.

Bonten, M. J, R. J. Willems, and R. A. Weinstein. 2001. Vancomycin-resistant enterococci: why are they here, and where do they come from?', *Lancet Infect Dis* **1**: 314-25.

Borkow, G., and J. Gabbay. 2009. Copper an ancient remedy returning to fight microbial, fungal and viral infections. *Curr Chem Biol* **3**:272-278.

Boykin, D. W. 2002. Antimicrobial activity of the DNA minor groove binders furamidine and analogs. *J Braz Chem Soc* **13**:763-771.

Braga, T. M., P. E. Marujo, C. Pomba, and M. F. Lopes. 2011. Involvement, and dissemination, of the enterococcal small multidrug resistance transporter *qacZ* in resistance to quaternary ammonium compounds. *J Antimicrob Chemother* **66**:283-286.

Braga, T. M., C. Pomba, and M. F. Lopes. 2012. High-level vancomycin resistant *Enterococcus faecium* related to humans and pigs found in dust from pig breeding facilities. *Vet Microbiol* **161**:344-9.

Brantl, S. 2004. Plasmid Replication Control by Antisense RNAs, pp. 47-62. *In* B. E. Funnell and G. J. Phillips (ed.), *Plasmid Biology*. ASM Press, Washington, DC.

Brouwer, M. S., P. Mullany, and A. P. Roberts. 2010. Characterization of the conjugative transposon Tn6000 from *Enterococcus casseliflavus* 664.1H1 (formerly *Enterococcus faecium* 664.1H1). *FEMS Microbiol Lett* **309**:71-6.

Brown, M.R.W., and P. Gilbert. 1993. Sensitivity of biofilms to antimicrobial agents. *Journal of Applied Bacteriology* **74**:87S-97S.

Bruand, C., S. D. Ehrlich, and L. Janniere. 1991. Unidirectional theta replication of the structurally stable *Enterococcus faecalis* plasmid pAM beta 1. *Embo J* **10**:2171-2177.

Bruins, M. R., S. Kapil, and F. W. Oehme. 2000. Microbial Resistance to Metals in the Environment. *Ecotoxicol Environl Saf* **45**:198-207.

Buffet-Bataillon, S., P. Tattevin, M. Bonnaure-Mallet, and A. Jolivet-Gougeon. 2012. Review: Emergence of resistance to antibacterial agents: the role of quaternary ammonium compounds - a critical review. *Int J Antimicrob Agents* **67**:2837-2842.

Burgos, M. J., R. L. López, H. Abriouel, N. B. Omar, and A. Galvez A. 2009. Multilocus sequence typing of *Enterococcus faecalis* from vegetable foods reveals two new sequence types. *Foodborne Pathog Dis* **6**:321-7.

Burrus, V., and M. K. Waldor. 2004. Shaping bacterial genomes with integrative and conjugative elements. *Res Microbiol* **155**:376-86.

Byappanahalli, M. N., M. B. Nevers, A. Korajkic, Z. R. Staley, and V. J. Harwood. 2012. Enterococci in the environment. *Microbiol Mol Biol Rev* **76**:685-706.

Camargo, I. L., R. C. Zanella, M. C. Brandileone, A. C. Pignatari, G. H. Goldman, N. Woodford, and A. L. Darini. 2005. Occurrence of insertion sequences within the genomes and Tn1546-like elements of glycopeptide-resistant enterococci isolated in Brazil, and identification of a novel element, *ISEfa5*. *Int J Med Microbiol* **294**:513-519.

Campos, J., J. Mourão, N. Pestana, L. Peixe, C. Novais, and P. Antunes. 2013. Microbiological quality of ready-to-eat salads: an underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. *Int J Food Microbiol* **166**:464-470.

Carias, L. L., S. D. Rudin, C. J. Donskey, and L. B. Rice. 1998. Genetic linkage and cotransfer of a novel, *vanB*-containing transposon (Tn5382) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. *J Bacteriol* **180**:4426-34.

Castillo-Rojas, G., M. Mazari-Hiriart, S. Ponce de León, R. I. Amieva-Fernández, R. A. Agis-Juárez, J. Huebner, Y. López-Vidal. 2013. Comparison of *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from water and clinical samples: antimicrobial susceptibility and genetic relationships. *PLoS One* **8**:e59491.

Cerf, O., B. Carpentier, and P. Sanders. 2010. Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "resistance" has different meanings. *Int J of Food Microbiol* **136**:247-254.

Cetinkaya, Y., P. S. Falk, C. G. Mayhall. 2002. Effect of gastrointestinal bleeding and oral medications on acquisition of vancomycin-resistant *Enterococcus faecium* in hospitalized patients. *Clin Infect Dis* **35**:935-942.

Chaplin, C. E. 1952. Bacterial Resistance to Quaternary Ammonium Disinfectants. *J Bacteriol* **63**:453-458.

Chow, J. W. 2000. Aminoglycoside Resistance in Enterococci. *In* Elioupoulos, G. M. (ed.). Antimicrobial resistance. *Inf Dis Soc Am* **31**:586-589.

Chowdhury, S. A., S. R. Nallapareddy, C. A. Arias, and B. E. Murray. 2013. The Majority of a Collection of US Endocarditis *Enterococcus faecalis* Isolates from 1974 to 2004 Lack Capsular Genes and Belong to Diverse, Non-Hospital Associated Lineages. J Clin Microbiol [Epub ahead of print].

Clark, N. C., L. M. Weigel, J. B. Patel, and F. C. Tenover. 2005. Comparison of Tn1546-like elements in vancomycin-resistant *Staphylococcus aureus* isolates from Michigan and Pennsylvania. Antimicrob Agents Chemother **49**:470-2.

Clewell, D. B., F. Y. An, B. A. White, and C. Gawron-Burke. 1985. *Streptococcus faecalis* sex pheromone (cAM373) also produced by *Staphylococcus aureus* and identification of a conjugative transposon (Tn918). J Bacteriol **162**:1212-20.

Clewell, D. B., M. V. Francia, S. E. Flannagan, and F. Y. An. 2002. Enterococcal plasmid transfer: sex pheromones, transfer origins, relaxases, and the *Staphylococcus aureus* issue. Plasmid **48**:193-201.

Clewell, D. B., and M. V. Francia. 2004. Conjugation in Gram-Positive Bacteria, p. 227-256. In B. E. Funnell and G. J. Phillips (ed.), Plasmid Biology ASM Press, Washington, DC.

Collins, M.D., C Ash, J. A. E. Farrow, S. Wallbanks, and A. M. Williams. 1989a. 16S Ribosomal ribonucleic acid sequence analyses of lactococci and related taxa. Description of *Vagococcus fluvialis* gen. nov., sp. nov. J Appl Bacteriol **67**:453–460.

Cooper, T. F., and J. A. Heinemann. 2000. Post segregational killing does not increase plasmid stability but acts to mediate the exclusion of competing plasmids. Proc Natl Acad Sci U S A **97**:12643-12648.

Coque, T. M., R. Willems, R. Canton, R. Del Campo, and F. Baquero. 2002a. High occurrence of *esp* among ampicillin-resistant and vancomycin-susceptible *Enterococcus faecium* clones from hospitalized patients. J Antimicrob Chemother **50**:1035-1038.

Coque, T. M. 2002b. Enterococcal Virulence, p. 301-354. In M. S. Gilmore, D. B. Clewell, P. Couvalin, G. M. Dunny, B. E. Murray, and L. B. Rice (ed.), The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance. ASM Press, Washington, D. C.

Coque, T. M., R. J. Willems, J. Fortún, J. Top, S. Diz, E. Loza, R. Cantón, and F. Baquero. 2005. Population structure of *Enterococcus faecium* causing bacteremia in a Spanish university hospital: setting the scene for a future increase in vancomycin resistance? *Antimicrob Agents Chemother* **49**:2693-2700.

Coque, T. M. 2008. Evolution of Pathogenic Enterococci, p. 501-521. In F. Baquero, C. Nombela, G. H. Cassell, and J. A. Gutiérrez Fuentes (ed.), *Evolutionary Biology of Bacterial and Fungal Pathogens*. ASM Press, Washington, D.C.

Coque, T. M, A. R. Freitas, C. Novais, L. Peixe, Luísa, and F. Baquero. 2011. Mobile Genetic Elements and Lateral Genetic Transfer in Enterococci. *In* Semedo-Lemsaddek, T., M. T. Barreto-Crespo, and R. Tenreiro R (ed.). *Enterococcus and Safety*. Series: Advances in Food Safety and Food Microbiology. USA, Nova Science Publishers.

Cordina, C., R. Hill, A. Deshpande, J. Hood, and T. Inkster. 2012 Tigecycline-resistant *Enterococcus faecalis* associated with omeprazole use in a surgical patient. *J Antimicrob Chemother* **67**:1806-1807.

Correa, J. E., A. De Paulis, S. Predari, D. O. Sordelli, and P. E. Jeric. 2008. First report of *qacG*, *qacH* and *qacJ* genes in *Staphylococcus haemolyticus* human clinical isolates. *J Antimicrob Chemother* **62**:956-960.

Courvalin, P. 2006. Vancomycin resistance in gram-positive cocci. *Clin Infect Dis* **42**:S25-34.

Courvalin, P. 2008. Evolution of Glycopeptide Resistance, p. 279-295. *In* F. Baquero, C. Nombela, G. H. Cassell, and J. A. Gutiérrez-Fuentes (ed.), *Evolutionary Biology of Bacterial and Fungal Pathogens*, vol. 24. ASM Press, Washington, DC.

Criado, R., J. Gutierrez, A. Budin-Verneuil, P. E. Hernandez, A. Hartke, L. M. Cintas, Y. Auffray, and A. Benachour. 2008. Molecular analysis of the replication region of the pCIZ2 plasmid from the multiple bacteriocin producer strain *Enterococcus faecium* L50. *Plasmid* **60**:181-9.

Davies, I. J., A. P. Roberts, D. Ready, H. Richards, M. Wilson, and P. Mullany. 2005. Linkage of a novel mercury resistance operon with streptomycin resistance on a conjugative plasmid in *Enterococcus faecium*. *Plasmid* **54**:26-38.

Deák, T. 2011. A survey of current taxonomy of common foodborne bacteria. Part II. Gram-positive phyla of *Firmicutes* and *Actinobacteria*. *Acta Alimentaria* **40**:95-116.

de Been, M., W. van Schaik, L. Cheng, J. Corander, and R. J. Willems. 2013 Recent recombination events in the core genome are associated with adaptive evolution in *Enterococcus faecium*. *Genome Biol Evol* **5**:1524-1535.

de Graef, E. M., A. Decostere, E. De Leener, H. Goossens, M. Baele, and F. Haesebrouck. 2007. Prevalence and mechanism of resistance against macrolides, lincosamides, and streptogramins among *Enterococcus faecium* isolates from food-producing animals and hospital patients in Belgium. *Microb Drug Resist* **13**:135-141.

de la Cruz, F., and J. Davies. 2000. Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends Microbiol* **8**:128-133.

de la Hoz, A. B., S. Ayora, I. Sitkiewicz, S. Fernandez, R. Pankiewicz, J. C. Alonso, and P. Ceglowski. 2000. Plasmid copy-number control and better-than-random segregation genes of pSM19035 share a common regulator. *Proc Natl Acad Sci U S A* **97**:728-33.

De Leener, E., A. Martel, A. Decostere, F. Haesebrouck. 2004. Distribution of the *erm* (B) gene, tetracycline resistance genes, and Tn1545-like transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. *Microb Drug Resist* **10**:341-345.

del Solar, G., M. Moscoso, and M. Espinosa. 1993. Rolling circle-replicating plasmids from gram-positive and gram-negative bacteria: a wall falls. *Mol Microbiol* **8**:789-96.

del Solar, G., R. Giraldo, M. J. Ruiz- Echevarria, M. Espinosa, and R. Diaz- Orejas. 1998. Replication and control of circular bacterial plasmids. *Microbiol Mol Biol Rev* **62**:434-64.

Denyer, S.P. 1995. Mechanism of action of antibacterial biocides. *Int Biodet* **36**:227–244.

Depardieu, F., I. Podglajen, R. Leclercq, E. Collatz, and P. Courvalin. 2007. Modes and modulations of antibiotic resistance gene expression. *Clin Microbiol Rev* **20**:79-114.

Di Cesare, A., G. M. Luna, C. Vignaroli, S. Pasquaroli, S. Tota, P. Paroncini, and F.

Biavasco. 2013. Aquaculture can promote the presence and spread of antibiotic-resistant Enterococci in marine sediments. PLoS One **8**:e62838.

Direção Geral de Saúde (DGS). 2013. Portugal – Controlo da Infecção e Resistências aos Antimicrobianos em números – 2013. Programa de Prevenção e Controlo de Infecções e de Resistência aos Antimicrobianos. ISSN: 2183-072X. <https://www.dgs.pt/estatisticas-de-saude/estatisticas-de-saude/publicacoes/portugal-controlo-da-infecao-e-resistencia-aos-antimicrobianos-em-numeros-2013-pdf.aspx>. [Consultado em 7 de Janeiro de 2014].

Direção Geral de Veterinária (DGV). 2010. Relatório Nacional De Monitorização Do Consumo De Antimicrobianos Portugal. <http://www.apifarma.pt/Documentos%20ENews/DGV%20%20Relatório%20Anual%20Consumo%20Antimicrobianos.pdf>. [Consultado em 7 de Janeiro de 2014].

Directive 98/8/EC. Concerning the placing of biocidal products on the market. Official Journal of the European Communities of the European Parliament and the Council (16 February 1998). O J L123:1-63.

Directive 70/524/EEC. Concerning additives in feeding-stuff. Official Journal of the European Communities of the European Parliament and the Council (23 November 1970). O J L **270**:1–17.

Doud, C. W., H. M. Scott, and L. Zurek. 2013. Role of House Flies in the Ecology of *Enterococcus faecalis* from Wastewater Treatment Facilities. Microb Ecol Dec 14. [Epub ahead of print].

Dubois-Brissonnet, F., C. Malgrange, L. Guerin-Mechin, B. Heyd, and J. Y. Leveau. 2001. Changes in fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by growth conditions: Consequences of resistance to quaternary ammonium compounds. Microbiol **106**:97–110.

Donabedian, S. M., M. B. Perry, N. Abdujamilova, M. J. Gordoncillo, A. Naqvi, K. C. Reyes, M. J. Zervos, and P. Bartlett. 2010. Characterization of vancomycin-resistant *Enterococcus faecium* isolated from swine in three Michigan countries. J Clin Microbiol **48**:4156-4160.

Dombrádi, Z., O. Dobay, K. Nagy, A. Kozák, V. Dombrádi, and J. Szabó. 2012. Prevalence of vanC vancomycin-resistant enterococci in the teaching hospitals of the University of Debrecen, Hungary. *Microb Drug Resist* **18**:47-51.

European Centre for Disease Prevention and Control (ECDC). 2013a. Surveillance of antimicrobial consumption in Europe, 2010. Stockholm.

European Centre for Disease Prevention and Control (ECDC). 2013b. Annual Epidemiological Report 2013. Reporting on 2011 surveillance data and 2012 epidemic intelligence data. Stockholm: ECDC; 2013.

European Centre for Disease Prevention and Control (ECDC). 2013c. Point prevalence survey of healthcare- associated infections and antimicrobial use in European acute care hospitals. Stockholm: ECDC; 2013.

EFSA, ECDC, EMEA. 2009. Joint Opinion on antimicrobial resistance (AMR) focused on zoonotic infections. Scientific Opinion of the European Centre for Disease Prevention and Control; Scientific Opinion of the Panel on Biological Hazards; Opinion of the Committee for Medicinal Products for Veterinary Use; Scientific Opinion of the Scientific Committee on Emerging and Newly Identified Health Risks. *EFSA J* **7**:1372.

EFSA (European Food Safety Authority). 2012a. Antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in the European Union in 2010. European Union Summary Report. *Journal* **10**:2598.

EFSA (European Food Safety Authority). 2012b. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Scientific Opinion on the safety and efficacy of copper compounds (E4) as feed additives for all animal species: cupric sulphate pentahydrate based on a dossier submitted by Manica S.p.A. *EFSA Journal* **10**:2969. [38 pp.] doi:10.2903/j.efsa.2012.2969.

EFSA Panel on Contaminants in the Food Chain (CONTAM). 2012c. Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. *EFSA Journal* **10**:2985.

Espinosa, M., S. Cohen, M. Couturier, G. del Solar, R. Díaz-Orejas, R. Giraldo, L. Jánniere, C. Miller, M. Osborn, and C. M. Thomas. 2000. Plasmid replication and copy number control, p. 1-47. In C. M. Thomas (ed.), *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. Harwood Academic Press, Amsterdam, The Netherlands.

European Medicines Agency (EMA). 2012. Sales of veterinary antimicrobial agents in 19 EU/EEA countries in 2010. (EMA/88728/2012).

Feil, E. J., and B. G. Spratt. 2001. Recombination and the population structures of bacterial pathogens. *Annu Rev Microbiol* **55**:561-590.

Feil E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt. 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol* **186**:1518–1530.

Finney, L. A., and T. V. O'Halloran. 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Science* **300**:931-936.

Fisher, K. and C. Phillips. 2009. The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology* **155**:1749-57.

Flahaut, S., J. Frere, P. Boutibonnes, and Y. Auffray. 1996. Comparison of the bile salts and sodium dodecyl sulfate stress responses in *Enterococcus faecalis*, *Appl Environ Microbiol* **62**:2416-20.

Fraise, A. P. 2002. Biocide abuse and antimicrobial resistance--a cause for concern? *The J Antimicrob Chemother* **49**:11-12.

Frazer, A. D. and M. B. Edin. 1930. Tellurium in the treatment of syphilis. *Lancet* **216**:133-134.

Francia, M. V., A. Varsaki, M. P. Garcillan-Barcia, A. Latorre, C. Drainas, and F. de la Cruz. 2004. A classification scheme for mobilization regions of bacterial plasmids. *FEMS Microbiol Rev* **28**:79 100.

Franz, C. M., A. B. Muscholl-Silberhorn, N. M. Yousif, M. Vancanneyt, J. Swings, and W. H. Holzapfel. 2001. Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Appl Environ Microbiol* **67**:4385-9.

Freitas, A. R., C. Novais, P. Ruiz-Garbajosa, T. M. Coque, L. Peixe. 2009a. Dispersion of multidrug-resistant *Enterococcus faecium* isolates belonging to major clonal complexes in different Portuguese settings. *Appl Environ Microbiol* **75**: 4904-4908.

Freitas, A. R., C. Novais, P. Ruiz-Garbajosa, T. M. Coque, and L. Peixe. 2009b. Clonal expansion within clonal complex 2 and spread of vancomycin-resistant plasmids among different genetic lineages of *Enterococcus faecalis* from Portugal. *J Antimicrob Chemother* **63**:1104-1111.

Freitas, A. R., A. P. Tedim, C. Novais, P. Ruiz-Garbajosa, G. Werner, J. A. Laverde-Gomez, R. Canton, L. Peixe, F. Baquero, and T. M. Coque. 2010. Global spread of the *hyl*(Efm) colonization-virulence gene in megaplasmids of the *Enterococcus faecium* CC17 polyclonal subcluster. *Antimicrob Agents Chemother* **54**:2660-5.

Freitas, A. R, T. M. Coque, C. Novais, A. M. Hammerum, C. H. Lester, M. J. Zervos, S. Donabedian, L. B. Jensen, M. V. Francia, F. Baquero, and L. Peixe. 2011a. Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. *J Clin Microbiol* **49**:925-931.

Freitas, A. R, C. Novais, R. Correia, M. Monteiro, T. M. Coque, and L. Peixe. 2011b. Non-susceptibility to tigecycline in enterococci from hospitalised patients, food products and community sources. *Int J Antimicrob Agents* **38**:174-176.

Freitas, A. R, C. Novais, A. P. Tedim, M. V. Francia, F. Baquero, L. Peixe, and T. M. Coque. 2013. Microevolutionary events involving narrow host plasmids influences local fixation of vancomycin-resistance in *Enterococcus* populations. *PLoS One* **8**:e60589.

Frost, L. S., R. Leplae, A. O. Summers, A. Toussaint . 2005. Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol* **3**:722-732.

Galimand, M., E. Schmitt, M. Panvert, B. Desmolaize, S. Douthwaite, Y. Mechulam, and P. Courvalin. 2011. Intrinsic resistance to aminoglycosides in *Enterococcus faecium* is conferred by the 16S rRNA m5C1404-specific methyltransferase EfmM RNA **17**:251-62.

Galloway-Penã, J., J. H. Roh, M. Latorre, X. Qin, and B. E. Murray. 2012. Genomic and SNP Analyses Demonstrate a Distant Separation of the Hospital and Community-Associated Clades of *Enterococcus faecium*. PLoS One **7**:e30187.

Garcia-Migura, L., H. Hasman, and L. B. Jensen. 2009. Presence of pRI1: a small cryptic mobilizable plasmid isolated from *Enterococcus faecium* of human and animal origin. Curr Microbiol **58**:95-100.

Garcillan-Barcia, M. P., and F. de la Cruz. 2008. Why is entry exclusion an essential feature of conjugative plasmids? Plasmid **60**:1-18.

Garcillan-Barcia, M. P., M. V. Francia, and F. de la Cruz. 2009. The diversity of conjugative relaxases and its application in plasmid classification. FEMS Microbiol Rev **33**:657-87.

Garnier, F., S. Taourit, P. Glaser, P. Courvalin, and M. Galimand. 2000. Characterization of transposon Tn1549, conferring VanB-type resistance in *Enterococcus* spp. Microbiol **146**(Pt 6):1481-1489.

Gilbert, P., and A. J. McBain. 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin Microbiol reviews **16**:189-208.

Gilbert, P., and L. E. Moore. 2005. Cationic antiseptics: Diversity of action under a common epithet. J Appl Microbiol **99**:703–715.

Gillespie, M. T., J. W. May, and R. A. Skurray. 1986. Plasmid-encoded resistance to acriflavine and quaternary ammonium compounds in methicillin-resistant *Staphylococcus aureus*. FEMS Microbiol Lett **34**:47-51.

Gnanadhas, D. P, S. A. Marathe, and D. Chakravorty. 2012. Biocides-resistance, cross-resistance mechanisms and assessment. Expert Opin Investig Drugs **22**:191-206.

Gonçalves, A., G. Igrejas, H. Radhouani, S. Correia, R. Pacheco, T. Santos, R. Monteiro, A. Guerra, F. Petrucci-Fonseca, F. Brito, C. Torres, and P. Poeta. 2013. Antimicrobial resistance in faecal enterococci and *Escherichia coli* isolates recovered from Iberian wolf. *Lett Appl Microbiol* **56**:268-274.

Gordillo, M. E., K. V. Singh, and B. E. Murray BE. 1993. Comparison of ribotyping and pulsed-field gel electrophoresis for subspecies differentiation of strains of *Enterococcus faecalis*. *J Clin Microbiol* **31**:1570-1574.

Grady, R., and F. Hayes. 2003. Axe-Txe, a broad-spectrum proteic toxinantitoxin system specified by a multidrug-resistant, clinical isolate of *Enterococcus faecium*. *Mol Microbiol* **47**:1419-32.

Grass, G., K. Thakali, P. E. Klebba, D. Thieme, A. Müller, G. F. Wildner, and C. Rensing. 2004. Linkage between catecholate siderophores and the multicopper oxidase CueO in *Escherichia coli*. *J Bacteriol* **186**:5826–5833.

Gaze, W. H., N. Abdouslam, P. M. Hawkey, and E. M. H. Wellington. Incidence of Class 1 Integrons in a Quaternary Ammonium Compound-Polluted Environment. 2005. *Antimicrob Agents Chemother* **49**:1802-1807.

Grindley, N. D. F. 2001. The movement of Tn3-like elements: transposition and cointegrate resolution, p. 272-302. *In* N. Craig, M. Craigie, M. Gellert, and A. M. Lambowitz (ed.), *Mobile DNA II*. ASM Press, Washington, DC.

Guardabassi, L., P. T. Bronnum, R. Dano, A. Forslund, and A. Dalsgaard. 2002. Dissemination of vancomycin-resistant enterococci harboring *vanA* through disposal of waste derived from industrial production of vancomycin. *Microb Drug Resist* **8**:401-406.

Guardabassi, L., B. Perichon, J. van Heijenoort, D. Blanot, and P. Courvalin. 2005. Glycopeptide resistance *vanA* operons in *Paenibacillus* strains isolated from soil. *Antimicrob Agents Chemother* **49**:4227-33.

Guardabassi, L., and Y. Agerso. 2006. Genes homologous to glycopeptide resistance *vanA* are widespread in soil microbial communities. *FEMS Microbiol Lett* **259**:221-5.

Guérin-Méchin, L., F. Dubois-Brissonnet, B. Heyd, and J. Y. Leveau. 1999. Specific variations of fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by Quaternary Ammonium Compounds and relation with resistance to bactericidal activity. *J Appl Microbiol* **87**:735-742.

Gupta, S., M. C. Maiden. 2001. Exploring the evolution of diversity in pathogen populations. *Trends Microbiol* **9**:181-185.

Halliwell, B., and J.M. Gutteridge. 1984. Free radicals, lipid peroxidation, and cell damage. *Lancet* **2**:1095.

Halvorsen, E. M., J. J. Williams, A. J. Bhimani, E. A. Billings, and P. J. Hergenrother. 2011. Txe, an endoribonuclease of the enterococcal Axe-Txe toxin-antitoxin system, cleaves mRNA and inhibits protein synthesis. *Microbiol* **157**:387-397.

Hammad, A. M., T. Shimamoto, and T. Shimamoto. 2014. Genetic characterization of antibiotic resistance and virulence factors in *Enterococcus* spp. from Japanese retail ready-to-eat raw fish. *Food Microbiol* **38**:62-66.

Harrison, J. J., H. Ceri, C. A. Stremick, and R. J. Turner. 2004. Biofilm susceptibility to metal toxicity. *Environ Microbiol* **6**:1220-1227.

Hasman, H., and F. M. Aarestrup. 2002. *tcrB*, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. *Antimicrob Agents Chemother* **46**:1410–1416.

Hasman, H., and F. M. Aarestrup. 2005 The *tcrB* gene is part of the *tcrYAZB* operon conferring copper resistance in *Enterococcus faecium* and *Enterococcus faecalis*. *Microbiol* **151**:3019–3025.

Hasman, H., A. G. Villadsen, and F. M. Aarestrup. 2005. Diversity and stability of plasmids from glycopeptide-resistant *Enterococcus faecium* (GRE) isolated from pigs in Denmark. *Microb Drug Resist* **11**:178-84

Hasman, H., I. Kempf, B. Chidaine, R. Cariolet, A. K. Ersbøll, H. Houe, H. C. Bruun Hansen, and F. M. Aarestrup. 2006. Copper resistance in *Enterococcus faecium*, mediated by the *tcrB* gene, is selected by supplementation of pig feed with copper sulfate.

Appl Environ Microbiol **72**:5784–5789.

Heaton, M. P., L. F. Discotto, M. J. Pucci, and S. Handwerger. 1996. Mobilization of vancomycin resistance by transposon-mediated fusion of a VanA plasmid with an *Enterococcus faecium* sex pheromone-response plasmid. *Gene* **171**:9-17.

Hegstad, K., S. Langsrud, B. T. Lunestad, A. A. Scheie, M. Sunde, and S. P. Yazdankhah. 2010. Does the Wide Use of Quaternary Ammonium Compounds Enhance the Selection and Spread of Antimicrobial Resistance and Thus Threaten Our Health? *Microbial Drug Resist* **16**:91-104.

Heikens, E., M. J. Bonten, and R. J. Willems. 2007. Enterococcal surface protein Esp is important for biofilm formation of *Enterococcus faecium* E1162. *J Bacteriol* **189**:8233–8240.

Heikens, E., W. van Schaik, H. L. Leavis, M. J. Bonten, and R. J. Willems. 2008. Identification of a novel genomic island specific to hospital-acquired clonal complex 17 *Enterococcus faecium* isolates. *Appl Environ Microbiol* **74**:7094-7097.

Heikens, E., M., K. V. Singh, K. D. Jacques-Palaz, M. van Luit-Asbroek, E.A. Oostdijk, J. Bonten, B. E., Murray, and R. J. Willems. 2011. Contribution of the enterococcal surface protein Esp to pathogenesis of *Enterococcus faecium* endocarditis. *Microbes Infect* **13**:1185-1190.

Hidron, A. I., J. R. Edwards, J. Patel, T. C. Horan, D. M. Sievert, D. A. Pollock, S. K. Fridkin. 2008. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infect. Control Hosp Epidemiol* **29**:996-1011.

Hodges, N. D. C. 1889. The value of mercuric Chloride as a disinfectant. *Science* **13**:62-64.

Hodgkinson, V., and M. J. Petris. 2012. Copper homeostasis at the host-pathogen interface. *J Biol Chem* **287**:13549-13555.

Hollenbeck, B. L. and L. B. Rice. 2012. Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virul* **3**:421-433.

Homan, W. L., D. Tribe, S. Poznanski, M. Li, G. Hogg, E. Spalburg, J. D. Van Embden, and R. J. Willems. 2002. Multilocus sequence typing scheme for *Enterococcus faecium*. *J Clin Microbiol* **40**:1963-1971.

Hood, M. I., and E. P. Skaar. 2012. Nutritional immunity: transition metals at the pathogen-host interface. *Nat Rev Microbiol* **10**:525–537.

Huh, J. Y., W. G. Lee, K. Lee, W. S. Shin, and J. H. Yoo. 2004. Distribution of insertion sequences associated with Tn1546-like elements among *Enterococcus faecium* isolates from patients in Korea. *J Clin Microbiol* **42**:1897-902.

Hufnagel, M., V. J. Carey, L. Baldassarri, R. R. Reinert, and J. Huebner. 2006. Distribution of four capsular serotypes of *Enterococcus faecalis* among clinical isolates from different geographical origins and infection sites. *Infection* **34**:22-25.

Huycke, M. M. 2002. Physiology of Enterococci. In M. S. Gilmore, D. B. Clewell, P. Couvalin, G. M. Dunny, BE Murray, and L. B. Rice (ed.), *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. ASM Press, Washington, D. C.

Ishikawa, S., Y. Matsumura, F. Yoshizako, and T. Tsuchido. 2002. Characterization of a cationic surfactant-resistant mutant isolated spontaneously from *Escherichia coli*. *J Appl Microbiol* **92**:261–26.

Iversen, A., I. Kuhn, A. Franklin, and R. Mollby. 2002. High prevalence of vancomycin-resistant enterococci in Swedish sewage. *Appl Environ Microbiol* **68**:2838-4282.

Jain, R., M. C. Riviera, J. E. Moore, and J. A. Lake. 2003. Horizontal gene transfer accelerates genome innovation and evolution. *Mol Bio Evol* **20**:1598-1602.

Jensen, L. B., L. Garcia-Migura, A. J. Valenzuela, M. Lohr, H. Hasman, and F. M. Aarestrup. 2010. A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods* **80**:25-43.

Johnston, L., and L. A. Jaykus. 2004 Antimicrobial resistance of *Enterococcus* species isolated from produce. *Appl Environ Microbiol* **70**:3133-3137.

Jureen, R., J. Top, S. C. Mohn, S. Harthug, N. Langeland, and R. J. Willems. 2003. Molecular characterization of ampicillin-resistant *Enterococcus faecium* isolates from hospitalized patients in Norway. *J Clin Microbiol* **4**:2330-2336.

Kak, V., and J. W. Chow. 2002. Acquired Antibiotic Resistance in Enterococci, p. 355-383. *In* M. S. Gilmore, Clewell, P. Couvalin, G. M. Dunny, BE Murray, and L. B. Rice (ed.), *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. ASM Press, Washington, D. C.

Karlin, K. D. 1993. Metalloenzymes, structural motifs, and inorganic models. *Science* **261**:701-708.

Kaufmann, M. E. 1998. Pulsed-Field Gel Electrophoresis, p. 17-31. *In* N. Woodford and A. P. Johnson (ed.), *Molecular Bacteriology: protocols and clinical applications*. Humana Press, Inc, Totowa, N.J.

Kawalec, M., Z. Pietras, E. Daniłowicz, A. Jakubczak, M. Gniadkowski, W. Hryniewicz, and R. J. Willems. 2007. Clonal structure of *Enterococcus faecalis* isolated from Polish hospitals: characterization of epidemic clones. *Clin Microbiol* **45**:147-53.

Kazama, H., H. Hamashima, M. Sasatsu, and T. Ara. 1998. Distribution of the antiseptic-resistance gene *qacE delta 1* in gram-positive bacteria. *FEMS Microbiol Lett* **165**:295-299.

Keyes, E. L. 1920. The treatment of gnorrhoea of the male urethra. *JAMA* **75**:1325-1329.

Khan, S. A. 1997. Rolling-circle replication of bacterial plasmids. *Microbiol Mol Biol Rev* **61**:442-55.

Khan, S. A. 2004. Rolling-Circle Replication, p. 63-78. *In* B. E. Funnell and G. J. Phillips (ed.), *Plasmid Biology*. ASM Press, Washington, DC.

Khan, S. A. 2005. Plasmid rolling-circle replication: highlights of two decades of research.

Plasmid **53**:126-36.

Khan, M. A., M. van der Wal, D. J. Farrell, L. Cossins, A. van Belkum, A. Alaidan, and J. P. Hays. 2008. Analysis of *VanA* vancomycin-resistant *Enterococcus faecium* isolates from Saudi Arabian hospitals reveals the presence of clonal cluster 17 and two new Tn1546 lineage types. J Antimicrob Chemother **62**:279-283.

Kilpper-Bälz, R. and K. H. Schleifer. 1981. DNA-rRNA hybridization studies among staphylococci and some other Gram-positive bacteria. FEMS Microbiol Lett **10**:357–362.

Kilpper-Bälz, R., G. Fischer, and K. H. Schleifer. 1982. Nucleic acid hybridization of group N and group D streptococci. Cur Microbiol **7**:245–250.

Kilpper-Bälz, R. and Schleifer, K.H. 1984. Nucleic acid hybridization and cell wall composition studies of pyogenic streptococci. FEMS Microbiol Lett **24**:355–364.

Kim, S. W., E. J. Jeong, H. S. Kang, J. I. Tak, W. Y. Bang, J. B. Heo, J. Y. Jeong, G. M. Yoon, H. Y. Kang, and J. D. Bahk. 2006. Role of RepB in the replication of plasmid pJB01 isolated from *Enterococcus faecium* JC1. Plasmid **55**:99-113.

Kim, B.E., T. Nevitt, and D.J. Thiele. 2008. Mechanisms for copper acquisition, distribution and regulation. Nat Chem Biol **4**:176–185.

Kim, D. S., K. V. Singh, S. R. Nallapareddy, X. Qin, D. Panesso, C. A. Arias, and B. E. Murray. 2010. The *fms21* (*pilA*)-*fms20* locus encoding one of four distinct pili of *Enterococcus faecium* is harboured on a large transferable plasmid associated with gut colonization and virulence. J Med Microbiol **59**:505-507.

Kim, J., S. Lee, and S. Choi. 2012. Copper resistance and its relationship to erythromycin resistance in *Enterococcus* isolates from bovine milk samples in Korea. J Microbiol **50**:540–354.

Klare, I., C. Konstabel, S. Mueller-Bertling, G. Werner, B. Strommenger, C. Kettlitz, S. Borgmann, B. Schulte, D. Jonas, A. Serr, A. M. Fahr, U. Eigner, and W. Witte. 2005. Spread of ampicillin/vancomycin-resistant *Enterococcus faecium* of the epidemic-virulent clonal complex-17 carrying the genes *esp* and *hyl* in German hospitals. Eur J Clin Microbiol Infect Dis **24**:815-25.

Klein, G. 2003. Taxonomy, ecology and antibiotic resistance of enterococci from food and gastro-intestinal tract. *Int J Food Microbiol* **88**:123-131.

Kozitskaya, S., S. H. Cho, K. Dietrich, R. Marre, K. Naber, and W. Ziebuhr. 2004. The bacterial insertion sequence element IS256 occurs preferentially in nosocomial *Staphylococcus epidermidis* isolates: association with biofilm formation and resistance to aminoglycosides. *Infect Immun* **72**:1210-5.

Kozłowicz, B. K., M. Dworkin, and G. M. Dunny. 2006. Pheromone-inducible conjugation in *Enterococcus faecalis*: a model for the evolution of biological complexity? *Int J Med Microbiol* **296**:141-7.

Kurenbach, B., C. Bohn, J. Prabhu, M. Abudukerim, U. Szewzyk, and E. Grohmann. 2003. Intergeneric transfer of the *Enterococcus faecalis* plasmid pIP501 to *Escherichia coli* and *Streptomyces lividans* and sequence analysis of its tra region. *Plasmid* **50**:86-93.

Kuch, A., R. J. Willems, G. Werner, T. M. Coque, A. M. Hammerum, A. Sundsfjord, I. Klare, P. Ruiz-Garbajosa, G. S. Simonsen, M. van Luit-Asbroek, W. Hryniewicz, and E. Sadowy. 2012. Insight into antimicrobial susceptibility and population structure of contemporary human *Enterococcus faecalis* isolates from Europe. *J Antimicrob Chemother* **67**:551-558.

Lambert, P. A. 2004. Mechanisms of action of biocides. pp139-153. *In* Fraise, A. L, and P. Maillard, Jr., (ed.) Russell, Hugo and Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization. 4th ed. United Kingdom: Blackwell Science Ltd.

Lancaster, H., R. Bedi, M. Wilson, and P. Mullany. 2005. The maintenance in the oral cavity of children of tetracycline-resistant bacteria and the genes encoding such resistance. *J Antimicrob Chemother* **56**:524-31.

Lancefield, R.C. 1933. A serological differentiation of human and other groups of hemolytic streptococci. *J Exp Med* **57**:571–595.

Larsen, J., H. C. Schönheyder, C. H. Lester, S. S. Olsen, L. J. Porsbo, L. Garcia-Migura, L. B. Jensen, M. Bisgaard, and A. M. Hammerum. 2010. Porcine-origin gentamicin-resistant *Enterococcus faecalis* in humans, Denmark. *Emerg Infect Dis*

16:682-684.

Latorre, M., F. Olivares, A. Reyes-Jara, G. López, and M. González. 2011. CutC is induced late during copper exposure and can modify intracellular copper content in *Enterococcus faecalis*. *Biochem Biophys Res Commun* **406**:633-637.

Latorre, M., J. Galloway-Peña, J. H. Roh, M. Budinich, A. Reyes-Jara, B. E. Murray, A. Maass, and M. González. 2014. *Enterococcus faecalis* reconfigures its transcriptional regulatory network activation at different copper levels. *Metallomics*. [Epub ahead of print].

Laverde Gomez, J. A., W. van Schaik, A. R. Freitas, T. M. Coque, K. E. Weaver, M. V. Francia, W. Witte, and G. Werner. 2011. A multiresistance megaplasmid pLG1 bearing a *hylEfm* genomic island in hospital *Enterococcus faecium* isolates. *Int J Med Microbiol* **301**:165-175.

Leavis, H., J. Top, N. Shankar, K. Borgen, M. Bonten, J. van Embden, and R. J. Willems. 2004. A novel putative enterococcal pathogenicity island linked to the *esp* virulence gene of *Enterococcus faecium* and associated with epidemicity. *J Bacteriol* **186**:672-82.

Leavis, H. L., M.J. Bonten, and R. J. Willems. 2006 Identification of high- risk enterococcal clonal complexes: global dispersion and antibiotic resistance. *Curr Opin Microbiol* **9**:454-460.

Leavis, H. L., R. J. Willems, W. J. van Wamel , F. H. Schuren , M. P. Caspers , and M.J. Bonten . 2007. Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant subspecies of *E. faecium*. *PLoS Pathog* **3**:75–96.

LeBlanc, D. J., J. M. Inamine, and L. N. Lee. 1986. Broad geographical distribution of homologous erythromycin, kanamycin, and streptomycin resistance determinants among group D streptococci of human and animal origin. *Antimicrob Agents Chemother* **29**:549-55.

Leblanc, D. 2006. *Enterococcus*, p.175-204. In M. Dworkin, S. falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.). *The Prokaryotes: a Handbook on the Biology of Bacteria: Firmicutes, Cyanobacteria*. Springer, NY, USA.

Lebreton, F., W. van Schaik, A. M. McGuire, P. Godfrey, A. Griggs, V. Mazumdar, J. Corander, L. Cheng, S. Saif, S. Young, Q. Zeng, J. Wortman, B. Birren, R. J. Willems, A. M. Earl, M. S. Gilmore. 2013. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *MBio* 4:4.

Leclercq, R., and P. Courvalin. 2005. *Enterococcus*, p. 299-313. In D. G. White, M. N. Alekshun and P. F. McDermott (ed.), *Frontiers in Antimicrobial Resistance*. ASM Press, Washington, D. C.

Leendertse, M., E. Heikens, L. M. Wijnands, M. van Luit-Asbroek, G. J. Teske, J. J. Roelofs, M. J. Bonten, T. van der Poll, and R. J. Willems. 2009. Enterococcal surface protein transiently aggravates *Enterococcus faecium*-induced urinary tract infection in mice. *J Infect Dis* 200:1162–1165.

Lemire, J. A., J. J. Harrison, and R. J. Turner. 2013. Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nat Rev Microbiol* 11:371-384.

Lester, C. H., D. Sandvang, S. S. Olsen, H. C. Schönheyder, J. O. Jarlöv, J. Bangsbo, D. S. Hansen, T. G. Gensen, N. Frimodt-Møller, and A. M. Hammerum. DANRES Study Group. 2008. Emergence of ampicillin-resistant *Enterococcus faecium* in Danish hospitals. *J Antimicrob Chemother* 62:1203-1206.

Li, Y., C. Canchaya, F. Fang, E. Raftis, K. A. Ryan, J. P. van Pijkeren, D. van Sinderen, and P. W. O'Toole. 2007. Distribution of megaplasms in *Lactobacillus salivarius* and other lactobacilli. *J Bacteriol* 189:6128-39.

Liebert, C. A., J. Wireman, T. Smith, and A. O. Summers. 1997. Phylogeny of Mercury Resistance (*mer*) Operons of Gram-Negative Bacteria Isolated from the Fecal Flora of Primates. *Appl Environ Microbiol* 63:1066–1076.

Linder, M. C., and M. Hazegh Azam. 1996. Copper biochemistry and molecular biology. *Am J Clin Nutr* 63:797S–811S.

Liu, Y., B. Cao, L. Gu, K. Liu, and Z. Feng. 2011. Successful control of vancomycin-resistant *Enterococcus faecium* nosocomial outbreak in a teaching hospital in China. *Am J Infect Control* 40:568-571.

Liu, Y., Y. Wang, C. Wu, Z. Shen, S. Schwarz, X. D. Du, L. Dai, W. Zhang, Q. Zhang, and J. Shen. 2012. First report of the multidrug resistance gene *cfr* in *Enterococcus faecalis* of animal origin. *Antimicrob Agents Chemother* **56**:1650-1654.

Liu, Y., Y. Wang, S. Schwarz, Y. Li, Z. Shen, Q. Zhang, C. Wu, and J. Shen. 2013. Transferable multiresistance plasmids carrying *cfr* in *Enterococcus* spp. from swine and farm environment *Antimicrob Agents Chemother* **57**:42-48.

Long, K.S., J. Poehlsgaard, C. Kehrenberg, S. Schwarz, and B. Vester. 2006. The Cfr rRNA methyltransferase confers resistance to Phenicol, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. *Antimicrob Agents Chemother* **50**:2500-2505.

Lopez, M., Y. Saenz, B. Rojo-Bezarez, S. Martinez, R. del Campo, F. Ruiz-Larrea, M. Zarazaga, and C. Torres. 2009. Detection of *vanA* and *vanB2*-containing enterococci from food samples in Spain, including *Enterococcus faecium* strains of CC17 and the new singleton ST425. *Int J Food Microbiol* **133**:172-178.

López, M., M. J. Alvarez-Martínez, F. Marco, and C. Torres. 2013. Glycopeptide-resistant *Enterococcus faecium*. Analysis of the resistance genotype, virulence and genetic lines. *Enferm Infecc Microbiol Clin* **31**:10-14.

Loughlin, M. F., M. V. Jones, and P. A. Lambert. 2002. *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. *J Antimicrob Chemother* **49**:631–639.

Ludwig, W., E. Seewaldt, R. Kilpper-Bälz, K. H. Schleifer, L. Magrum, C. R. Woese, G. E. Fox, and E. Stackebrandt. 1985. The phylogenetic position of *Streptococcus* and *Enterococcus*. *J Gen Microbiol* **131**:543–551.

Lutsenko, S. and J. H. Kaplan. 1995. Organization of P-type ATPases: Significance of structural diversity. *Biochem* **34**:15607-15613.

Ma, D., D. N. Cook, J. E. Hearst, and H. Nikaido. 1994. Efflux pumps and drug resistance in gram-negative bacteria. *Trends Microbiol* **2**:489-93.

Macedo, A. S., A. R. Freitas, C. Abreu, E. Machado, L. Peixe, J. C. Sousa, and C. Novais. 2011. Characterization of antibiotic resistant enterococci isolated from untreated waters for human consumption in Portugal. *Int J Food Microbiol* **145**:315-319.

MacKinnon, M. G., M. A. Drebot, and G. J. Tyrrell. 1997. Identification and characterization of IS1476, an insertion sequence-like element that disrupts VanY function in a vancomycin-resistant *Enterococcus faecium* strain. *Antimicrob Agents Chemother* **41**:1805-7.

Maclellan, S. R., C. D. Sibley, and T. M. Finan. 2004. Second chromosomes and megaplasids in bacteria, p. 529- 542. In B. E. Funnell and G. J. Phillips (ed.), *Plasmid Biology*. ASM Press, Washington, DC.

McGann, P., Y. I. Kwak, A. Summers, J. F. Cummings, P. E. Waterman, and E. P. Lesho. 2011. Detection of *qacA/B* in clinical isolates of methicillin-resistant *Staphylococcus aureus* from a regional healthcare network in the eastern United States. *Infect Contr Hosp Epidemiol* **32**:1116-1119.

Magnani, D., and M. Solioz. 2007. How bacteria handle copper, pp. 259-285. In Nies, D. H., and S. Silver (ed) *Molecular Microbiology of heavy-metals*. Springer; Heidelberg.

Magnani, D., O. Barré, S. D. Gerber, and M. Solioz. 2008. Characterization of the CopR regulon of *Lactococcus lactis* IL1403. *J Bacteriol* **190**:536–545.

Mahillon, J., and M. Chandler. 1998. Insertion sequences. *Microbiol Mol Biol Rev* **62**:725-74.

Mahillon, J., C. Leonard, and M. Chandler. 1999. IS elements as constituents of bacterial genomes. *Res Microbiol* **150**:675-87.

Maillard, J. Y. 2002. Bacterial target sites for biocide action. *J Appl Microbiol* **92**:16S–27S.

Maillard J. Y. 2005. Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. *Therapeutics and clinical risk management* **1**:307-320.

Magos, L., and T. W. Clarkson. 2006. Overview of the clinical toxicity of mercury. *Ann Clin Biochem* **43**:257–268.

Mahbub, A. M, N. Kobayashi, M. Ishino, A. Sumi, K. Kobayashi, N. Uehara, and N. Watanabe. 2005. Detection of a novel *aph(2'')* allele (*aph[2'']-Ie*) conferring high-level gentamicin resistance and a spectinomycin resistance gene *ant(9)-Ia* (*aad 9*) in clinical isolates of enterococci. *Microb Drug Resist* **11**:239-247.

Malathum, K. Singh, K. V., Weinstock, G. M, and Murray, B. E. 1998. Repetitive Sequence-Based PCR versus Pulsed-Field Gel Electrophoresis for Typing of *Enterococcus faecalis* at the Subspecies Level. *J Clin Microbiol* **36**: 211–215.

Malik, A., E. K. Celik, C. Bohn, U. Bockelmann, K. Knobel, and E. Grohmann. 2008. Detection of conjugative plasmids and antibiotic resistance genes in anthropogenic soils from Germany and India. *FEMS Microbiol Lett* **279**:207-16.

Manson, J. M., L. E. Hancock, and M. S. Gilmore. 2010. Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other traits. *Proc Natl Acad Sci U S A* **107**:12269-12274.

Martinez-Bueno, M., E. Valdivia, A. Galvez, and M. Maqueda. 2000. pS86, a new theta-replicating plasmid from *Enterococcus faecalis*. *Curr Microbiol* **41**:257-261.

Mathema, V. B., B. C. Thakuri, and M. Sillanpää. 2011. Bacterial mer operon-mediated detoxification of mercurial compounds: a short review. *Arch Microbiol* **193**:837-844.

Matsushima, A., S. Takakura, M. Yamamoto, Y. Matsumura, M. Shirano, M. Nagao, Y. Ito, Y. Iinuma, T. Shimizu, N. Fujita, and S. Ichiyama. 2012. Regional spread and control of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* in Kyoto, Japan. *Eur J Clin Microbiol Infect Dis* **31**:1095-1100.

McBride, S. M., V. A. Fishetti, D. J. Leblanc, R. C. Moellering, Jr, and M. S. Gilmore. 2007. Genetic diversity among *Enterococcus faecalis*. *PLoS One* **2**:e582.

McDonnell, G., and A. D. Russell. 1999. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev* **12**:147-179.

McIntosh, D., M. Cunningham, B. Ji, F. A. Fekete, E. M. Parry, S. E. Clark, Z. B. Zalinger, I. C. Gilg, G. R. Danner, K. A. Johnson, M. Beattie, and R. Ritchie. 2008. Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida subsp. salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. *J Antimicrob Chemother* **61**:1221-8.

Marinho, C., N. Silva, S. Pombo, T. Santos, R. Monteiro, A. Gonçalves, J. Micael, P. Rodrigues, A. C. Costa, G. Igrejas, and P. Poeta. 2013. Echinoderms from Azores islands: an unexpected source of antibiotic resistant *Enterococcus* spp. and *Escherichia coli* isolates. *Mar Pollut Bull* **69**:122-127.

Merlin, C., J. Mahillon, A. Nesvera, and A. Toussaint. 2000. Gene recruiters and transporters: The modular structure of bacterial mobile elements, p. 363-409. *In* C. M. Thomas (ed.), *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. Harwood Academic Publishers, Amsterdam, The Netherlands.

Mindlin, S. Z., I. A. Bass, E. S. Bogdanova, Z. M. Gorlenko, E. S. Kalyaeva, M. A. Petrova , and V. G. Nikiforov. 2002. Horizontal Transfer of Mercury Resistance Genes in Environmental Bacterial Populations. *Molecular Biology* **36**:160-170.

Mindlin, S. Z., and M. A. Petrova. 2013. Mercury resistance Transposons, pp. 33-52. *In* Roberts, A. P., and P. Mullany, *Bacterial Integrative Mobile Genetic Elements* (ed.). Lande Bioscience.

Mitrakul, K., C. Y. Loo, C. V. Hughes, and N. Ganeshkumar. 2004. Role of a *Streptococcus gordonii* copper-transport operon, copYAZ, in biofilm detachment. *Oral Microbiol Immunol* **19**:395-402.

Moken, M. C., L. M. McMurry, and S. B. Levy. 1997. Selection of multiple-antibiotic-resistant (mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the mar and acrAB loci. *Antimicrob Agents Chemother* **41**:2770-2772.

Molbak, L., A. Tett, D. W. Ussery, K. Wall, S. Turner, M. Bailey, and D. Field. 2003. The plasmid genome database. *Microbiology* **149**:3043-3045.

Møller AK, Barkay T, Hansen MA, Norman A, Hansen LH, Sørensen SJ, Boyd ES, Kroer N. 2013. Mercuric reductase genes (*merA*) and mercury resistance plasmids in High Arctic snow, freshwater and sea-ice brine. *FEMS Microbiol Ecol*. In press.

Moreno F., M. R., P. Sarantinopoulos, E. Tsakalidou, and L. De Vuyst. 2006. The role and application of enterococci in food and health. *Int. J Food Microbiol* **106**:1-24.

Moritz, E. M., and P. J. Hergenrother. 2007. Toxin-antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci. *Proc Natl Acad Sci U S A* **104**:311-316.

Murray, B. E. 1990. The life and times of *Enterococcus*. *Clin Microbiol Rev* **3**:46-65.

Murray, B. E. 1992. Beta-lactamaseproducing enterococci. *Antimicrob Agents Chemother* **36**:2355-2359.

Nascimento, A. M., and E. Chartone-Souza. 2003. Operon mer: bacterial resistance to mercury and potential for bioremediation of contaminated environments. *Genet Mol Res.* **2**:92-101.

Nes, I. F., D. B. Diep, and H. Holo. 2007. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. *J bacterial* **189**:1189- 1198.

Nies DH. 1999. Microbial heavy-metal resistance. *Appl Microbiol Biotechnol Review* **51**:730-50.

Nishihara, T., T. Okamoto, and N. Nishiyama. 2000. Biodegradation of didecyldimethylammonium chloride by *Pseudomonas fluorescens* TN4 isolated from activated sludge. *J Appl Microbiol* **88**:641-7.

Novais, C., T. M. Coque, J. C. Sousa, F. Baquero, and L. Peixe. 2004. Portuguese Resistance Study Group. Local genetic patterns within a vancomycin-resistant *Enterococcus faecalis* clone isolated in three hospitals in Portugal. *Antimicrob Agents Chemother* **48**:3613-3617.

Novais, C., T. M. Coque, P. Boerlin, I. Herrero, M. A. Moreno, L. Dominguez, and L.

Peixe. 2005a. Vancomycin-resistant *Enterococcus faecium* clone in swine, Europe. Emerg Infect Dis **11**:1985-1987.

Novais, C., T. M. Coque, H. Ferreira, J. C. Sousa, and L. Peixe. 2005b. Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. Appl Environ Microbiol **71**:3364-3368.

Novais, C., T. M. Coque, M. J. Costa, J. C. Sousa, F. Baquero, and L. V. Peixe. 2005c. High occurrence and persistence of antibiotic-resistant enterococci in poultry food samples in Portugal. J Antimicrob Chemother **56**:1139-1143.

Novais, C., T. M. Coque, J. C. Sousa, and L. V. Peixe. 2006. Antimicrobial resistance among faecal enterococci from healthy individuals in Portugal. Clin Microbiol Infect **12**:1131-1134.

Novais, C., A. R. Freitas, J. C. Sousa, F. Baquero, T. M. Coque, and L. V. Peixe. 2008. Diversity of Tn1546 and its role in the dissemination of vancomycin-resistant enterococci in Portugal. Antimicrob Agents Chemother **52**:1001-8.

Novais, C., A. R. Freitas, F. Baquero, L. Peixe, and T. M. Coque. 2010. Enterococci from human, animal and environmental sources are reservoirs of Gram Positive conjugative transposons, 3rd International ASM Conference on Enterococci. American Society for Microbiology, Portland, Oregon, USA.

Odermatt, A., and M. Solioz. 1995. Two trans-acting metalloregulatory proteins controlling expression of the copper-ATPases of *Enterococcus hirae*. J Biol Chem **270**:4349-4354.

Ojo, K. K., D. Tung, H. Luis, M. Bernardo, J. Leitao, and M. C. Roberts. 2004. Gram-positive *merA* gene in gram-negative oral and urine bacteria. FEMS Microbiol Lett **238**:411-416.

Ortega Morente, E., M. A. Fernández-Fuentes, M. J. Grande Burgos, H. Abriouel, R. Pérez Pulido, and A. Gálvez. 2013. Biocide tolerance in bacteria. Int J Food Microbiol **172C**:49-56.

Osborn, A. M., K. D. Bruce, P. Strike, and D. A. Ritchie. 1997. Distribution, diversity

and evolution of the bacterial mercury resistance (mer) operon. FEMS Microbiol Rev. **19**:239-262.

Osborn, A. M., and D. Boltner. 2002. When phage, plasmid and transposons collide: genomic islands, and conjugative- and mobilizable-transposons as a mosaic continuum. Plasmid **48**:202-212.

Palazzo, I. C., A. Pitondo-Silva, C. E. Levy, and A. L. da Costa Darini. 2011. Changes in vancomycin-resistant *Enterococcus faecium* causing outbreaks in Brazil. J Hosp Infect **79**:70-74.

Palmer, K. L., V. N. Kos, and M. S. Gilmore. 2010. Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. Curr Opin Microbiol **13**:632-639.

Patel, S. N., N. Memari, D. Shahinas, B. Toye, F. B. Jamieson, and D. j. Farrell. 2013. Linezolid resistance in *Enterococcus faecium* isolated in Ontario, Canada. Diagn Microbiol Infect Dis **77**:350-353.

Patterson, J. E., S. M. Colodny, and M. J. Zervos. 1988. Serious infection due to beta-lactamase-producing *Streptococcus faecalis* with high-level resistance to gentamicin. J Infect Dis **158**:1144-1145.

Paulsen, I. T., L. Banerjee, G. S. Myers, K. E. Nelson, R. Seshadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouiri, T. Utterback, D. Radune, K. A. Ketchum, B. A. Dougherty, and C. M. Fraser. 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science **299**:2071-2074.

Pendleton, J. N., S. P. Gorman, and B. F. Gilmore. 2013. Clinical relevance of the ESKAPE pathogens. Expert Rev Anti Infect Ther **11**:297-308.

Perichon, B., and P. Courvalin. 2009. VanA-type vancomycin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **53**:4580-7.

Poole, K. 2005. Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* **56**:20–51.

Porcheron, G., A. Garénaux, J. Proulx, M. Sabri, and C. M. Dozois. 2013. The fate of not sensing phosphate: downregulation of type 1 fimbriae through activation of the Pho regulon reduces *E. coli* urinary tract virulence. *Front Cell Infect Microbiol* **3**:90.

Poeta, P., D. Costa, J. Rodrigues, C. Torres. 2006. Antimicrobial resistance and the mechanisms implicated in faecal enterococci from healthy humans, poultry and pets in Portugal. *Int J Antimicrob Agents* **27**:131-137.

Portmann, R., K. R. Poulsen, R. Wimmer, and M. Solioz. 2006. CopY-like copper inducible repressors are putative 'winged helix' proteins. *Biometals* **19**:61-70.

Quintiliani, R., Jr., and P. Courvalin. 1996. Characterization of Tn1547, a composite transposon flanked by the IS16 and IS256-like elements, that confers vancomycin resistance in *Enterococcus faecalis* BM4281. *Gene* **172**:1-8.

Rademacher, C., and B. Masepohl. 2012. Copper-responsive gene regulation in bacteria. *Microbiol* **158**: 2451–64.

Radhouani, H., G. Igrejas, A. Gonçalves, R. Pacheco, R. Monteiro, R. Sargo, F. Brito, C. Torres, and P. Poeta. 2013. Antimicrobial resistance and virulence genes in *Escherichia coli* and enterococci from red foxes (*Vulpes vulpes*). *Anaerobe* **23**:82-6.

Raze, D., O. Dardenne, S. Hallut, M. Martinez-Bueno, J. Coyette and J. M. Ghuysen. 1998. The gene encoding the low-affinity penicillin-binding protein 3r in *Enterococcus hirae* S185R is borne on a plasmid carrying other antibiotic resistance determinants. *Antimicrob Agents Chemother* **42**:534-539.

Rice, L.B., and S. H. Marshall. 1994a. Insertions of IS256-like element flanking the chromosomal beta-lactamase gene of *Enterococcus faecalis* CX19. *Antimicrob Agents Chemother* **38**:693–701.

Rice, L. B., L. L. Carias, and S. H. Marshall. 1995. Tn5384, a composite enterococcal mobile element conferring resistance to erythromycin and gentamicin whose ends are directly repeated copies of IS256. *Antimicrob Agents Chemother* **39**:1147-53.

Rice, L. B. 1998a. Tn916 family conjugative transposons and dissemination of antimicrobial resistance determinants. *Antimicrob Agents Chemother* **42**:1871-7.

Rice LB, Carias LL. 1998b. Transfer of Tn5385, a composite, multiresistance chromosomal element from *Enterococcus faecalis*. *J Bacteriol* **180**:714-721.

Rice, L. B., L. Carias, S. Rudin, C. Vael, H. Goossens, C. Konstabel, I. Klare, S. R. Nallapareddy, W. Huang, and B. E. Murray. 2003. A potential virulence gene, *hylEfm*, predominates in *Enterococcus faecium* of clinical origin. *J Infect Dis* **187**:508-512.

Rice, L. B., S. Bellais, L. L. Carias, R. Hutton-Thomas, R. A. Bonomo, P. Caspers, M. G. Page, and L. Gutmann. 2004. Impact of specific *pbp5* mutations on expression of beta-lactam resistance in *Enterococcus faecium*. *Antimicrob Agents Chemother* **48**:3028-32.

Rice, L. B., L. L. Carias, S. Marshall, S. D. Rudin, and R. Hutton-Thomas. 2005. Tn5386, a novel Tn916-like mobile element in *Enterococcus faecium* D344R that interacts with Tn916 to yield a large genomic deletion. *J Bacteriol* **187**:6668-6677.

Rice, L. B., V. Lakticova, L. Carias, S. Rudin, R. Hutton, and S. H. Marshall. 2009. Transferable capacity for gastrointestinal colonization in *Enterococcus faecium* in a mouse model. *J Infect Dis* **199**:342-349.

Rice, L. B. 2012. Mechanisms of resistance and clinical relevance of resistance to β -lactams, glycopeptides, and fluoroquinolones. *Mayo Clin Proc* **87**:198-208.

Ridenhour, M. B., H. M. Fletcher, J. E. Mortensen, and L. Daneo-Moore. 1996. A novel tetracycline-resistant determinant, *tet(U)*, is encoded on the plasmid pKQ10 in *Enterococcus faecium*. *Plasmid* **35**:71-80.

Ridge, P. G., Y. Zhang, and V. N. Gladyshev. 2008. Comparative genomic analyses of copper transporters and cuproproteomes reveal evolutionary dynamics of copper utilization and its link to oxygen. *PLoS One* **3**:e1378.

Roberts, M. C., J. Sutcliffe, P. Courvalin, L. B. Jensen, J. Rood, H. Seppala. 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob Agents Chemother* **43**:2823-2830.

Roberts, A. P., and P. Mullany. 2009. A modular master on the move: the Tn916 family of mobile genetic elements. *Trends Microbiol* **17**:251-8.

Roberts, A. P., and P. Mullany. 2011. The Tn916-like genetic elements; a diverse group of modular mobile elements. *FEMS Microbiol Rev*:*In press*.

Rollins, L. D., L. N. Lee, and D. J. LeBlanc. 1985. Evidence for a disseminated erythromycin resistance determinant mediated by Tn917-like sequences among group D streptococci isolated from pigs, chickens, and humans. *Antimicrob Agents Chemother* **27**:439-44.

Rosvoll, T. C., T. Pedersen, H. Sletvold, P. J. Johnsen, J. E. Sollid, G. S. Simonsen, L. B. Jensen, K. M. Nielsen, and A. Sundsfjord. 2009. PCR-based plasmid typing in *Enterococcus faecium* strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. *FEMS Immunol Med Microbiol* **58**:254-68.

Rosvoll, T. C., B. L. Lindstad, T. M. Lunde, K. Hegstad, B. Aasnaes, A. M. Hammerum, C. H. Lester, G. S. Simonsen, A. Sundsfjord, and T. Pedersen. 2012. Increased high-level gentamicin resistance in invasive *Enterococcus faecium* is associated with *aac(6')Ie-aph(2)Ia*-encoding transferable megaplasmids hosted by major hospital-adapted lineages. *FEMS Immunol Med Microbiol* **66**:166-176.

Ruiz-Garbajosa, P., M. J. Bonten, D. A. Robinson, J. Top, S. R. Nallapareddy, C. Torres, T. M. Coque TM, R. Cantón, F. Baquero, B. E. Murray, R. del Campo, and R. J. Willems. 2006. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. *J Clin Microbiol* **44**:2220-2228.

Ruiz-Garbajosa, P., M. de Regt, M. Bonten, F. Baquero, T. M. Coque, R. Canton, R. HJ, and R. J. Willems. 2011. High-density fecal *Enterococcus faecium* colonization in hospitalized patients is associated with the presence of the polyclonal subcluster CC17. *Eur J Clin Microbiol Infect Dis* **31**:519-522.

Russell, A. D., and I. Chopra. 1990. Understanding Antibacterial Action and Resistance. Second ed. Ellis Horwood, Chichester, UK.

Russell, A.D., and M. J. Day. 2002. Antibiotic and biocide resistance in bacteria.

Microbios **85**:45-65.

Russell, A. D., and N. J. Russell. 1995. Biocides: activity, action and resistance. Society for General Microbiology Symposium no. 53. Cambridge University Press, Cambridge, pp. 327–365.

Russell, A. D. 2003. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. *Lancet Infect Dis* **3**:794-803.

Samanovic, M. I., Ding C, Thiele DJ K. H. Darwin. 2012. Copper in microbial pathogenesis: meddling with the metal. *Cell Host Microbe* **11**:106–115.

Samuel, J., H. Coutinho, A. Galloway, C. Rennison, M. E. Kaufmann, and N. Woodford. 2008. Glycopeptide-resistant *Enterococcus raffinosus* in a haematology unit: an unusual cause of a nosocomial outbreak. *J Hosp Infect* **70**:294-296.

Sandvang, D., F. M. Aarestrup, and L. B. Jensen. 1998. Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiol Lett* **160**:37-41.

Santagati, M., F. Campanile, and S. Stefani. 2012. Genomic diversification of enterococci in hosts: the role of the mobilome. *Frontiers in Microbiol* **3**:95.

Santos, T., N. Silva, G. Igrejas, P. Rodrigues, J. Micael, T. Rodrigues, R. Resendes, A. Gonçalves, C. Marinho, D. Gonçalves, R. Cunha, and P. Poeta. 2013. Dissemination of antibiotic resistant *Enterococcus* spp. and *Escherichia coli* from wild birds of Azores Archipelago. *Anaerobe* **24**:25-31.

Sarti, M., F. Campanile, C. Sabia, M. Santagati, R., and S. Stefani. 2012. Polyclonal diffusion of beta-lactamase-producing *Enterococcus faecium*. *J Clin Microbiol* **50**:169-172.

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2009. Scientific Committee on Emerging and Newly Identified Health Risks. Assessment of the Antibiotic Resistance Effects of Biocides. Brussels.

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2010. Research strategy to address the knowledge gaps on the antimicrobial resistance effects of biocides. Adopted this opinion at its 7th plenary of 17 March 2010.

Schell, M. A., M. Karmirantzou, B. Snel, D. Vilanova, B. Berger, G. Pessi, M. C. Zwahlen, F. Desiere, P. Bork, M. Delley, R. D. Pridmore, and F. Arigoni. 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc Natl Acad Sci U S A* **99**:14422-7.

Schleifer, K.H., Kraus, J., Dvorak, C., Kilpper-Bälz, R., Collins, M.D. and Fischer, W. 1985. Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov. *Sys Appl Microbiol* **6**:183–195.

Schwarz, F. V., V. Perreten, and M. Teuber. 2001. Sequence of the 50-kb conjugative multiresistance plasmid pRE25 from *Enterococcus faecalis* RE25. *Plasmid* **46**:170-87.

Seettulsingh, P. S., J. F. Tomayko, P. E. Coudron, S. M. Markowitz, C. Skinner, K. V. Singh, and B. E. Murray. 1996. Chromosomal DNA Restriction Endonuclease Digestion Patterns of β -Lactamase-Producing *Enterococcus faecalis* Isolates Collected from a Single Hospital over a 7-Year Period. *J Clin Microbiol* **34**:1892–1896.

Seputiene, V., A. Bogdaite, M. Ruzauskas, and E. Suziedeliene. 2012. Antibiotic resistance genes and virulence factors in *Enterococcus faecium* and *Enterococcus faecalis* from diseased farm animals: pigs, cattle and poultry. *Pol J Vet Sci* **15**:431-438.

Shaw, J. H., and D. B. Clewell. 1985. Complete nucleotide sequence of macrolide-lincosamide-streptogramin B-resistance transposon Tn917 in *Streptococcus faecalis*. *J Bacteriol* **164**:782-96.

Sheldon, A. T. Jr. 2005. Antiseptic "resistance": real or perceived threat? *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America* **40**:1650-1656.

Sherman, J.M. (1937) The streptococci. *Bacteriological Reviews*, **1**: 3–97.

Sidhu, M.S., S. Langsrud, and A. Holck. 2001. Disinfectant and antibiotic resistance of lactic acid bacteria isolated from the food industry. *Microb Drug Resist* **7**:73-83.

Sidhu, M., E. Heir, T. Leegaard, K. Wiger, and A. Holck. 2002. Frequency of Disinfectant Resistance Genes and Genetic Linkage with β -Lactamase Transposon Tn552 among Clinical Staphylococci. *Antimicrob Agents and Chemother* **46**:2797–803.

Siguier, P., J. Filee, and M. Chandler. 2006. Insertion sequences in prokaryotic genomes. *Curr Opin Microbiol* **9**:526-31.

Silver, S. 1996. Bacterial resistances to toxic metal ions – a review. *Gene* **179**:9-19.

Simjee, S., D. G. White, J. Meng, D. D. Wagner, S. Qaiyumi, S. Zhao, J. R. Hayes, and P. F. McDermott. 2002. Prevalence of streptogramin resistance genes among *Enterococcus* isolates recovered from retail meats in the Greater Washington DC área. *J Antimicrob Chemother* **50**:877-82.

Singh, K. V., G. M. Weinstock, and B. E. Murray. 2002. An *Enterococcus faecalis* ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. *Antimicrob Agents Chemother* **46**:1845-50.

Singh, S. K., G. Grass, C. Rensing, and W. R. Montfort. 2004. Cuprousoxi- dase activity of CueO from *Escherichia coli*. *J Bacteriol* **186**:7815–7817.

Singh, S. K., S. A. Roberts, S. F. McDevitt, A. Weichsel, G. F. Wildner, G. B. Grass, C. Rensing, and W. R. Montfort. 2011. Crystal structures of multicopper oxidase CueO bound to copper(I) and silver(I): functional role of a methionine-rich sequence *J Biol Chem* **286**:37849-57.

Sitthisak, S., K. Howieson, C. Amezola, and R. K. Jayaswal. 2005. Characterization of a multicopper oxidase gene from *Staphylococcus aureus*. *Appl Environ Microbiol* **71**:5650-5653.

Skipington, E., and M. A. Ragan. 2011. Lateral genetic transfer and the construction of genetic exchange communities. *FEMS Microbiol Rev* **35**:707-735.

Skurnik, D., R. Ruimy, D. Ready, E. Ruppe, C. Bernède-Bauduin, F. Djossou, D. Guillemot, G. B. Pier, and A. Andremont. 2010. Is exposure to mercury a driving force for the carriage of antibiotic resistance genes? *J Med Microbiol* **59**:804-807.

Slater, F. R., M. J. Bailey, A. J. Tett, and S. L. Turner. 2008. Progress towards understanding the fate of plasmids in bacterial communities. *FEMS Microbiol Ecol* **66**:3-13.

Sletvold, H., P. J. Johnsen, G. S. Simonsen, B. Aasnaes, A. Sundsfjord, and K. M. Nielsen. 2007. Comparative DNA analysis of two *vanA* plasmids from *Enterococcus faecium* strains isolated from poultry and a poultry farmer in Norway. *Antimicrob Agents Chemother* **51**:736-9.

Sletvold, H., P. J. Johnsen, O. G. Wikmark, G. S. Simonsen, A. Sundsfjord, and K. M. Nielsen. 2010. Tn1546 is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal *Enterococcus faecium* lineages. *J Antimicrob Chemother* **65**:1894-906.

Smillie, C., M. P. Garcillan-Barcia, M. V. Francia, E. P. Rocha, and F. de la Cruz. 2010. Mobility of plasmids. *Microbiol Mol Biol Rev* **74**:434-452.

Smith, J. M., N. H. Smith, M. O' Rourke, and B. G. Spratt. 1993. How clonal are bacteria? *Proc natl Acad Sci U S A* **90**:4384-4388.

Smith, J. M., E. J. Feil, and N. H. Smith. 2000. Population structure and evolutionary dynamics of pathogenic bacteria. *Bioessays* **22**:1115-1122.

Soge, O. O., N. K. Beck, T. M. White, D. B. No, and M. C. Roberts. 2008. A novel transposon, Tn6009, composed of a Tn916 element linked with a *Staphylococcus aureus* mer operon. *J Antimicrob Chemother* **62**:674-80.

Solioz, M., and C. Vulpe. 1996. CPx-type ATPases: a class of P-type ATPases that pump heavy metals. *Trends Biochem Sci* **21**:237-241.

Solioz, M., and J. V. Stoyanov. 2003. Copper homeostasis in *Enterococcus hirae*. *FEMS Microbiol Rv* **27**:183-195.

Solioz, M., H. K. Abicht, M. Mermoud, and S. Mancini. 2010. Response of gram-positive bacteria to copper stress. *J Biol Inorg Chem* **15**:3-14.

Solioz, M., M. Mermoud, H. K. Abicht, and S. Mancini. 2011. Responses of Lactic Acid

Bacteria to heavy Metal Stress, pp163-195. In Tsakalidou, E., and K. Papadimitriou (ed). Stress Responses of Lactic Acid Bacteria. Springer. Science Business Media, LLC.

Song, X., J. Sun, T. Mikalsen, A. P. Roberts, and A. Sundsfjord. 2013.Characterisation of the plasmidome within *Enterococcus faecalis* isolated from marginal periodontitis patients in Norway. PLoS One **8**:e62248.

Sousa, J. C. 2006. *Manual de Antibióticos Antibacterianos*. Porto, II Ed, Universidade Fernando Pessoa.

Stapleton, .P, R. Pike, P. Mullany, V. Lucas, G. Roberts, R. Rowbury, M. Wilson, and H. Richards. 2004. Mercuric resistance genes in gram-positive oral bacteria. FEMS Microbiol Lett **236**:213-220.

Stickler, D., A. L. Fraise, and P. Maillard. 2004. Bacterial resistance: Intrinsic resistance of gram-negative bacteria, p. 154-169. In A. D. Russell, W. B. Hugo and G. A. J. Ayliffe (ed.), Principles and Practice of Disinfection, Preservation & Sterilization. Blackwell Science Ltd, United Kingdom.

Stoyanov, J. V., J. L. Hobman, N. L. Brown. 2001. CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. Mol Microbiol **39**:502-511.

Strausak, D., and M. Solioz. 1997. CopY is a copper-inducible repressor of the *Enterococcus hirae* copper ATPases. J Biol Chem **272**:8932-8936.

Suller, M. T., and A. D. Russell. 1999. Antibiotic and biocide resistance in methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcus. J Hosp Infect **43**:281-291.

Summers, A.O., J. Wireman, M. J., Vimy, F. L. Lorscheider, B. Marshall, S. B. Levy, S. Bennett, and L. Billard. 1993. Mercury released from dental "silver" fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. Antimicrob Agents Chemother **37**:825-834.

Sung, K., S. A. Khan, and M. S. Nawaz. 2008. Genetic diversity of Tn1546-like elements in clinical isolates of vancomycin-resistant enterococci. Int J Antimicrob Agents **31**:549-554.

Tabata, A., H. Nagamune, T. Maeda, K. Murakami, Y. Miyake, and H. Kourai. 2003. Correlation between resistance of *Pseudomonas aeruginosa* to quaternary ammonium compounds and expression of outer membrane protein OprR. *Antimicrob Agents Chemother* **47**:2093–2099.

Tanimoto, K., and Y. Ike. 2008. Complete nucleotide sequencing and analysis of the 65-kb highly conjugative *Enterococcus faecium* plasmid pMG1: identification of the transfer-related region and the minimum region required for replication. *FEMS Microbiol Lett* **288**:186-195.

Taylor, D. E., A. Gribbeel, T. D. Lawley, and D. M. Traez. 2004. Antibiotic resistance plasmids, p. 473-489. In B. E. Funnell and G. J. Phillips (ed.), *Plasmid Biology*. American Society for Microbiology, Washington.

Teixeira, L. M., R. R. Facklam, A. G. Steigerwalt, N. E. Pigott, V. L. Merquior, and D. J. Brenner. 1995. Correlation between phenotypic characteristics and DNA relatedness within *Enterococcus faecium* strains. *J Clin Microbiol* **33**:1520-1523.

Teixeira, L. M., and R. Facklam. 2003. *Enterococcus*, p. 422-433. In B. E. Murray (ed.) *Manual of Clinical Microbiology*. ASM Press, Washington DC.

Teuber, M., F. Schwarz, and V. Perreten. 2003. Molecular structure and evolution of the conjugative multiresistance plasmid pRE25 of *Enterococcus faecalis* isolated from a raw-fermented sausage. *Int J Food Microbiol* **88**:325-9.

Tezel, U., and S. G. Pavlostathis. 2011. Role of quaternary ammonium compounds on antimicrobial resistance in the environment, pp. 349-379. In Keen P. L., and M. H. M. M. Monforts (ed). *Antimicrobial Resistance in the Environment*. John Wiley & Sons, Inc.

Thaker, M., P. Spanogiannopoulos, and G. D. Wright. 2010. The tetracycline resistome. *Cell. Mol Life Sci* **67**:419–431.

Thomas, C. M. 2004. Evolution and population genetics of bacterial plasmids, p. 509-528. In B. E. Funnell and G. J. Phillips (ed.), *Plasmid Biology*. American Society for Microbiology, Washington DC.

Thomas, C. M., and K. M. Nielsen. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol* **3**:711-21.

Thompson, J. K., and M. A. Collins. 2003. Completed sequence of plasmid pIP501 and origin of spontaneous deletion derivatives. *Plasmid* **50**:28-35.

Torres, C., C. Tenorio, A. Portillo, M. García, C. Martínez, R. Del Campo, F. Ruiz-Larrea, and M. Zarazaga. 2003. Intestinal colonization by vanA- or vanB2-containing enterococcal isolates of healthy animals in Spain. *Microb Drug Resist* **1**:S47-52.

Thumu, S. C., and P. M. Halami. 2013. Phenotypic expression, molecular characterization and transferability of erythromycin resistance genes in *Enterococcus* spp. isolated from naturally fermented food. *J Appl Microbiol* doi: 10.1111/jam.12386. [Epub ahead of print].

Tomayko, J. F., and B. E. Murray. 1995 analysis of *Enterococcus faecalis* isolates from intercontinental sources by multilocus enzyme electrophoresis and pulsed-field gel electrophoresis. *J Clin microbial* **33**:2903-2907.

Tomich, P. K., F. Y. An, S. P. Damle, and D. B. Clewell. 1979. Plasmid-related transmissibility and multiple drug resistance in *Streptococcus faecalis* subsp. *zymogenes* strain DS16. *Antimicrob Agents Chemother* **15**:828-30.

Tomich, P. K., F. Y. An, and D. B. Clewell. 1980. Properties of erythromycin-inducible transposon Tn917 in *Streptococcus faecalis*. *J Bacteriol* **141**:1366-74.

Tomita, H., C. Pierson, S. K. Lim, D. B. Clewell, and Y. Ike. 2002. Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in *Enterococcus faecium*. *J Clin Microbiol* **40**:3326-3333.

Tomita, H., K. Tanimoto, S. Hayakawa, K. Morinaga, K. Ezaki, H. Oshima, and Y. Ike. 2003. Highly conjugative pMG1- like plasmids carrying Tn1546-like transposons that encode vancomycin resistance in *Enterococcus faecium*. *J Bacteriol* **185**:7024-7028.

Tomita, H., and Y. Ike. 2005. Genetic analysis of transfer-related regions of the

vancomycin resistance *Enterococcus* conjugative plasmid pHTbeta: identification of oriT and a putative relaxase gene. J Bacteriol 187:7727-37.

Van Melder, L., and M. Saavedra De Bast. 2009. Bacterial toxin-antitoxin systems: more than selfish entities? PLoS Genet 5:e1000437.

Top, J., R. J. Willems, S. van der Velden, M. Asbroek, and M. Bonten. 2008. Emergence of clonal complex 17 *Enterococcus faecium* in The Netherlands. J Clin Microbiol 46:214-219.

Toussaint, A., and C. Merlin. 2002. Mobile elements as a combination of functional modules: Plasmid 47:28-35.

Trun, N., and J. Trempy. 2004. Fundamental Bacterial Genetics. Blackwell Science Ltd.

Truong-Bolduc, Q. C., J. Strahilevitz, and D. C. Hooper. 2006. NorC, a new efflux pump regulated by MgrA of *Staphylococcus aureus*. Antimicrob Agents Chemother 50:1104-1107.

Tylecote, R. F. 1992. A History of Metallurgy. Institute of Materials. London.

Urwin, R., and M. C. Maiden. 2003. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol 11:479-487.

Valdezate, S., C. Labayru, A. Navarro, M. A. Mantecon, M. Ortega, T. M. Coque, M. Garcia, and J. A. Saez-Nieto. 2009. Large clonal outbreak of multidrug-resistant CC17 ST17 *Enterococcus faecium* containing Tn5382 in a Spanish hospital. J Antimicrob Chemother 63:17-20.

Van Melder, L., and M. Saavedra De Bast. 2009. Bacterial toxin-antitoxin systems: more than selfish entities? PLoS Genet 5:e1000437.

van Schaik, W., and R. J. Willems. 2010a. Genome-based insights into the evolution of enterococci. Clin Microbiol Infect 16:527-532.

Vats, N., and S. F. Lee. 2001. Characterization of a copper-transport operon, copYAZ, from *Streptococcus mutans*. Microbiology 147:653-662.

Vickerman, M. M., S. E. Flannagan, A. M. Jesionowski, K. A. Brossard, D. B. Clewell, and C. M. Sedgley. 2010. A genetic determinant in *Streptococcus gordonii* Challis encodes a peptide with activity similar to that of enterococcal sex pheromone cAM373, which facilitates intergeneric DNA transfer. *J Bacteriol* **192**:2535-45.

Vijayakrishnan, R., and A. Rapose. 2012. Fatal *Enterococcus durans* aortic valve endocarditis: a case report and review of the literature. *BMJ Case Rep* 8.

Waar, K., A. B. Muscholl-Silberhorn, R. J. Willems, M. J. Slooff, H. J. Harmsen, and J. E. Degener. 2002. Genogrouping and incidence of virulence factors of *Enterococcus faecalis* in liver transplant patients differ from blood culture and fecal isolates. *J Infect Dis* **185**:1121-7.

Waldron, K. J., and N. J. Robinson. 2009a. How do bacterial cells ensure that metalloproteins get the correct metal? *Nat Rev Microbiol* **7**:25-35.

Warnes, S. L., S. M. Green, H. T. Michels, C. W. Keevil. 2010. Biocidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNAs. *Appl Environ Microbiol* **76**:5390-5401.

Warnes, S. L., and C. W. Keevil. 2011. Mechanism of Copper Surface toxicity in vancomycin-resistant enterococci following wet or dry surface contact. *Appl Environ Microbiol* **77**:6049-6059.

Warnes, S. L., C. J. Highmore, C. W. Keevil. 2012. Horizontal transfer of antibiotic resistance genes on abiotic touch surfaces: implications for public health. *MBio* **3** pii: e00489-12.

Weaver, K. E., L. B. Rice, and G. Churchward. 2002. Plasmids and Transposons, p. 219-263. In M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, and L. B. Rice (ed.), *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. ASM Press, Washington, DC.

Weaver, K. E., S. M. Kwong, N. Firth, and M. V. Francia. 2009. The RepA_N replicons of Gram-positive bacteria: a family of broadly distributed but narrow host range plasmids. *Plasmid* **61**:94-109.

Weigel, L. M., D. B. Clewell, S. R. Gill, N. C. Clark, L. K. McDougal, S. E. Flannagan, J. F. Kolonay, J. Shetty, G. E. Killgore, and F. C. Tenover. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* **302**:1569-71.

Werner, G., I. Klare, and W. Witte. 1999. Large conjugative *vanA* plasmids in vancomycin-resistant *Enterococcus faecium*. *J Clin Microbiol* **37**:2383-4.

Werner, G., B. Hildebrandt, and W. Witte. 2001. Aminoglycoside-streptothricin resistance gene cluster *aadE-sat4-aphA-3* disseminated among multiresistant isolates of *Enterococcus faecium*. *Antimicrob Agents Chemother* **45**:3267-9.

Werner, G., B. Hildebrandt, and W. Witte. 2003. Linkage of *erm(B)* and *aadE-sat4-aphA-3* in multiple-resistant *Enterococcus faecium* isolates of different ecological origins. *Microb Drug Resist* **9 Suppl 1**:S9-16.

Werner, G., B. Strommenger, and W. Witte. 2008a. Acquired vancomycin resistance in clinically relevant pathogens. *Future Microbiol* **3**:547-62.

Werner, G., S. Gfrörer, C. Fleige, W. Witte, and I. Klare. 2008b. Tigecycline-resistant *Enterococcus faecalis* strain isolated from a German intensive care unit patient. *J Antimicrob Chemother* **61**:1182-1183.

Werner, G., C. Fleige, B. Ewert, J. A. Laverde-Gomez, I. Klare, and W. Witte. 2010. High-level ciprofloxacin resistance among hospital-adapted *Enterococcus faecium* (CC17). *Int J Antimicrob Agents* **35**:119-125.

Werner, G., C. Fleige, U. Geringer, W. van Schaik, I. Klare, and W. Witte. 2011. IS element *IS16* as a molecular screening tool to identify hospital-associated strains of *Enterococcus faecium*. *BMC Infect Dis* **11**:80.

Werner, G., I. Klare, C. Fleige, U. Geringer, W. Witte, H. M. Just, and R. Ziegler. 2012. Vancomycin-resistant vanB-type *Enterococcus faecium* isolates expressing varying levels of vancomycin resistance and being highly prevalent among neonatal patients in a single ICU. *Antimicrob Resist Infect Control* **1**:21.

Werner, G., T. M. Coque, C. M. Franz, E. Grohmann, K. Hegstad, L. Jensen, W. van Schaik, K. Weaver. 2013. Antibiotic resistant enterococci-tales of a drug resistance gene

trafficker. Int J Med Microbiol **303**:360-379.

Willems, R. J., W. Homan, J. Top, M. van Santen-Verheuver, D. Tribe, X. Manziros, C. Gaillard, C. M. Vandenbroucke-Grauls, E. M. Mascini, E. van Kregten, J. D. van Embden, and M. J. Bonten. 2001. Variant *esp* gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospitals. Lancet **357**:853-855.

Willems, R. J., J. Top, M. van Santen, D. A. Robinson, T. M. Coque, F. Baquero, H. Grundmann, and M. J. Bonten. 2005. Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. Emerg Infect Dis **11**:821-828.

Willems, R.J. and W. van Schaik. 2009. Transition of *Enterococcus faecium* from commensal organism to nosocomial pathogen. Future Microbiol **4**:1125-1135.

Willems, R. J., van Schaik, J. Top, D. R. Riley, J. Boekhorst, J. E. P. Vrijenhoek, C. M. E. Schapendonk, A. P. A. Hendrickx, I. J. Nijman, M. J. M. Bonten, H. Tettelin, and R. J. L. Willems. 2010. Pyrosequencing-based comparative genome analysis of the nosocomial pathogen *Enterococcus faecium* and identification of a large transferable pathogenicity island. BMC Genomics **11**:239.

Willems, R. J., W. P. Hanage, D. E. Bessen, and E. J. Feil. 2011. Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance. FEMS Microbiol Rev **35**:872-900.

Willems, R. J., J. Top, W. van Schaik, H. Leavis, M. Bonten, J. Sirén, W. P. Hanage, and J. Corander. 2012. Restricted Gene Flow among Hospital Subpopulations of *Enterococcus faecium*. MBio **3**:e00151-12.

Wilson, I. G., and G. G. McAfee. 2002. Vancomycin-resistant enterococci in shellfish, unchlorinated waters, and chicken. Int J Food Microbiol **79**:143-51.

Witte, W. 2000. Ecological impact of antibiotic use in animals on different complex microflora: environment. Int J Antimicrob Agents **14**:321-325.

Wood, B.J. and W.H.N Holzapfel. 1995. The Genera of Lactic Acid Bacteria. **2**:327-367. Chapman & Hall.

Wozniak, R. A., and M. K. Waldor. 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* **8**:552-63.

Ye, H. F., M. Zhang, M. O'Donoghue, and M. Boost. 2012. Are *qacG*, *qacH* and *qacJ* genes transferring from food isolates to carriage isolates of staphylococci? *J Hosp Infect* **80**:95-6.

Yoshida, Y., S. Furuta, and E. Niki.1993. Effects of metal chelating agents on the oxidation of lipids induced by copper and iron. *Biochim Biophys Acta* **1210**:81-88.

Zhang, X., J. E. Vrijenhoek, M. J. Bonten, R. J. Willems, and W. van Schaik. 2011. A genetic element present on megaplasms allows *Enterococcus faecium* to use raffinose as carbon source. *Environ Microbiol* **13**:518-28.

Zhang, X., F. L. Paganelli, D. Bierschenk, A. Kuipers, M. J. Bonten, R. J. Willems, and W. van Schaik. 2012. Genome-wide identification of ampicillin resistance determinants in *Enterococcus faecium*. *PLoS Genet* **8**:e1002804.

Zhu, L., J. Elguindi, C. Rensing, and S. Ravishankar. 2012. Antimicrobial activity of different copper alloy surfaces against copper resistant and sensitive *Salmonella enterica*. *Food Microbiol* **30**:303-310.

Zscheck, K.K., and B. E. Murray. 1990. Evidence for a staphylococcal-like mercury resistance gene in *Enterococcus faecalis*. *Antimicrob Agents Chemother* **34**:1287-1289.

Zuniga, M., I. Pardo, and S. Ferrer. 2003. Conjugative plasmid pIP501 undergoes specific deletions after transfer from *Lactococcus lactis* to *Oenococcus oeni*. *Arch Microbiol* **180**:367-373.

CAPÍTULO 2

Objetivos e Lista de trabalhos

A resistência aos antibióticos é considerada um dos problemas de saúde pública e económicos mais relevantes da atualidade (25,000 mortes/ano-União Europeia). Medidas políticas e intervenções no terreno (focadas na limitação do uso de antibióticos) foram adotadas em diferentes regiões e setores para controlar este problema, mas sem o sucesso esperado.

Sendo necessárias novas estratégias de intervenção para a contenção da disseminação da resistência aos antibióticos, a investigação científica no tópico “*fatores promotores da resistência aos antibióticos na interface da saúde humana-animal-ambiente*” foi considerada prioritária pela Comissão Europeia e o 7º Programa Quadro de Financiamento Europeu à Investigação. Entre estes fatores, está o impacto do uso de diversos biocidas (incluídos em produtos alimentares, desinfetantes e antissépticos) na seleção e dinâmica de elementos genéticos móveis associados à resistência a antibióticos.

Com efeito, a acumulação de elementos genéticos adquiridos contribui para uma melhor sobrevivência e adaptação dos microrganismos a novos ambientes e hospedeiros. Em *Enterococcus* spp. parece ter promovido o sucesso de determinadas linhagens clonais em diferentes nichos ecológicos e, possivelmente, contribuído para que se tornasse um importante patógeno nosocomial a nível mundial. Contudo, a maioria dos estudos existentes tem-se centrado no impacto da aquisição de resistência a antibióticos sobre a evolução genómica e adaptabilidade de *Enterococcus* spp. a diferentes nichos ecológicos, designadamente aos sujeitos a uma elevada pressão seletiva associada ao uso de antibióticos.

Neste contexto, a elaboração desta tese foi baseada na seguinte **HIPÓTESE**:

A aquisição de genes que conferem tolerância a biocidas contribui para a evolução genómica de Enterococcus spp. resistentes a antibióticos, favorecendo a sua sobrevivência e adaptação a diferentes hospedeiros e comunidades ecológicas.

Estabeleceu-se como **OBJETIVO GERAL:**

*Caracterizar o contexto ecológico e genético da tolerância a biocidas em *Enterococcus* spp. resistentes a antibióticos.*

Para alcançar este objetivo foram incluídos 920 *Enterococcus* spp. de diferentes nichos ecológicos (humano, animal, ambiental, alimentos) obtidos durante um período de 16 anos (1997-2012) e com características fenotípicas e genotípicas variadas. Para além da avaliação da resistência a antibióticos, o estudo incidiu sobre a tolerância a compostos de cobre, mercúrio e de amónio quaternário, largamente utilizados em variadas atividades antropogénicas e disseminados em diferentes ambientes.

Os seguintes **OBJETIVOS ESPECÍFICOS** foram estabelecidos:

1. *Analisar a ocorrência e dispersão de genes associados à tolerância ao cobre, mercúrio e compostos de amónio quaternário em *Enterococcus* spp. de origem humana, animal, ambiental e alimentos.* Pretendeu-se verificar se *Enterococcus* spp., de diferentes comunidades ecológicas e hospedeiros, constituem reservatórios importantes de genes de tolerância a biocidas, favorecendo a sobrevivência e persistência de determinadas estirpes com resistência múltipla aos antibióticos em diferentes ambientes. Esta informação é importante para se estabelecerem medidas futuras, que minimizem o impacto do uso de biocidas na epidemiologia da resistência aos antibióticos.

2. *Conhecer a estrutura populacional de *E. faecium* e *E. faecalis* portadores de genes de tolerância ao cobre, mercúrio e compostos de amónio quaternário resistentes a antibióticos.* Pretendeu-se avaliar se a capacidade de adquirir e acumular genes que conferem tolerância a biocidas ocorre, mais frequentemente, em linhagens clonais predominantes em determinadas comunidades ecológicas. Uma melhor sobrevivência destas linhagens, em nichos e hospedeiros particulares, favorece o seu envolvimento em eventos de transmissão horizontal e, consequentemente, a aquisição de diferentes características adaptativas, nomeadamente a resistência a antibióticos.

3. *Correlacionar a presença de genes de tolerância ao cobre, mercúrio e compostos de amónio quaternário com fenótipos/genótipos particulares de resistência a antibióticos.*

Pretendeu-se compreender se os biocidas podem selecionar/manter determinados perfis de resistência a antibióticos em diferentes comunidades ecológicas. Foi também avaliado o impacto da transmissão horizontal na co-dispersão de genes de resistência a antibióticos e que conferem tolerância a biocidas, bem como a diversidade dos elementos genéticos móveis associados.

4. *Conhecer o ambiente genético dos genes de tolerância ao cobre, mercúrio e compostos de amónio quaternário nos isolados estudados e em genomas disponíveis em bases de dados.*

Pretendeu-se verificar se em *Enterococcus* spp. genes associados à tolerância a biocidas estão incluídos em organizações genéticas multimodulares, o que poderá favorecer a sua participação na estruturação de elementos genéticos, potencialmente, associados a patogenicidade e resistência a antibióticos. Pretendeu-se também avaliar a contribuição de outros géneros bacterianos, pertencentes às mesmas comunidades ecológicas, na evolução genómica da tolerância a biocidas e resistência a antibióticos em *Enterococcus* spp.

Lista de trabalhos que integram a tese.

Os resultados obtidos nesta tese originaram 6 artigos (3 publicações em revistas internacionais, 1 artigo submetido para publicação e 2 artigos em preparação), organizados em função dos objectivos específicos propostos.

I. Resistência a antibióticos no ambiente de produção animal: determinantes genéticos e linhagens clonais.

O estudo de *Enterococcus* spp. de suiniculturas foi importante dado que o sulfato de cobre é usado como aditivo alimentar na produção intensiva de suínos (em concentrações superiores às usadas noutros tipos de produção animal) e os compostos de amónio quaternário são usados como desinfetantes de superfícies. Assim, foi necessário caracterizar a epidemiologia de *Enterococcus* spp. resistentes a antibióticos, em diferentes suiniculturas portuguesas, de forma a selecionar os isolados de interesse para posteriores estudos no âmbito da tolerância aos biocidas.

- Novais C, Freitas AR, Silveira E, Antunes P, Silva R, Coque TM, Peixe L. 2013. Spread of multidrug-resistant *Enterococcus* to animals and humans: an underestimated role for the pig farm environment. *Journal of Antimicrobial Chemotherapy*, 2013; 68(12): 2746-2754.

II. Dispersão de genes associados a tolerância a metais em *Enterococcus* spp. resistentes a antibióticos de diferentes comunidades ecológicas e linhagens clonais.

- Silveira E, Freitas AR, Antunes P, Barros M, Campos J, Coque TM, Peixe L, Novais C. Co-transfer of resistance to high concentrations of copper and first-line antibiotics among *Enterococcus* from different origins (humans, animals, the environment and foods) and clonal lineages. *Journal of Antimicrobial Chemotherapy*. Aceite para publicação (doi:10.1093/jac/dkt479).
- Silveira E, Barros M, Freitas AR, Antunes P, Coque TM, Peixe L, Novais C. *Enterococcus* spp from different origins and clonal lineages carry two *merA* genes found in several *Firmicutes* members and are co-transferred with antibiotic resistance and copper tolerance. (Em preparação)

III. Modularização de plataformas genéticas móveis portadoras de genes que codificam para tolerância a metais, compostos de amônio quaternário e/ou resistência a antibióticos.

- Silveira E, Barros M, Freitas A R, Antunes P, Coque TM, Peixe L, Novais C. Antibiotic resistance, copper and mercury tolerance genes are carried by *RepA_N* *pLG1*-like megaplasms among *Enterococcus* from several origins and clones. (Em preparação).
- Silveira E, Marques P, Barros M, Cardoso N, Freitas A R, Mourão J, Coque TM, Antunes P, Peixe L, Novais C. First description of a multidrug-resistant ST17 *Enterococcus faecium* carrying a transferable *Inc18*-like::*qacZ* plasmid. (Submetido para publicação).
- Novais C, Freitas AF, Silveira E, Baquero F, Peixe L, Roberts AP, Coque TM. 2012. Different genetic supports for the *tet(S)* gene in *Enterococci*. *Antimicrobial Agents and Chemotherapy*, 2012; 56(11):6014-6018.

CAPÍTULO 3

Resultados e Discussão

3.1. Resistência a antibióticos no ambiente de produção animal: determinantes genéticos e linhagens clonais

Publicações

3.1.1. *Spread of multidrug-resistant Enterococcus to animals and humans: an underestimated role for the pig farm environment.*

Neste trabalho foi avaliada a disseminação de *Enterococcus* spp. resistentes a antibióticos em suínos e ambiente de suiniculturas, mostrando-se que está associada quer à dispersão clonal de isolados com resistência múltipla quer a transferência horizontal. Da larga coleção de *Enterococcus* spp englobados neste trabalho, em que Eduarda Silveira participou na caracterização clonal e nos estudos de transferência horizontal de genes de resistência a antibióticos, foram selecionados isolados com características fenotípicas e genotípicas diversas para estudos subsequentes relativos à caracterização da tolerância a biocidas.

Spread of multidrug-resistant *Enterococcus* to animals and humans: an underestimated role for the pig farm environment.

Carla Novais¹, Ana R. Freitas¹, Eduarda Silveira¹, Patrícia Antunes^{1,3}, Ricardo Silva⁴,
Teresa M. Coque^{2,5,6}, Luísa Peixe¹

¹REQUIMTE, Laboratório de Microbiologia. Faculdade de Farmácia, Universidade do Porto, Porto, Portugal;

²Fundación para la Investigación Biomédica del Hospital Universitario Ramón y Cajal (FiBioHRYC, IRYCIS),

Madrid, Spain; ³Faculdade de Ciências da Nutrição e Alimentação. Universidade do Porto, Porto, Portugal;

⁴Faculdade Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal; ⁵Unidad de Resistencia a

Antibióticos y Virulencia Bacteriana asociada al Consejo Superior de Investigaciones Científicas (CSIC),

Madrid, Spain; ⁶CIBER en Epidemiología y Salud Pública (CIBERESP), Madrid, Spain.

Journal of Antimicrobial Chemotherapy. 2013; 68(12): 2746-2754.

Spread of multidrug-resistant *Enterococcus* to animals and humans: an underestimated role for the pig farm environment

Carla Novais^{1†}, Ana R. Freitas^{1,2}, Eduarda Silveira¹, Patrícia Antunes^{1,3}, Ricardo Silva⁴, Teresa M. Coque^{2,5,6} and Luísa Peixe^{1*†}

¹REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; ²Fundación para la Investigación Biomédica del Hospital Universitario Ramón y Cajal (FiBioHRYC, IRYCIS), Madrid, Spain; ³Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Porto, Portugal; ⁴Faculdade Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal; ⁵Unidad de Resistencia a Antibióticos y Virulencia Bacteriana asociada al Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain; ⁶CIBER en Epidemiología y Salud Pública (CIBERESP), Madrid, Spain

*Corresponding author. Tel: +351-220428580; Fax: +351-22693390; E-mail: lpeixe@ff.up.pt

†Carla Novais and Luísa Peixe contributed equally to the direction, preparation and writing of the manuscript.

Received 11 December 2012; returned 10 April 2013; revised 21 May 2013; accepted 15 June 2013

Objectives: The aim of this study was to discover the potential role of the pig farm environment in the spread of multidrug-resistant (MDR) *Enterococcus* strains, including high-risk clones, to animals and humans.

Methods: *Enterococcus* isolates were recovered from a variety of samples ($n=82$; swine, feed/medicines/antiseptics and pig farm facilities) from six Portuguese farms, most using antibiotics. Antimicrobial susceptibility/conjugation assays were performed by standard procedures, bacterial identification/screening of antibiotic resistance genes were performed by PCR and clonality was determined using PFGE/multilocus sequence typing.

Results: *Enterococcus* isolates resistant to antibiotics ($n=473$) were recovered from samples of different origin (swine, feed/antiseptics, animal residues and pig farm facilities), but only the clinically relevant species *Enterococcus faecium* ($n=171$) and *Enterococcus faecalis* ($n=78$) were included for further comprehensive molecular analysis. Isolates resistant to vancomycin, ampicillin, tetracyclines, erythromycin and aminoglycosides were better recovered in Slanetz–Bartley medium with these antibiotics present than in media not supplemented with antibiotics ($P<0.05$). *E. faecium* was more frequently resistant to ampicillin, ciprofloxacin or nitrofurantoin and *E. faecalis* to tetracyclines, chloramphenicol or aminoglycosides ($P<0.05$). Glycopeptide and erythromycin resistance rates were similar in both species. The transfer of resistance to several antibiotics, including vancomycin and ampicillin, was demonstrated. Clones associated with human infections were detected in different samples from the same farm [*E. faecium* from sequence type (ST) 78 lineage and *E. faecalis* ST16; manure, waste lagoons, faeces and drinking water] and in geographically distant farms [*E. faecium* clonal complex (CC) 5; *E. faecalis* CC21 and ST16].

Conclusions: The pig farm environment has an underestimated potential role in the transmission of MDR *Enterococcus* to animals and, possibly, to humans. The continuous contact of swine with MDR *Enterococcus* by different routes (e.g. feed, dust, air and rooms) might decrease the impact of restrictive antibiotic use policies and reinforces the need for different and preliminary interventions at the husbandry management level.

Keywords: swine, *vanA*, high-risk clones, co-resistance, indirect contamination routes

Introduction

Animal infectious diseases can greatly affect the cost of food production, with antimicrobial agents commonly used on farms.^{1,2} The pervasive selection of antibiotic-resistant bacteria in the animals' guts facilitates the persistence and spread of multidrug-resistant (MDR) strains to humans via the food chain,³

occurring by direct (e.g. food; farm workers in contact with animals or the pig farm environment) and indirect (animal waste handling, wind, surface and ground waters) routes.^{4–9} Most of the studies addressing the occurrence of MDR bacteria in the animal setting are focused on direct routes and do not provide a comprehensive analysis of the genetic elements and clones involved in the spread of antibiotic resistance.^{5,6,8,10–13}

Enterococcus spp. are used as a bioindicator of faecal contamination and antibiotic resistance among animals and foods.³ Different countries have reported the occurrence of antibiotic-resistant enterococci isolated from swine,^{3,10,12–14} with descriptions of strains and genetic elements of relevance in the clinical setting at the local^{15,16} or global scale.¹⁷ In recent decades, *Enterococcus* has become one of the main causes of nosocomial infections worldwide, owing to the wide dissemination of the high-risk clonal complexes (CCs) of *Enterococcus faecium* and *Enterococcus faecalis*.^{18–20} Recently, strains belonging to high-risk CCs associated with human infections have been recovered from farm and companion animals (*E. faecium* from the formerly designated CC17; *E. faecalis* CC2) and strains from CCs commonly found among animals have also been detected in humans [*E. faecium* CC5; *E. faecalis* sequence type (ST) 16].^{21,22}

This study addresses the potential role of the pig farm environment in the transmission of MDR *E. faecium* and *E. faecalis* (namely high-risk CCs) to farm animals and, by indirect routes, to humans.

Methods

Study design

Six pig farms located in the north, centre and south of Portugal, arbitrarily designated by roman numerals, were analysed from April 2006 to December 2007. Farms I–III, V and VI used intensive production methods (pigs are housed indoors and rely mostly on rations for food) and farm IV used extensive methods (pigs remain outside and rely primarily on foraging and grazing for food). Information concerning the use of antibiotics was available for four farms. The animals were treated either with tetracyclines (farms V and VI), gentamicin (farm II) or tetracycline plus tylosin followed by amoxicillin (farm III). Adult animals were also feed with Oreprox[®], (supplement including copper and zinc) on farm III. Antibiotics were replaced by the probiotic formula Farmaflor[®] (complex of lactic bacteria for the prevention of digestive nutritional disorders among swine) each 6 months on farms V and VI.

Farms III, V and VI were periodically disinfected when they were free of animals, and reproductive females were washed before entering the maternity rooms. Virkon[®] and Mistral[®] (contain copper; also used to disinfect piglets' skin) were used on farm III and quicklime and other products for which information was not available on farms V and VI.

Eighty-two samples were classified according to their origin: (i) swine ($n=20$; faeces, nostril/surface swabs from piglets aged 2–4 weeks or young adults/adult animals aged ≥ 6 months); (ii) feed/antiseptic ($n=22$; feed, water or antiseptic); (iii) residues ($n=17$; waste lagoons, residual waters and manure); and (iv) pig farm facilities ($n=23$; air, water, walls/floors after disinfection, dust, soil and food distribution equipment).

Sample processing and antibiotic susceptibility testing

Samples were enriched in buffered peptone water (1/10) for 16 h (37°C) and a 0.1 mL aliquot was plated onto Slanetz–Bartley agar plates without antibiotics and supplemented with 16 mg/L of tetracycline, 1000 mg/L of streptomycin, 125 mg/L of gentamicin, 8 mg/L of erythromycin, 6 mg/L of vancomycin or 16 mg/L of ampicillin. From each sample, only one colony per morphology and plate was selected for further studies.

Antibiotic susceptibility to vancomycin, teicoplanin, ampicillin, tetracycline, minocycline, erythromycin, quinupristin/dalfopristin, ciprofloxacin, chloramphenicol, nitrofurantoin and to high concentrations of gentamicin and streptomycin was determined using the disc diffusion method following CLSI guidelines.²³ *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as control strains. For practical purposes, *Enterococcus* classified as resistant or with an intermediate

level of resistance according to CLSI guidelines were considered as resistant. MDR was considered when the isolates were resistant to three or more antibiotics from different families.²⁴

Identification and characterization of antibiotic resistance genes

Genus identification was performed by Gram's stain, catalase test, bile-aesculin hydrolysis and growth in 6.5% NaCl. Identification of *E. faecium*, *E. faecalis*, *Enterococcus durans* and *Enterococcus hirae*, and detection of genes conferring resistance to glycopeptides [*vanA*, *vanB*, *vanC1* and *vanC2*], tetracyclines [*tet(M)*, *tet(L)*, *tet(O)*, *tet(S)*, *tet(K)*, *tet(W)*, *tet(X)*, *tet(Q)*, *tet(32)*, *tetA(P)* and *tetB(P)*], macrolides [*erm(A)*, *erm(B)* and *erm(C)*] and aminoglycosides [*pac(6')*-Ie-aph(2'')-Ia, *aph(2'')-Ib*, *aph(2'')-Ic* and *aph(2'')-Id*] was performed using PCR as previously described.^{10,25–29}

Clonal relatedness

Representative *E. faecium* and *E. faecalis* from different farms and antibiotic resistance phenotypes were analysed by PFGE and multilocus sequence typing (MLST) as described (<http://www.mlst.net>).^{26,30} Of note, STs previously included in CC17 were recently separated in three different lineages (ST17, ST18 and ST78).²²

Conjugation assays

Filter mating assays were performed at a donor:recipient ratio of 1:1 using *E. faecalis* JH2–2, *E. faecium* BM4105RF (both resistant to rifampicin and fusidic acid) or *E. faecium* GE-1 (resistant to tetracycline, rifampicin and fusidic acid) as receptor strains.¹⁷

Statistical analysis

Differences among the rates of antibiotic resistance and among isolates recovered from different plates or samples were analysed by the Fisher exact test ($\alpha=0.05$) using GraphPad Prism software, version 6.0a.

Results

Bacterial diversity and antibiotic resistance background

Enterococci ($n=473$) were recovered from 98% of the samples studied (80/82). They were identified as *E. faecium* ($n=171$), *E. faecalis* ($n=78$), *E. hirae* ($n=73$), *Enterococcus gallinarum* ($n=14$), *Enterococcus casseliflavus* ($n=5$) and other *Enterococcus* spp. ($n=132$). *E. faecium* was the predominant species on most of the farms (II, IV, V and VI). Isolates resistant to tetracycline (occurring in 96%–100% of samples), erythromycin (96%–100%), quinupristin/dalfopristin (83%–100%), ciprofloxacin (85%–100%), nitrofurantoin (55%–100%), high levels of streptomycin (65%–94%), high levels of gentamicin (60%–82%) or chloramphenicol (60%–77%) were recovered from all groups of samples. Resistance to glycopeptides (vancomycin, teicoplanin; 5%–17%) was detected in swine (faeces, hoof) and piggery facilities (maternity soil and exterior soil from the extensive production piggery IV). Ampicillin-resistant isolates (9%–35%) were detected in samples from diverse sources, including animals (faeces and nostril), medicine and feed, animal residues (e.g. waste lagoons, manure and residual water) and piggery facilities (maternity soil and sty) (see Table S1, available as Supplementary data at JAC Online, for more detailed information). No correlation was detected between the use of a particular antibiotic by a specific farm and

the occurrence of higher rates of resistance to that antibiotic compared with other farms ($P > 0.05$; data not shown).

Among the 473 *Enterococcus* isolates recovered, only the predominant and clinically relevant species *E. faecium* and *E. faecalis*, were included for further analysis. Table 1 shows antibiotic-resistant isolates from these two species recovered from media supplemented or not with antibiotics. Isolates resistant to ampicillin or gentamicin were better detected using agar plates containing each of these two compounds ($P < 0.05$), in contrast to isolates resistant to other antibiotics, which were recovered from media containing antibiotics from different families ($P > 0.05$). Nevertheless, every time an antibiotic was used to supplement Slanetz–Bartley medium, isolates resistant to that antibiotic were better recovered than in media without any supplement ($P < 0.05$).

The analysis of the data by species revealed that *E. faecium* was more frequently resistant to ampicillin, ciprofloxacin or nitrofurantoin and *E. faecalis* to tetracycline, minocycline, chloramphenicol, high concentrations of gentamicin or high concentrations of streptomycin ($P < 0.05$). Resistance to glycopeptides or erythromycin was similar in both species ($P > 0.05$). MDR *Enterococcus* (99% of the *E. faecalis* and 60% of the *E. faecium*) isolates were frequently recovered on the farms studied.

The diversity and occurrence of antibiotic resistance genes is also shown in Table 1. High-level resistance to glycopeptides was due to the presence of *vanA* (four *E. faecium* and one *E. faecalis*). Genes coding for resistance to tetracyclines [*tet*(M), *tet*(L), *tet*(S)], aminoglycosides [*aac*(6')-Ie-aph(2'')-Ia] and showing the MLS_B phenotype *erm*(B) were detected in isolates recovered from plates supplemented with different antibiotics. *Enterococcus* carrying *tet*(M), *tet*(L) or *erm*(B) and exhibiting a susceptible phenotype to either tetracyclines or erythromycin were detected in 3% and 2% of the isolates, respectively.

Clonal relatedness

Fifty-three *E. faecium* and 28 *E. faecalis* were classified among 35 *E. faecium* and 13 *E. faecalis* PFGE types. They corresponded to a diversity of STs, some of them belonging to the main CCs of *E. faecalis* (one CC2 and three CC21) and *E. faecium* [nine CC5 (two included in ST18 and four in ST78 clonal lineages), four CC22 and two CC9] (Table 2). The *E. faecalis* CC2 (ST6) isolate was detected in a water sample from farm II and the *E. faecalis* CC21 (ST21, ST224) isolates in air and manure from farms II and VI. *E. faecium* ST132 and ST431 (ST18 clonal lineage) and ST393 (ST78 clonal lineage)²² were recovered from environmental samples from farm II; the CC5 (ST150, ST185) isolates from farms IV, V and VI, both in swine and environmental samples; the *E. faecium* CC22 isolates from antiseptic and drinking water from farms III and IV; and *E. faecium* CC9 from pig farm air and feed from farms V and VI (Table 2). Most of these clones were recovered from plates supplemented with antibiotics.

Ten clones (four *E. faecalis* and six *E. faecium*) were detected in different samples from the same farm. Among these, horizontal spread among animals of different ages and among animals or the farm environment was detected within farm III (*E. faecalis* clones SN204, SN205; *E. faecium* clone SN221, SN226). Five clones (three *E. faecalis* and two *E. faecium*) were also isolated from different farms throughout the study, and occurred in different sample types, including pig faeces, animal residues (solid manure, waste lagoon) and feed.

Conjugation assays

Conjugative transfer of resistance to one or more antibiotics varied between 18% and 80% depending on the plating method used (Table 3). A diversity of antibiotic resistance phenotypes was observed. Specific strains (Table 3) were able to transfer distinct antibiotic resistance determinants when different antibiotics were used in plates to recover transconjugants, although resistance to tetracycline and/or erythromycin was transferred in most cases. *E. faecium* and *E. faecalis* transferred vancomycin resistance and transconjugants were recovered from plates supplemented with vancomycin or tetracycline. Ampicillin resistance was transferred from few *E. faecium* belonging to ST393 and ST431 and only when this antibiotic was used to select ampicillin-resistant transconjugants.

Discussion

The recovery of MDR *E. faecalis* and *E. faecium* (some associated with high-risk CCs causing human infections) from animals and environmental samples from intensive and extensive farms highlights the potential impact of indirect routes on the transmission of both antibiotic resistance traits and successful clones with relevance in human medicine.

The occurrence on all farms of MDR isolates containing antibiotic resistance genes that can be co-transferred is of concern, as MDR gives a great opportunity for microorganism survival under different selective pressures, namely when one (farms V and VI) or more (farm III) antimicrobial agents are used.³¹ The occurrence of resistance to glycopeptides, which were banned for animal use in Europe in 1998,³² and the transfer of ampicillin resistance among *Enterococcus* are of note.³³ This study is the first documenting transferable ampicillin resistance among isolates of swine origin, despite the frequent use of β -lactams in the animal production setting.²

The detection of host-specific high-risk CCs such as *E. faecium* CC5 (mainly associated with swine and previously described to be spread among swine, healthy humans and hospitalized patients from European countries and the USA in the last decade) *E. faecium* from ST18/ST78 clonal lineages and *E. faecalis* CC2 earlier isolated in both animals and humans^{17,20} is worrying, as it might result in a zoonotic potential for these major clones. Transfer of these strains from animals to humans, or vice versa, as seems to have happened with CC398 *Staphylococcus aureus*³⁴ cannot be ruled out. Also, the survival of *Enterococcus* under different chemical conditions within the animal setting³⁵ might have contributed to the isolation of these strains from samples collected across pig farms.

The high-frequency occurrence of MDR isolates in piggeries (e.g. environment, drinking water and feed) and the colonization of pigs from young to adult ages can facilitate the spread and persistence of common strains within farm facilities and the colonization of new animals, as previously observed by Heuer et al.³⁶ in poultry flocks. Moreover, the recovery of the same clone from swine of different ages and the detection of MDR isolates, including high-risk CCs, in samples of drinking water (supposedly disinfected with UV and chlorine), air and dust and from disinfected rooms and in an antiseptic sample collected after being used in a piglet, indicate ineffective disinfection and the survival of antibiotic-resistant *Enterococcus* spp. despite cleaning measures. Animal transport,³⁷ common pig

Table 1. Resistance to 12 antibiotics of *E. faecium* and *E. faecalis* recovered from different piggy samples

Basis of isolate selection	Isolates recovered in different selective media (n)	Samples (n)	Piggery	No. of isolates with antibiotic resistance and the genes											
				VAN	TEC	AMP	TET	MIN	ERY	Q/D	CIP	CHL	GEN	STR	NIT
No antibiotic	<i>E. faecalis</i> (10)	residues (manure, waste lagoon, residual water; 7), medicine/antiseptic (2), piglet/adult feed (5), drinking water (1), animal (faeces, nostril, skin; 9), piggery facilities (soil, feed distributor device, air, dust; 16).	II, III, IV, V, VI	0	0	0	10	9	8	NR	6	1	3	6	0
	<i>E. faecium</i> (48)			0	0	3	35	34	43	28	32	8	1	10	19
Vancomycin (6 mg/L)	<i>E. faecalis</i> (2)	residues (manure, residual water; 2), animal (6 month pig faeces; 1), piggery facilities (exterior soil; 1).	III, IV, V, VI	0	0	0	2	2	2	NR	0	0	1	2	0
	<i>E. faecium</i> (5)			4	4	1	4	4	5	4	4	1	0	0	4
Ampicillin (16 mg/L)	<i>E. faecium</i> (22)	residues (manure, waste lagoon, residual water; 5), animal (piglet/adult faeces; 3), piggery facilities (dust; 1).	II, III, V, VI	vanA – 4				tet(M) – 6; tet(L) – 2		erm(B) – 4		aac(6')-Ie-(aph2'')-Ia – 1			
	0			0	13	20	20	18	12	18	2	1	10	11	
Erythromycin (8 mg/L)	<i>E. faecalis</i> (14)	residues (manure, waste lagoon, residual water; 45), medicine (1), antiseptic (1), piglet/adult feed (7), drinking water (2), animal (piglet/adult faeces, piglet skin, nostril, hoof; 11), piggery facilities (dust, soil and ventilation of clean, disinfected and empty rooms, water from maternity soil, soil of a pig-occupied room, exterior soil; 10).	I, II, III, IV, V, VI	1	1	0	14	14	14	NR	6	6	9	9	0
	<i>E. faecium</i> (39)			1	1	5	36	36	39	24	28	1	5	18	19
Tetracycline (16 mg/L)	<i>E. faecalis</i> (14)	residues (manure, waste lagoon; 3), drinking water (2), piglet feed (2), piggery facilities (water from maternity soil, dust, soil of a pig-occupied room (5)	I, II, III, IV, V, VI	vanA – 2				tet(M) – 44; tet(L) – 3		erm(B) – 40		aac(6')-Ie-(aph2'')-Ia – 14			
	0			0	0	14	13	10	NR	9	7	6	9	0	
<i>E. faecium</i> (45)	0	0	6	44	44	41	17	29	3	0	14	27			
Streptomycin (1000 mg/L)	<i>E. faecalis</i> (14)	residues (manure, waste lagoon, residual water; 8), medicine/antiseptic (2), adult feed (2), drinking water (1) animal (piglet faeces; 1), piggery facilities (water from maternity soil, exterior soil; 3).	I, II, III, V, VI	vanA – 2				tet(M) – 51; tet(L) – 39; tet(S) – 1		erm(B) – 26		aac(6')-Ie-(aph2'')-Ia – 4			
	0			0	0	14	14	14	NR	8	10	5	14	1	
<i>E. faecium</i> (5)	0	0	1	4	5	5	4	2	1	1	3	4			
Gentamicin (125 mg/L)	<i>E. faecalis</i> (24)	residues (manure, waste lagoon, residual water; 11), piglet/adult feed (7), drinking water (2) animal (adult/piglet faeces; 6), piggery facilities (water from soil, dust; 2).	I, II, III, V, VI	vanA – 4				tet(M) – 16; tet(L) – 13 tet(S) – 3		erm(B) – 15		aac(6')-Ie-(aph2'')-Ia – 5			
	0			0	0	24	24	24	NR	18	19	23	18	2	
<i>E. faecium</i> (7)	0	0	0	7	7	7	4	5	7	7	4	3			
aac(6')-Ie-aph(2'')-Ia – 29															

Table 2. Epidemiological features and clonal relationship of representative *E. faecalis* and *E. faecium* from the piggeries studied

CC ^a	ST	PFGE (no. of subtypes)	No. of isolates	Date of isolation/region	Source (n)/piggery	Antibiotic resistance profile ^b	Antibiotic resistance genes ^b
<i>E. faecalis</i>							
CC2	6	HB6	1	2007/Sth	water/II	VAN, TEC, TET, MIN, ERY, CIP, CHL, GEN	<i>vanA</i> , <i>tet(M)</i> , <i>aac6'-Ie-aph2''-Ia</i>
CC21	21	HX6	1	2007/Nth	air from piggery facility/VI	TET, MIN, ERY, GEN, STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>aac6'-Ie-aph2''-Ia</i>
		SN206 (HX7)	1	2007/Nth	liquid manure/VI	TET, MIN, ERY, GEN, STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aac6'-Ie-aph2''-Ia</i>
Singleton	224	SN205 (1)	1	2006/Sth	solid manure/II	TET, ERY, CHL, GEN, STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aac6'-Ie-aph2''-Ia</i>
	16	SN205 (5)	7	2006-07/Sth, Ct, Nth	piglet/adult pig faeces (3)/III; solid manure (2)/IV; waste lagoon/I; adult pig feed/VI; drinking water/III	TET, [MIN], ERY, [CHL], GEN, [STR]	<i>tet(M)</i>, [tet(L)], [erm(B)], <i>aac6'-Ie-aph2''-Ia</i>
	200	SN201	1	2006/Sth	solid manure/II	TET, ERY	<i>tet(M)</i>
	35	SN204	5	2006/Sth, Ct	piglet/adult pig feed (2)/II, III; adult pig faeces/III, drinking water/III, facilities dust/III	TET, [MIN], ERY, [CIP], [CHL], [GEN], STR	<i>tet(M)</i>, <i>tet(L)</i>, [erm(B)], <i>aac6'-Ie-aph2''-Ia</i>
	445	SN242	3	2006/Ct	adult pig feed/III, solid manure/III, residual water/III	TET, [MIN], ERY, [CHL], STR, [NIT]	<i>tet(M)</i>, <i>tet(L)</i>, <i>tet(S)</i>, <i>erm(B)</i>
Unk		SN200	1	2006/Sth	waste lagoon/I	TET, STR	<i>tet(M)</i> , <i>tet(L)</i>
		SN202 (1)	2	2006/Sth	solid manure/II, waste lagoon/II	TET, ERY, GEN, [STR]	[tet(M)], [erm(B)], <i>aac6'-Ie-aph2''-Ia</i>
		SN220	1	2006/Ct	adult pig feed/III	TET, ERY	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>
		SN237	2	2006/Sth, Ct	waste lagoon/II, antiseptic/III	TET, MIN, ERY, CHL, [GEN], STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , [aac6'-Ie-aph2''-Ia]
		SN239	1	2006/Sth	solid manure/II	TET, ERY, CHL, GEN, STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>aac6'-Ie-aph2''-Ia</i>
		SN240	1	2006/Ct	solid manure/III	TET, ERY, CHL, GEN, STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aac6'-Ie-aph2''-Ia</i>
<i>E. faecium</i>							
ST18 clonal lineage	132	H119 (1)	1	2007/Sth	water/II	VAN, TEC, AMP, ERY, GEN	<i>vanA</i> , <i>aac6'-Ie-aph2''-Ia</i>
	431	SN208 (1)	1	2007/Sth	facilities dust/II	AMP, TET, MIN, ERY, CIP, STR, NIT	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>
ST78 clonal lineage	393	SN208	4	2006/Sth	solid manure/II, waste lagoon (3)/II	AMP, TET, [MIN], ERY, [CIP], STR, NIT	[tet(M)], [tet(L)], [erm(B)]
CC5	150	SN216	1	2007/Nth	adult pig faeces/V	AMP, TET, ERY, CIP, STR	<i>tet(M)</i> , <i>erm(B)</i>
	185	A (4)	8	2007/Sth, Nth	soil (4)/IV, solid manure (2)/IV, adult pig diarrhoeal faeces/VI, food-distributing equipment/V	[VAN], [TEC], TET, MIN, [ERY], [Q/D], [NIT], [STR]	[vanA], [tet(M)], [tet(L)], [erm(B)]
CC9	433	SN214	1	2007/Nth	air from piggery facility/V	TET, MIN	<i>tet(M)</i> , <i>tet(L)</i>
	437	SN219	1	2007/Nth	adult pig feed/VI	TET, MIN, ERY, CIP, [CHL], STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>
CC22	32	SN210; SN213	2	2007/Sth, Ct	antiseptic/III, drinking water/IV	TET, MIN, [CIP], [NIT]	<i>tet(M)</i> , <i>tet(L)</i> , <i>tet(S)</i>

Continued

Table 2. Continued

CC ^a	ST	PFGE (no. of subtypes)	No. of isolates	Date of isolation/region	Source (n)/piggery	Antibiotic resistance profile ^b	Antibiotic resistance genes ^b
Singleton	435	SN217	1	2007/Nth	adult pig faeces/V	TET, ERY, CIP, GEN, STR	<i>tet(M)</i> , <i>erm(B)</i> , <i>aac6'-Ie-aph2''-Ia</i>
	436	SN220	1	2007/Nth	adult pig feed/V	TET, MIN, CIP	<i>tet(M)</i>
	30	SN221 (1)	3	2006/Ct	piglet faeces/III, facilities dust/III, piglet feed/III	TET, [MIN], ERY, [CIP], [GEN], [STR]	[tet(M)], tet(L), [erm(B)], [aac6'-Ie-aph2''-Ia]
	428	SN207	1	2006/Sth	waste lagoon/I	TET, MIN, ERY, GEN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aac6'-Ie-aph2''-Ia</i>
	429	SN209	1	2006/Sth	solid manure/II	TET, ERY, STR	<i>tet(L)</i> , <i>erm(B)</i>
	430	SN211	2	2007/Sth	piglet feed/II, facilities dust/II	AMP, TET, MIN, ERY, [CIP], STR, NIT	[tet(M)], tet(L), erm(B)
	432	SN212	1	2007/Nth	liquid manure/IV	TET, MIN, ERY, Q/D	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>
Unk	434	SN215	2	2007/Nth	air from piggery facility/V	TET, ERY, STR	<i>tet(M)</i> , <i>tet(L)</i>
	443	SN243	1	2007/Nth	young adult pig faeces/V	VAN, TEC, TET, ERY, NIT	<i>vanA</i> , <i>tet(M)</i> , <i>erm(B)</i>
	new	SN223	1	2006/Sth	waste lagoon/II	TET, MIN, ERY, STR, NIT	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>
		SN222	1	2006/Sth	waste lagoon/I	TET, MIN	<i>tet(M)</i> , <i>erm(B)</i>
		SN224	1	2006/Sth	waste lagoon/II	TET, MIN, ERY, CIP	<i>tet(M)</i> , <i>tet(L)</i>
		SN246	1	2006/Sth	water/II	TET	<i>tet(M)</i>
		SN232	1	2007/Sth	residual water/II	TET, MIN, ERY, CIP, GEN, STR	<i>tet(L)</i> , <i>erm(B)</i>
		SN225	1	2006/Ct	antiseptic/III	TET, ERY, CHL	<i>tet(S)</i>
		SN226 (2)	3	2006/Ct	antiseptic/III, facilities dust/III, adult pig faeces/III	TET, ERY, [Q/D], [CIP], STR	tet(M), tet(L), erm(B)
		SN227	1	2006/Ct	piglet faeces/III	TET	<i>tet(L)</i> , <i>erm(B)</i>
		SN231	1	2006/Ct	residual water/III	TET, ERY, Q/D	<i>tet(M)</i> , <i>erm(B)</i>
		SN248	1	2006/Ct	solid manure/III	TET, MIN, ERY, Q/D, CHL, GEN, STR, NIT	<i>tet(M)</i> , <i>tet(L)</i>
		SN249	1	2007/Sth	soil/IV	–	–
		SN244	1	2007/Sth	soil/IV	TET, MIN, Q/D, NIT	<i>tet(M)</i>
		SN230	2	2007/Nth	waste lagoon/V, solid manure/V	TET, [MIN], ERY, STR, NIT	tet(M), tet(L), erm(B)
		SN233	1	2007/Nth	air from piggery facility/V	TET	<i>tet(M)</i>
		SN236	1	2007/Nth	facilities wall/V	TET, MIN	<i>tet(M)</i> , <i>erm(B)</i>
		SN245	1	2007/Nth	piggery facilities/V	–	<i>erm(B)</i>
		SN234	1	2007/Nth	drinking water/VI	TET, MIN, ERY, STR	<i>tet(M)</i> , <i>erm(B)</i>
		SN235	1	2007/Nth	facilities floor/VI	TET, MIN, ERY, CIP, STR, NIT	<i>erm(B)</i>

GEN, high-level resistance to gentamicin; STR, high-level resistance to streptomycin; Unk, unknown; Sth, south; Ct, center; Nth, north.

–, Absence of antibiotic resistance.

Rows in bold represent common clones detected in different samples from the same piggery farm. Rows in grey represent clones spread in different farms.

^aCCs are shown according to eBURST clustering. MLST was performed in representative clones from the different piggeries.

^bThe variable presence of a given resistance phenotype/genotype among isolates belonging to the same clonal lineage is indicated by brackets.

breeding lines sold to different countries, commercial trade in living animals³⁸ and wild animals (e.g. birds, contacting swine or waste lagoons)³⁹ could be other factors explaining the spread of particular MDR clones to the different farms. Also,

MDR strains detected in manure samples sold for land application by some of the farms studied might contribute to their dispersion to other food products consumed by man, stressing the potential of indirect routes for MDR strain dissemination.^{9,40}

Table 3. Transfer of antibiotic resistance from wild *E. faecium* and *E. faecalis* using media supplemented with different antibiotics for transconjugant selection

Antibiotic used for selection	Positive matings (%)	Wild strains with positive mating assays ^a	Species	CC/ST (no. of isolates)	Antibiotic resistance phenotype transferred ^b
Ampicillin (10 mg/L)	5/18 (28)	71 ^{c,d} , 133 ^c , 109 ^{c,d} , 148 ^{c,d,e} , 512 ^a	<i>E. faecium</i>	SN208-ST393 (2)/ST431 (1) ^f	AMP
Gentamicin (500 mg/L)	4/22 (18)	122 ^g 636 ^g 722 ^{e,g} 77 ^c	<i>E. faecalis</i> <i>E. faecium</i>	SN237-ST224 SN206-CC21/ST21 A5-ST185/CC5	GEN, ERY GEN, ERY, TET, STR GEN GEN, ERY, STR
Vancomycin (6 mg/L)	4/5 (80)	558 ^c , 573 ^c 707 ^c 444 ^g	<i>E. faecium</i> <i>E. faecium</i> <i>E. faecalis</i>	SN243-ST443 HB6-CC2/ST6	VAN, TEC, [GEN] VAN, TEC, ERY VAN, TEC, ERY, CHL
Erythromycin (8 mg/L)	14/32 (44)	133 ^c 168 ^h , 341 ^h , 427 ^{e,h} 180 ^h , 545 ^h , 592 ^h	<i>E. faecium</i> <i>E. faecium</i> <i>E. faecium</i>	SN208-ST393 ^f SN226 SN231, SN212-ST432, SN215-ST434	ERY, STR ERY, TET, [Q/D], [GEN] ERY
Tetracycline (6 mg/L)	25/60 (42)	765 ^h , 455 ^{h,i} , 559 ^h , 594 ^{h,i} 122 ^c	<i>E. faecium</i> <i>E. faecalis</i>	SN216-ST150(1) SN237-ST224	ERY, TET ERY, TET, Q/D, CHL, GEN, STR
		338 ^g	<i>E. faecalis</i>		ERY, TET
		45 ^g	<i>E. faecalis</i>		ERY, TET, Q/D, CHL, STR
		11 ^h	<i>E. faecium</i>		TET, [ERY], [GEN]
		168 ^h , 341 ^h , 71 ^{d,h} , 92 ^h , 96 ^h	<i>E. faecium</i>	SN226 (2), SN223 (1), SN208-ST393(1) ^f	TET, ERY, STR
		223 ^h	<i>E. faecium</i>	SN221-ST30	TET, ERY, [STR]
		255 ^h , 523 ^h , 594 ^{h,i}	<i>E. faecium</i>	SN216 (1)	TET, ERY
		534 ^h , 765 ^h	<i>E. faecium</i>	A3.1' (1)	TET, ERY, Q/D
		536 ^h , 583 ^h	<i>E. faecium</i>		TET
		557 ^{d,h} , 558 ^h , 573 ^h	<i>E. faecium</i>	A5-ST185/CC5	TET, VAN
		32 ^{d,g,j} , 48 ^g , 340 ^g , 369 ^g	<i>E. faecalis</i>	SN242-ST445(1); SN201/ ST200 (1)	TET
		45 ^g	<i>E. faecalis</i>	SN239	TET, [ERY], CHL, [GEN], [STR]
		122 ^g , 46 ^{g,i}	<i>E. faecalis</i>	SN237-ST224; SN205.4-ST224/CC21	TET, ERY, CHL, STR
		224 ^{d,g,i}	<i>E. faecalis</i>		TET, ERY

GEN, high-level resistance to gentamicin; STR, high-level resistance to streptomycin.

^aStrains in bold originated transconjugants in separate mating assays, which were recovered in media supplemented with different antibiotics.

^bThe variable presence of a given resistance phenotype among transconjugants from isolates belonging to the same clonal lineage or transconjugants from one wild strain is indicated by brackets.

^cRecipient strain: *E. faecium* GE1.

^dNegative conjugation in mating assays with erythromycin.

^eNegative conjugation in mating assays with tetracycline.

^fST393 belongs to the ST78 clonal lineage and ST431 belongs to the ST18 clonal lineage.

^gRecipient strain: *E. faecalis* JH2-2.

^hRecipient strain: *E. faecium* BM4105RF.

ⁱNegative conjugation in mating assays with ampicillin.

^jNegative conjugation in mating assays with gentamicin.

In conclusion, this study highlights the role of pig farms in the transmission dynamics of antibiotic resistance, complementing previous reports mainly focused on the analysis of animals faeces, animal meat or pig-farm dust.^{3,10,12,13,41} The results of the study also stress the need for multilevel strategies to contain

the spread of antibiotic resistance within pig farms, including the control of waste spreading, manure sale, air quality, hygiene and disinfection.⁴² The implementation of periodic animal and environment surveillance studies are critical for addressing the persistence of successful clones with zoonotic potential, including strains

carrying both antibiotic resistance determinants and putative virulence factors.

Acknowledgements

We are grateful to Ana Isabel Dias and Ana Cristina Nogueira (Fernando Pessoa University) for technical support, and to Dr Adam P. Roberts (UCL Eastman Dental Institute, London, UK) and Dr Fatemeh Rafii (National Center for Toxicological Research, Arkansas, USA) for the *tet*(O), *tet*(X), *tet*(W), *tet*(32), *tet*(Q) and *tet*(A(P), *tet*B(P) positive controls.

Funding

This work was supported by Fundação para a Ciência e a Tecnologia, which belongs to the Ministry of Science, Technology and Innovation of Portugal (grant numbers POCI/AMB/61814/2004 and PEst-C/EQB/LA0006/2011). E. S. is supported by a PhD fellowship from Fundação para a Ciência e a Tecnologia (grant number SFRH/BD/63955/2009; POPH-QREN). Work in the laboratory of T. M. C. is funded by grants from the European Union (EVOTAR-LSHM-2011-282004), the Ministry of Economy and Competitiveness of Spain (PI12/01581) and the regional government of Madrid (S2010/BMD2414_PROMPT-CM).

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

References

- 1 Aarestrup FM, Oliver Duran C, Burch DG. Antimicrobial resistance in swine production. *Anim Health Res Rev* 2008; **9**: 135–48.
- 2 European Medicines Agency. Trends in the Sales of Veterinary Antimicrobial Agents in Nine European Countries (2005–2009). http://www.ema.europa.eu/docs/en_GB/document_library/Report/2011/09/WC500112309.pdf (20 May 2013, date last accessed).
- 3 European Food Safety Authority and European Centre for Disease Prevention and Control. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in the European Union in 2009. *EFSA J* 2011; **9**: 2154.
- 4 Phillips I, Casewell M, Cox T et al. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* 2004; **53**: 28–52.
- 5 Gibbs SG, Green CF, Tarwater PM et al. Airborne antibiotic resistant and nonresistant bacteria and fungi recovered from two swine herd confined animal feeding operations. *J Occup Environ Hyg* 2004; **1**: 699–706.
- 6 Sapkota AR, Curriero FC, Gibson KE et al. Antibiotic-resistant enterococci and fecal indicators in surface water and groundwater impacted by a concentrated swine feeding operation. *Environ Health Perspect* 2007; **115**: 1040–5.
- 7 Chee-Sanford JC, Mackie RI, Koike S et al. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *J Environ Qual* 2009; **38**: 1086–108.
- 8 Hölzel CS, Schwaiger K, Harms K et al. Sewage sludge and liquid pig manure as possible sources of antibiotic resistant bacteria. *Environ Res* 2010; **110**: 318–26.
- 9 Heuer H, Schmitt H, Smalla K. Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr Opin Microbiol* 2011; **14**: 236–43.
- 10 Aarestrup FM, Agerso Y, Gerner-Smidt P et al. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn Microbiol Infect Dis* 2000; **37**: 127–37.
- 11 Rizzotti L, Simeoni D, Cocconcetti P et al. Contribution of enterococci to the spread of antibiotic resistance in the production chain of swine meat commodities. *Food Prot* 2005; **68**: 955–65.
- 12 Kaszanyitzky EJ, Tenk M, Ghidán A et al. Antimicrobial susceptibility of enterococci strains isolated from slaughter animals on the data of Hungarian resistance monitoring system from 2001 to 2004. *Int J Food Microbiol* 2007; **115**: 119–23.
- 13 de Jong A, Thomas V, Simjee S et al. Pan-European monitoring of susceptibility to human-use antimicrobial agents in enteric bacteria isolated from healthy food-producing animals. *J Antimicrob Chemother* 2011; **67**: 638–51.
- 14 Ramos S, Igrejas G, Rodrigues J et al. Genetic characterization of antibiotic resistance and virulence factors in *vanA*-containing enterococci from cattle, sheep and pigs subsequent to the discontinuation of the use of avoparcin. *Vet J* 2012; **193**: 301–3.
- 15 Bogø JL, Willems RJ, van den Bogaard AE. Genetic characterization of glycopeptide-resistant enterococci of human and animal origin from mixed pig and poultry farms. *APMIS* 2003; **111**: 669–72.
- 16 Larsen J, Schønheyder HC, Lester CH et al. Porcine-origin gentamicin-resistant *Enterococcus faecalis* in humans, Denmark. *Emerg Infect Dis* 2010; **16**: 682–4.
- 17 Freitas AR, Coque TM, Novais C et al. Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. *J Clin Microbiol* 2011; **49**: 925–31.
- 18 Leavis HL, Bonten MJ, Willems RJ. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. *Curr Opin Microbiol* 2006; **9**: 454–60.
- 19 Willems RJ, Hanage WP, Bessen DE et al. Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance. *FEMS Microbiol Rev* 2011; **35**: 872–900.
- 20 Kuch A, Willems RJ, Werner G et al. Insight into antimicrobial susceptibility and population structure of contemporary human *Enterococcus faecalis* isolates from Europe. *J Antimicrob Chemother* 2012; **67**: 551–8.
- 21 Willems RJ, van Schaik W. Transition of *Enterococcus faecium* from commensal organism to nosocomial pathogen. *Future Microbiol* 2009; **4**: 1125–35.
- 22 Willems RJ, Top J, van Schaik W et al. Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *MBio* 2012; **3**: e00151–12.
- 23 Clinical Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement M100-S20*. CLSI, Wayne, PA, USA, 2010.
- 24 Magiorakos AP, Srinivasan A, Carey RB et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**: 268–81.

- 25 Arias CA, Robredo B, Singh KV et al. Rapid identification of *Enterococcus hirae* and *Enterococcus durans* by PCR and detection of a homologue of the *E. hirae* *mur-2* gene in *E. durans*. *J Clin Microbiol* 2006; **44**: 1567–70.
- 26 Novais C, Coque TM, Sousa JC et al. Local genetic patterns within a vancomycin-resistant *Enterococcus faecalis* clone isolated in three hospitals in Portugal. *Antimicrob Agents Chemother* 2004; **48**: 3613–7.
- 27 Patterson AJ, Colangeli R, Spigaglia P et al. Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. *Environ Microbiol* 2007; **9**: 703–15.
- 28 Spigaglia P, Barbanti F, Mastrantonio P. Tetracycline resistance gene *tet(W)* in the pathogenic bacterium *Clostridium difficile*. *Antimicrob Agents Chemother* 2008; **52**: 770–3.
- 29 Werner G, Gfrörer S, Fleige C et al. Tigecycline-resistant *Enterococcus faecalis* strain isolated from a German intensive care unit patient. *J Antimicrob Chemother* 2008; **61**: 1182–3.
- 30 Tenover FC, Arbeit RD, Goering R et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; **33**: 2233–9.
- 31 Cantón R, Ruiz-Garbajosa P. Co-resistance: an opportunity for the bacteria and resistance genes. *Curr Opin Pharmacol* 2011; **11**: 477–85.
- 32 Johnsen PJ, Townsend JP, Bøhn T et al. Retrospective evidence for a biological cost of vancomycin resistance determinants in the absence of glycopeptide selective pressures. *J Antimicrob Chemother* 2011; **66**: 608–10.
- 33 Rice LB, Carias LL, Rudin S et al. *Enterococcus faecium* low-affinity *pbp5* is a transferable determinant. *Antimicrob Agents Chemother* 2005; **49**: 5007–12.
- 34 Baquero F. On the shifting balance: the case of *Staphylococcus aureus* CC398. *MBio* 2012; **3**: e00078–12.
- 35 McLaughlin MR, Brooks JP, Adeli A. Temporal flux and spatial dynamics of nutrients, fecal indicators, and zoonotic pathogens in anaerobic swine manure lagoon water. *Water Res* 2012; **46**: 4949–60.
- 36 Heuer OE, Pedersen K, Jensen LB et al. Persistence of vancomycin-resistant enterococci (VRE) in broiler houses after the avoparcin ban. *Microb Drug Resist* 2002; **8**: 355–61.
- 37 Rule AM, Evans SL, Silbergeld EK. Food animal transport: a potential source of community exposures to health hazards from industrial farming (CAFOs). *J Infect Public Health* 2008; **1**: 33–9.
- 38 European Food Safety Authority. Analysis of the baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in holdings with breeding pigs, in the EU, 2008, Part B: factors associated with MRSA contamination of holdings. *EFSA J* 2010; **8**: 1597.
- 39 Blanco G, Lemus JA, Grande J. Microbial pollution in wildlife: linking agricultural manuring and bacterial antibiotic resistance in red-billed choughs. *Environ Res* 2009; **109**: 405–12.
- 40 Johnston LM, Jaykus LA. Antimicrobial resistance of *Enterococcus* species isolated from produce. *Appl Environ Microbiol* 2004; **70**: 3133–7.
- 41 Braga TM, Pomba C, Lopes MF. High-level vancomycin resistant *Enterococcus faecium* related to humans and pigs found in dust from pig breeding facilities. *Vet Microbiol* 2012; **161**: 344–9.
- 42 EFSA Panel on Biological Hazards (BIOHAZ). Scientific opinion on the public health risks of bacterial strains producing extended-spectrum β -lactamases and/or AmpC β -lactamases in food and food-producing animals. *EFSA J* 2011; **9**: 2322.

Supplementary data

Table S1. Distribution of antibiotic-resistant *Enterococcus* by the samples studied

Samples group	Antibiotic resistance ^a										Samples (n) ^b	Piggery		
	VAN	TEC	AMP	TET	MIN	ERY	Q/D	CIP	CHL	GEN			STR	NIT
SWINE														
rectal swab (6 month pigs)	+	+		+	+	+	+	+	+			+	1	V
infected hoof swab (adult reproductive pig)	+			+	+	+	+	+	+	+	+	+	1	V
faeces (adult reproductive pig; piglets)			+	+	+	+	+	+		+	+	+	2	III, V
nostril (adult male)			+	+	+	+	+	+				+	1	V
rectal swab (adult reproductive pig)			+	+	+	+	+	+			+	+	1	V
faeces (adult reproductive pig; piglets)				+	+	+	+	+	+	+	+	+	6	III, V, VI
(4); skin swab (piglets) (1); rectal swab (reproductive pig)				+	+	+	+	+		+	+	+		
skin swab (piglet), faeces (female reproductive pig);faeces (reproduction room)				+	+	+	+	+		+	+	+	3	V, VI
faeces (6 month pigs)				+	+	+	+	+				+	1	V
faeces				+	+	+	+	+	+	+	+		1	III
faeces (diarrhoea)				+	+	+	+	+			+		1	VI
faeces (gestation room; males room)				+	+	+	+	+					2	V
Total	10%	5%	25%	100%	100%	100%	95%	100%	45%	65%	75%	80%	20	
FEED/ANTISEPTIC/MEDICINE														
medicine, feed (piglets)			+	+	+	+	+	+	+	+	+	+	2	II
feed (reproductive pig; gestation room)				+	+	+	+	+	+	+	+	+	5	III, V, VI
(2), drinking water (2), antiseptic (1)														
feed (reproductive pig, piglets) (3), drinking water (1)				+	+	+	+	+	+	+	+		4	III
feed (reproductive room)				+	+	+	+		+	+	+		1	V
feed (reproductive pigs)				+	+	+	+	+			+	+	1	V
feed (fattening room)				+	+	+	+	+			+	+	1	V
drinking water (gestation room)				+	+	+	+	+			+	+	1	VI
feed + drinking water (feeder)				+	+	+	+	+			+		1	V
non-treated drinking water; treated water (chlorine, UV)				+	+	+	+	+					2	V
drinking water														
feed (reproductive room)				+	+	+	+	+			+	+	1	IV
Total	0%	0%	10%	100%	100%	100%	95%	85%	60%	60%	85%	55%	20	V

RESIDUES

waste lagoon (2), residual water (2), manure (1)	+	+	+	+	+	+	+	+	+	+	+	+	+	5	I, II, III
waste lagoon, residual water manure (5), residual water (2), waste lagoon) (1)	+	+	+	+	+	+	+	+	+	+	+	+	+	1	II
waste lagoon, manure manure	+	+	+	+	+	+	+	+	+	+	+	+	+	8	II, III, V
Total	0%	0%	35%	100%	100%	100%	100%	100%	100%	77%	82%	94%	100%	17	V, VI IV

PIGGERIES' FACILITIES

water (maternity soil)	+	+	+	+	+	+	+	+	+	+	+	+	+	1	II
water (maternity soil)	+	+	+	+	+	+	+	+	+	+	+	+	+	1	II
exterior soil	+	+	+	+	+	+	+	+	+	+	+	+	+	2	IV
dust (maternity sty)		+		+	+	+	+	+	+	+	+	+	+	1	II
water (maternity soil) (2); dust (maternity) (2); maternity environment (1)				+	+	+	+	+	+	+	+	+	+	5	II, III, V
feed distributor device															
air (gestation room),soil swab (disinfected empty room) (2), room wall swab (maternity),exterior soil disinfected room (ventilation)															
air (maternity room)				+	+	+	+	+	+	+	+	+	+	1	V
soil swab (disinfected empty room)				+	+	+	+	+	+	+	+	+	+	1	VI
air (fattening room)				+	+	+	+	+	+	+	+	+	+	1	VI
disinfected room (piglet nest)				+	+	+	+	+	+	+	+	+	+	1	VI
soil swab (disinfected empty room)				+	+	+	+	+	+	+	+	+	+	1	V
air (maternity)				+	+	+	+	+	+	+	+	+	+	1	VI
Total	17%	17%	9%	96%	96%	96%	83%	87%	35%	44%	65%	78%	23		IV, V, VI

VAN, vancomycin; TEC, teicoplanin; AMP, ampicillin; TET, tetracycline; MIN, minocycline; ERY, erythromycin; Q/D, quinupristin/dalfopristin; CIP, ciprofloxacin; CHL, chloramphenicol; GEN, high level resistance to gentamicin; STR, high level resistance to streptomycin; NIT, nitrofurantoin.

^aThe cells with ‘+’ represent the antibiotic resistance detected in each sample, independently if it is present in the same or different isolates (*E. faecium*, *E. faecalis*, *E. hirae*, *E. casseliflavus*, *E. gallinarum* and *Enterococcus* spp.). *Enterococcus* with resistance and intermediate resistance were considered together. *E. faecalis* resistant to quinupristin/dalfopristin was not included. Statistically significant differences were not detected (*P*>0.05) for the distribution of antibiotic-resistant *Enterococcus* among different sample groups.

^bEighty samples with enterococci out of 82 tested.

3.2. Dispersão de genes associados a tolerância a metais em *Enterococcus* spp. resistentes a antibióticos de diferentes comunidades ecológicas e linhagens clonais

Publicações e artigos em preparação:

3.2.1. *Co-transfer of resistance to high concentrations of copper and first-line antibiotics among Enterococcus from different origins (humans, animals, the environment and foods) and clonal lineages.*

3.2.2. *Enterococcus spp. from different origins and clonal lineages carry two merA genes found in several Firmicutes members and are co-transferred with antibiotic resistance and copper tolerance.*

Este capítulo engloba dois estudos relativos à dispersão de diferentes genes que codificam para tolerância ao cobre e mercúrio em *Enterococcus* spp. de diversas origens e linhagens clonais. Também é estabelecida uma relação entre os genes encontrados e fenótipos de resistência a antibióticos específicos, assim como evidenciada a relevância da transferência horizontal na co-dispersão de genes de tolerância a metais e resistência a antibióticos.

Estes dois estudos destacam-se de outros trabalhos por permitirem uma melhor compreensão do contexto epidemiológico em que se inserem os genes de tolerância ao mercúrio e cobre, nomeadamente pelo maior número e origem diversificada dos isolados estudados, por incluir a pesquisa genes descritos em outros géneros bacterianos pertencentes às mesmas comunidades de partilha genética em que *Enterococcus* spp estão inseridos e por avaliar a estrutura populacional de *E. faecium* e *E. faecalis* portadores dos genes estudados.

Co-transfer of resistance to high concentrations of copper and first-line antibiotics among *Enterococcus* from different origins (humans, animals, the environment and foods) and clonal lineages.

Eduarda Silveira¹, Ana R. Freitas¹, Patrícia Antunes^{1,2}, Mariana Barros¹, Joana Campos¹,
Teresa M. Coque^{3,4,5}, Luísa Peixe¹, Carla Novais¹

¹REQUIMTE, Laboratório de Microbiologia. Faculdade de Farmácia, Universidade do Porto, Porto, Portugal;

²Faculdade de Ciências da Nutrição e Alimentação. Universidade do Porto, Porto, Portugal; ³Fundación para la Investigación Biomédica del Hospital Universitario Ramón y Cajal (FiBioHRYC, IRYCIS), Madrid, Spain;

⁴Unidad de Resistencia a Antibióticos y Virulencia Bacteriana asociada al Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain; ⁵CIBER en Epidemiología y Salud Pública (CIBERESP), Madrid, Spain.

Co-transfer of resistance to high concentrations of copper and first-line antibiotics among *Enterococcus* from different origins (humans, animals, the environment and foods) and clonal lineages

Eduarda Silveira¹, Ana R. Freitas¹, Patrícia Antunes^{1,2}, Mariana Barros¹, Joana Campos¹, Teresa M. Coque³⁻⁵,
Luísa Peixe¹ and Carla Novais^{1*}

¹REQUIMTE, Laboratório de Microbiologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; ²Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Porto, Portugal; ³Servicio de Microbiología, Hospital Ramón y Cajal, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain; ⁴Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBER-ESP), Madrid, Spain; ⁵Unidad de Resistencia a Antibióticos y Virulencia Bacteriana asociada al Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain

*Corresponding author. Tel: +351-220-428-588; Fax: +351-226-093-390; E-mail: casilva@ff.up.pt

Received 17 June 2013; returned 29 September 2013; revised 10 October 2013; accepted 9 November 2013

Objectives: We studied the occurrence of diverse copper (Cu) tolerance genes from Gram-positive bacteria and their co-transfer with antibiotic resistance genes among *Enterococcus* from diverse sources.

Methods: *Enterococcus* ($n=922$) of several species and from human, animal, environment and food samples were included. Antimicrobial and CuSO_4 susceptibility and conjugation assays were performed by standard procedures, bacterial screening of Cu and antibiotic resistance genes by PCR, and clonality by PFGE/multilocus sequence typing.

Results: *tcrB* and *cueO* genes occurred in 15% ($n=137/922$) and 14% ($n=128/922$) of isolates, respectively, with the highest occurrence in piggeries ($P<0.05$). They were more frequent among *Enterococcus faecium* (*tcrB*: 23% versus 8% in *Enterococcus faecalis* and 12% in other species; *cueO*: 25% versus 5% and 9%, respectively; $P<0.05$). A correlation between phenotypic and genotypic assays was observed for most *E. faecium* (CuSO_4 MIC₅₀=24 mM in *tcrB/cueO*⁺ versus CuSO_4 MIC₅₀=12 mM in *tcrB/cueO*⁻), but not for other species. Co-transfer of Cu tolerance (associated with *tcrB*, *cueO* or an unknown mechanism) with erythromycin, tetracycline, vancomycin, aminoglycosides or ampicillin resistance was demonstrated. A variety of PFGE types was detected among isolates carrying Cu tolerance mechanisms, some identified in sequence types (STs) often linked to human infections (*E. faecium* from ST18 and ST78 clonal lineages and *E. faecalis* clonal complex 2).

Conclusions: Cu tolerance might contribute to the selection/maintenance of multidrug-resistant *Enterococcus* (including resistance to first-line antibiotics used to treat enterococcal infections) due to the use of Cu compounds (e.g. antiseptics/animal feed supplements). The distribution of the multicopper oxidase *cueO* and the co-transfer of ampicillin resistance along with Cu tolerance genes are described for the first time.

Keywords: *tcrB*, multicopper oxidase, horizontal transfer, biocides, metals

Introduction

Enterococcus are widespread in nature and able to survive a variety of physical and chemical challenges, often by the acquisition of diverse genetic elements, which participate in their adaptation to different hosts and/or environments.^{1,2} Over the last two decades, *Enterococcus faecium* and *Enterococcus faecalis* became increasingly important nosocomial pathogens worldwide, mostly associated with particular multidrug resistance clonal lineages resistant in the hospital environment.³ The selective pressure due to the

use of antibiotics in hospitals and farms has supported the maintenance of multidrug-resistant (MDR) successful clones in particular ecosystems, but co-selection of MDR isolates by other compounds widely used in these settings, such as metals or biocides, has been less explored.

Copper (Cu) is an essential metal for living organisms and plays a critical role in several cellular processes; thus, it is extensively used as a feed additive for the growth promotion of farm animals (e.g. pigs and poultry).⁴⁻⁶ Nonetheless, high concentrations of Cu are toxic for living cells, making this compound suitable to be used

for control of microorganism growth on surfaces.⁷ Several resistance mechanisms to overcome high concentrations of Cu, either intrinsic or acquired, have been described (e.g. efflux pumps and cellular detoxification), although only a few studies addressed the mechanisms associated with tolerance to Cu in *Enterococcus*.^{4,5,8–12} Cu homeostasis has been mostly analysed in *Enterococcus hirae* (*copYZAB* system; associated with a CuSO_4 MIC <8 mM)¹¹ and less in other species.¹³ Also, toxic levels of Cu can be overcome by the *tcyAZB* gene cluster (CuSO_4 MIC >8 mM), the only transferable CPx-type Cu ATPase gene described in *Enterococcus*. It was initially identified on the pA17sv1 plasmid, which harboured genes encoding resistance to macrolides [*erm*(B)] and glycopeptides (*vanA*).⁹ The distribution of the *tcyAZB* gene cluster among *Enterococcus* was scarcely evaluated in environments besides the animal setting and a few geographical locations.^{8,14–17} Also, the occurrence of other mechanisms against high Cu concentrations identified in *Enterococcus* spp. (e.g. the multicopper oxidase *cueO* identified in a single strain of *E. faecium*)¹⁸ or other genes carried by different Gram-positive bacteria (e.g. *Lactococcus*, *Listeria* and *Bacillus*)¹¹ commonly sharing the same ecosystems has never been explored.

The aim of this study was to obtain a snapshot of the occurrence of diverse Cu tolerance genes described in Gram-positive bacteria and their co-transfer with antibiotic resistance genes among *Enterococcus* from diverse sources.

Methods

Epidemiological background, species identification and study of antibiotic susceptibility

Nine hundred and twenty-two isolates were obtained from different sources (hospitalized and healthy humans, animals, foods, sewage and the environment) of the north, centre and south of Portugal during 1996–2012. Samples were collected from: (i) hospitalized humans (110 clinical isolates from different patients at five hospitals); (ii) healthy humans (126 isolates from faeces of 79 individuals); (iii) the environment (51 isolates from 16 water samples from hospital sewage and 3 isolates from 2 water samples of an urban river); (iv) food for human consumption (155 isolates from 75 poultry carcass lots of 11 brands sold across Portugal, 25 isolates from 7 lots of muscle/viscera of rainbow trout sold in four supermarkets and 2 samples from an aquaculture, and 21 isolates from 18 ready-to-eat salads of 7 brands); and (v) the animal production setting [232 isolates from 71 samples of six piggeries (animal faeces/skin, animal residues, feed/drinking water, air, walls/floors/equipment/dust of facilities) and 199 isolates from 41 samples of two trout aquacultures (water/sediment, feed samples)].

The feed available to fish in the trout aquacultures analysed in this study contained 3 ppm of CuSO_4 . In one of the piggeries (designated as Piggery III in Table 2), CuSO_4 was used for belly-bottom disinfection in piglets. In Portugal, commercialized feed for swine and poultry contains 25 and 5 ppm of CuSO_4 , respectively, which are within CuSO_4 concentrations allowed by the European Union in feed (maximum of 175 ppm).^{5,19}

Enterococcus were identified by standard methods (Gram, catalase, esculin hydrolysis and growth in 6.5% NaCl) and PCR (*E. faecium*, *E. faecalis*, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Enterococcus durans* and *E. hirae*).^{20,21} Susceptibility to vancomycin, teicoplanin, ampicillin, tetracycline, erythromycin, quinupristin/dalfopristin, ciprofloxacin, chloramphenicol, nitrofurantoin and high concentrations of gentamicin and streptomycin was determined by agar dilution or disc diffusion methods following CLSI guidelines.²² The search for genes encoding resistance to glycopeptides (*vanA*, *vanB*, *vanC1*, *vanC2*), macrolides [*erm*(A), *erm*(B),

erm(C)], tetracyclines [*tet*(M), *tet*(L), *tet*(O), *tet*(K), *tet*(S)], aminoglycosides [*aac*(6')-Ie-aph (2'')-Ia, *aadE*] and ampicillin (*pbp5*) was performed by PCR as described previously.^{20,23,24} The primers used for the search of *aadE* (*aadEF*, 5'-TCAGCGGCATATGTGCTATC-3'; *aadER*, 5'-TTCGAATTGTGACCCTTGAG-3') were designed according to the sequence described in plasmid pS177 (GenBank accession no. HQ115078.1). Part of the isolates analysed in this work corresponds to *Enterococcus* from previous surveillance studies and were selected from each source based on the following features: different samples, species and antibiotic resistance profiles,^{20,25–28} while those obtained between 2010 and 2012 have been firstly characterized in this study.

Study of Cu susceptibility

Genes previously associated with tolerance to high concentrations of Cu were searched for by PCR (Table S1, available as Supplementary data at JAC Online). The proteins encoded by the genes include two Cu export ATPases (*tcyB* and *copB*_{pDBORO} genes) and three multicopper oxidases (*cueO*, *mco*_{p5578} and *mco*_{pBMB171/PO245} genes). The primers and GenBank sequences used for primer design are described in Table S1. The following bacteria were used as positive controls in PCRs: *Bacillus thuringiensis* BMB171 (*mco*_{pBMB171/PO245} gene; GenBank accession no. CP001904.1), *Listeria monocytogenes* 08-5578 (*mco*_{p5578} gene; GenBank accession no. CP001603.1), *Lactococcus lactis* subsp. *cremoris* MB301 with pDBORO plasmid (*copB* gene; GenBank accession no. DQ089807.2) and *E. faecium* E289 (*tcyB* and *cueO* genes; this study). A positive amplicon from each PCR was sequenced and compared with known GenBank sequences to validate the method.

Isolates harbouring ($n=83$) or lacking ($n=96$) any of the genes studied, from different species, sources and dates of isolation and with diverse antibiotic resistance phenotypes, were selected for Cu tolerance phenotypic assays. The MICs were determined by the agar dilution method using Mueller–Hinton II agar plates supplemented with CuSO_4 adjusted to pH 7.2 (0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, 4, 8, 12, 16, 20, 24, 28, 32 and 36 mM) as described previously.²⁹ A 1 μL aliquot of a 10^7 cell suspension of each bacterium was applied to the CuSO_4 -supplemented medium.²⁹ The plates were incubated at 37°C and read at 24 h. The first CuSO_4 concentration without visible bacterial growth was considered as the MIC. *E. faecium* BM4105RF (negative for all genes tested; MIC of $\text{CuSO}_4=8–12$ mM) was used as a control strain.^{10,14}

Clonal relatedness

Clonality was established in representative *E. faecium* ($n=75$), *E. faecalis* ($n=20$), *E. hirae* ($n=4$), *E. casseliflavus* ($n=3$) and *E. gallinarum* ($n=3$) by PFGE (SmaI enzyme). Strains were selected based on the carriage of Cu tolerance genes, species, source, sample type, date of isolation and antibiotic resistance phenotypes.^{20,30} Multilocus sequence typing (MLST) was also performed for *E. faecium* ($n=45$) and *E. faecalis* ($n=14$) representative of different PFGE types (<http://www.mlst.net>).

Conjugation assays

Transfer of Cu resistance was analysed for isolates carrying *tcyB* and/or *cueO* (*E. faecium*, $n=34$; *E. faecalis*, $n=7$; and other species, $n=3$) or lacking known genes, but exhibiting high MIC values of CuSO_4 (≥ 16 mM, corresponding to a concentration above the MIC for control strain *E. faecium* BM4105RF; 21 *E. faecium*, 3 *E. faecalis*, 1 *E. hirae* and 3 *Enterococcus* spp.), which belonged to different clonal lineages. It was performed by the filter-mating method at a 1:1 donor:recipient ratio using *E. faecium* BM4105, *E. faecium* GE1 or *E. faecalis* JH2-2 as recipient strains (all resistant to rifampicin and fusidic acid; *E. faecium* GE1 also resistant to tetracycline). Transconjugants were recovered after incubation at 37°C for 24–48 h from plates of brain heart infusion agar supplemented with 30 mg/L rifampicin,

25 mg/L fusidic acid plus other antibiotics (8 mg/L tetracycline, 8 mg/L erythromycin, 6 mg/L vancomycin, 125 mg/L gentamicin or 8 mg/L ampicillin) or in Mueller–Hinton II agar supplemented with 30 mg/L rifampicin, 25 mg/L fusidic acid and 8–16 mM CuSO₄. Tolerance to Cu and co-transfer of antibiotic resistance was confirmed at the phenotypic and genotypic levels as described above.

Statistical analysis

Differences in the prevalence of Cu tolerance genes among species and ecological niches and the distribution of antibiotic resistance among *Enterococcus* with/without Cu tolerance genes were analysed using Fisher's exact test ($\alpha=0.05$) using GraphPad Prism software, version 6.0a.

Results

Prevalence of genes encoding Cu tolerance and epidemiological background

In this study, 348 *E. faecium*, 321 *E. faecalis*, 75 *E. hirae*, 29 *E. gallinarum*, 19 *E. casseliflavus*, 6 *E. durans* and 124 isolates that could

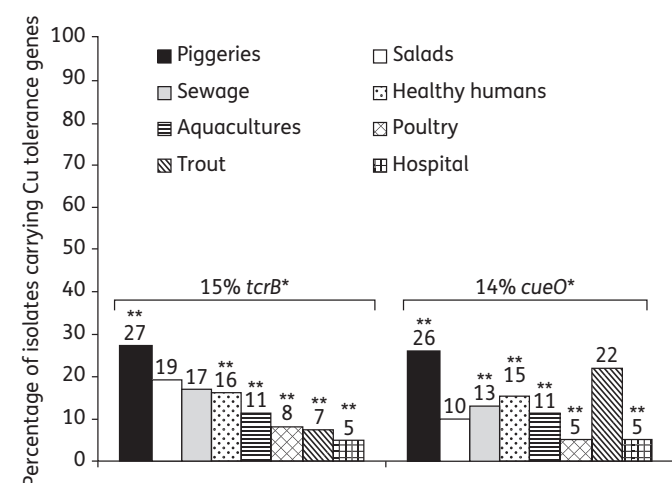


Figure 1. Distribution of *tcrB* and *cueO* genes among the *Enterococcus* spp. from different sources. *Occurrence of a given gene among all isolates studied. ** $P<0.05$ comparing piggeries with other niches.

only be identified to the genus level were included. The *tcrB* and *cueO* genes were detected in 15% ($n=137/922$) and 14% ($n=128/922$) of *Enterococcus*, respectively ($tcrB+cueO=101$, $tcrB=36$ and $cueO=27$, corresponding to 62%, 22% and 16% of the 164 isolates carrying Cu tolerance genes, respectively). These genes were spread in different environments, with the highest occurrence found in piggeries ($P<0.05$; Figure 1). Both of them were more frequently found among *E. faecium* than among other species (*tcrB*: 23%, 8% and 12% among *E. faecium*, *E. faecalis* and other species, respectively; *cueO*: 25%, 5% and 9% among *E. faecium*, *E. faecalis* or other species, respectively; $P<0.05$). None of the other genes searched for was detected.

Susceptibility to CuSO₄ and antibiotics

The MIC of CuSO₄ was variable according to the species and the presence/absence of Cu tolerance genes. *E. faecium* carrying *tcrB* and/or *cueO* showed MIC=12 to 36 mM for isolates carrying *tcrB* ($n=11$), MIC=8 to 36 mM for those containing *tcrB+cueO* ($n=33$) and MIC=12–24 mM for those carrying *cueO* ($n=19$). The CuSO₄ MIC₅₀ of these isolates was 24 mM, contrasting with those *E. faecium* without Cu tolerance genes (MIC₅₀=12 mM) (Table 1). For other species, the MIC₅₀ was similar between the group of isolates with and without Cu tolerance genes (*E. faecalis*, 32 mM; other species, 16 mM). The analysis of antibiotic resistance profiles revealed that *Enterococcus* isolates carrying *tcrB* and/or *cueO* were more resistant to erythromycin, tetracycline, high concentrations of streptomycin or nitrofurantoin ($P<0.05$; Figure 2).

Clonal relatedness

MDR isolates from different species and belonging to several clonal lineages were identified as carriers of *tcrB* and/or *cueO* genes. Seventy-five *E. faecium* isolates (representing 75% of *E. faecium* with *tcrB* and/or *cueO*) corresponded to 58 PFGE types. From these, 37 isolates representing different PFGE types were identified as sequence types (STs) previously included in the former designated clonal complex (CC) 17 and now belonging to ST18 (ST18, ST132, ST431 and ST670) and ST78 (ST133 and ST393) clonal lineages, as well as in CC5 (ST5, ST6, ST150 and ST185), CC9 (ST148), CC22 (ST32) and CC94 (ST94). Also, the STs ST19, ST30, ST430, ST695, ST101, ST123, ST184, ST421, ST428, ST432, ST434

Table 1. MICs of CuSO₄ for different *Enterococcus* species

Species	<i>tcrB/cueO</i>	No. of isolates	MIC (mM)										MIC ₅₀ (mM)
			4	8	12	16	20	24	28	32	36	>36	
<i>E. faecium</i>	+	63		1	3	11	7	12	15	11	3		24
	–	45	1	9	14	11	10						12
<i>E. faecalis</i>	+	10				2		1	1	2	3	1	32
	–	27		1	1	4	3	1	1	13	3		32
Other species	+	10 ^a		1	1	3	2			1		2	16
	–	24 ^b		5	5	4	2	1	2	5			16

^a*E. gallinarum* ($n=1$), *E. casseliflavus* ($n=3$), *E. hirae* ($n=3$) and *Enterococcus* spp. ($n=3$).

^b*E. gallinarum* ($n=3$), *E. casseliflavus* ($n=1$), *E. hirae* ($n=9$) and *Enterococcus* spp. ($n=11$).

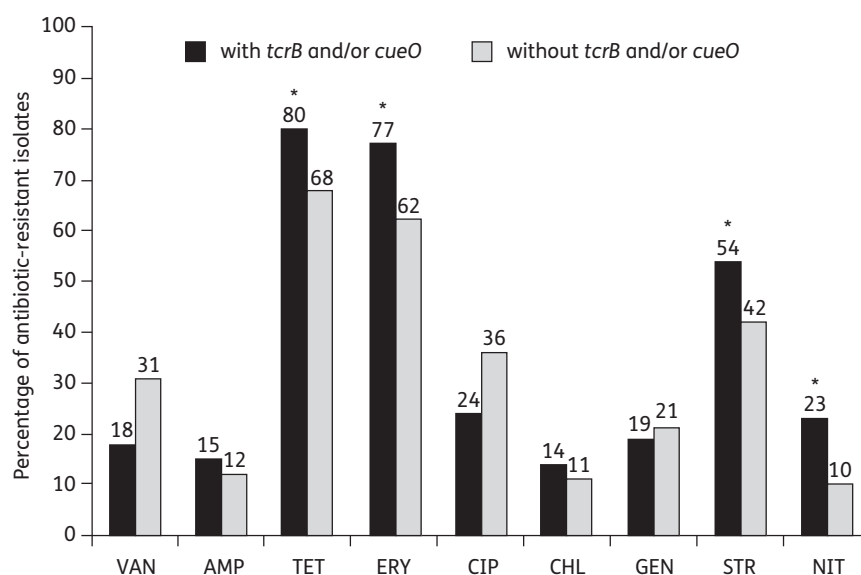


Figure 2. Distribution of antibiotic resistance among *Enterococcus* with ($n=164$) and without ($n=758$) *tcrB* and/or *cueO*. * $P<0.05$ (Fisher's exact test). VAN, vancomycin; AMP, ampicillin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; CHL, chloramphenicol; GEN, high concentrations of gentamicin; STR, high concentrations of streptomycin; NIT, nitrofurantoin.

and ST583, and new STs ST798, ST845, ST846, ST847, ST848 and ST859 not included in the clonal lineages described above were found (Table 2). *E. faecium* with CuSO_4 MIC ≥ 16 mM, but lacking known Cu tolerance genes ($n=8$) (six PFGE types), belonged to the ST18 (ST18 and ST132) clonal lineage and to ST125 and ST280. Twenty *E. faecalis* isolates (representing 71% of the *E. faecalis* with *tcrB* and/or *cueO*) were classified in 13 PFGE types belonging to CC2 (ST49, ST53, ST260 and ST288), CC21 (ST224) and four singletons (ST16, ST74, ST330 and ST445). Four *E. hirae* isolates, three *E. gallinarum* and three *E. casseliflavus* (representing 27%, 75% and 75% of the *E. hirae*, *E. gallinarum* and *E. casseliflavus* with *tcrB* and/or *cueO*) showed two, three and three PFGE types, respectively.

Conjugation assays

The transferability of *tcrB* and/or *cueO* was achieved for most of the strains studied (91%, $n=40/44$) and transconjugants could be obtained from medium supplemented with different antibiotics (50%–100%) and CuSO_4 (29%) (Table S2, available as Supplementary data at JAC Online).

All the transconjugants carrying *tcrB* and/or *cueO* presented higher CuSO_4 MICs (16–28 mM) than the recipient strains *E. faecium* GE1 (MIC=0.0156 mM) and *E. faecium* BM4105RF (MIC=8–12 mM). One transconjugant obtained from *E. faecalis* JH2-2 presented the same MIC as the recipient strain (Table S2). Resistance to erythromycin ($n=52$ transconjugants), tetracycline ($n=38$), high concentrations of streptomycin ($n=19$), vancomycin ($n=15$), high concentrations of gentamicin ($n=9$) and/or ampicillin ($n=2$) was also co-transferred with *tcrB*/*cueO* genes.

Enterococcus ($n=28$; 21 *E. faecium*, 3 *E. faecalis*, 1 *E. hirae* and 3 *Enterococcus* spp.; all niches) with CuSO_4 MIC ≥ 16 mM, but without *tcrB*/*cueO*, were also included in mating assays in order to evaluate if other transferable genes encoding Cu tolerance could be present.

Transconjugants of *E. faecium* GE1 obtained from matings with *E. faecium* isolates of clonal lineages ST18, ST280 and ST125 after plating in medium supplemented with ampicillin or Cu exhibited an increased CuSO_4 MIC in comparison with the recipient *E. faecium* GE1 strain (from 0.0156 to 16–20 mM). The same was detected in a *cueO*-carrying clinical *E. faecium* from the ST18 clonal lineage, whose transconjugant had a CuSO_4 MIC of 12 mM, but not the *cueO*. Resistance to antibiotics was also co-transferred to these transconjugants, namely to ampicillin ($n=8$), erythromycin ($n=4$), vancomycin ($n=4$), streptomycin ($n=1$) or gentamicin ($n=1$) (Table S2). As ampicillin transfer is a rare event, PFGE was performed to confirm the occurrence of true transconjugants, which showed PFGE type patterns similar to the recipient strain *E. faecium* GE1.

Discussion

This study documents the high occurrence of genes encoding tolerance to toxic concentrations of Cu among different *Enterococcus* clonal backgrounds, recovered from human, animal, environment and food samples, and often transferred with antibiotic resistance.

Enterococcus spp. were only reservoirs of *tcrB* and *cueO*. These genes have been previously found in other bacterial genera (Table S1), indicating horizontal transfer among microorganisms sharing common ecosystems, although the same could not be concluded for the other Cu tolerance genes searched for (*copB*_{PDBORO}, *mco*_{P5578} and *mco*_{PBMB171/P0245}). The higher prevalence of *tcrB* among piggeries than among other animal production settings where Cu is used at lower concentrations (e.g. poultry production farms or aquacultures) suggests strong selection within this environment. The *tcrB* rates found were lower than those reported for swine from Spain,⁸ Denmark^{8,14} and Australia,¹⁶ and higher than those for Sweden and the USA.^{8,15} However, comparative analysis should be taken cautiously because of differences in the

Table 2. Epidemiological features of *Enterococcus* carrying Cu tolerance genes representative of different species and sources

Species	Cu tolerance genes ^a	ST ^b	PFGE ^c	No. of isolates	Isolation date, region	Origin/source	Antibiotic resistance profile	Antibiotic resistance genes
<i>E. faecium</i>	<i>cueO</i>	ST32	SN210	1	2007, Ctr	Piggery III/antiseptic	TET, MIN	tet(M), tet(L), tet(S)
		ST428	SN207	1	2006, Sth	Piggery I/waste lagoon	TET, ERY, GEN	tet(M), tet(L), erm(B)
		ST670	90	1	1997, Ctr	Hospital A/unknown	TET, ERY, VAN, TEC, AMP, STR, Q/D	tet(M), erm(B), vanA, aadE
		ST94	T2	1	2010, Nth	Aquaculture B/feed	TET, ERY	tet(M), erm(B)
		ST6	CE	1	2001, Nth	Healthy human/faeces	TET, ERY, STR, NIT	tet(M), tet(L), erm(B), aadE
	<i>tcrB</i>	ST123	ET	1	2002, Nth	Sewage/water from Hospital D	TET, ERY, AMP, STR, CIP	tet(M), tet(L), erm(B), aadE
		ST132	C3	1	2001, Nth	Sewage/water from Hospital C	ERY, VAN, TEC, STR, CIP	erm(B), vanA, aadE
		ST148	16	1	2001, Nth	Butcher shop/poultry carcass	TET, ERY, GEN, AMP, STR, CIP, NIT	tet(M), tet(L), erm(B), aadE, aac(6')-Ie-qph (2'')-Ia
		ST798	CF	1	2001, Nth	Healthy human/faeces	TET, ERY, STR, CIP	tet(M), erm(B), aadE
		ST847	SN230	1	2007, Nth	Piggery V/waste lagoon	TET, ERY, STR	tet(M), tet(L), erm(B), aadE
<i>tcrB + cueO</i>	ST5	A	1	2002, Nth	Hospital B/pus	ERY, VAN, TEC	erm(B), vanA	
		EA, ES	2	2001, Nth	Sewage/water from Hospitals C and B	TET, ERY, VAN, TEC, AMP, CHL, GEN, STR, CIP, NIT	tet(M), tet(L), erm(B), vanA, aadE, aac(6')-Ie-qph (2'')-Ia	
	ST19	96	1	2001, Nth	Hospital B/urine	ERY, VAN, TEC, AMP	tet(M), tet(L), erm(B), vanA	
	ST30	T4	1	2011, Nth	Aquaculture A/water	TET, STR	tet(M), tet(L), aadE	
	ST101	T1	1	2010, Nth	Aquaculture A/water leaking into river	TET	tet(M), tet(L)	
		ST108	SN248	1	2006, Ctr	Piggery III/manure	TET, ERY, Q/D, CHL, GEN, STR, NIT	tet(M), tet(L), erm(B), aadE
		ST133	SN234	1	2006, Ctr	Piggery III/manure	TET, ERY, Q/D, CHL, GEN, STR, CIP	tet(M), erm(B)
		ST150	SN216	1	2006, Nth	Piggery VI/swine faeces	TET, Q/D, CHL, STR	tet(M), erm(B), aadE
		ST184	CH	1	2001, unknown	Healthy human/faeces	TET, ERY, STR, CIP	tet(M), tet(L), erm(B), aadE
		ST185	A5	2	2007, Sth	Piggery IV/soil	TET, VAN, TEC, Q/D, NIT	tet(M), vanA
ST393		SN208	2	2006, Sth	Piggery II/manure, waste lagoon	TET, ERY, AMP, STR, NIT	tet(M), tet(L), erm(B), aadE	
ST421		58, 59	2	1999, Nth	Butcher shop/poultry carcass	TET, ERY, VAN, TEC	tet(M), tet(L), erm(B), vanA	
ST430		SN211	1	2006, Sth	Piggery II/dust	TET, ERY, AMP, STR, CIP, NIT	tet(L), erm(B), aadE	
ST431		SN208.1	1	2006, Sth	Piggery II/dust	TET, ERY, AMP, STR, NIT	tet(M), tet(L), erm(B), aadE	
ST432		SN212	1	2007, Sth	Piggery IV/manure	TET, ERY, Q/D, GEN, STR	tet(M), tet(L), erm(B), aadE	
	unknown	ST434	SN215	1	2006, Nth	Piggery VI/air from facilities	TET, ERY, GEN, STR	tet(M), tet(L), erm(B), aadE
		ST583	ER	1	2001, Nth	Sewage/water from Hospital C	ERY, CIP	erm(B)
		ST695	SN225	1	2007, Ctr	Piggery III/antiseptic	TET, ERY, CHL	tet(S), erm(B)
		ST845	CG	1	2001, Nth	Healthy human/faeces	TET, ERY, STR, NIT	tet(M), tet(L), erm(B), aadE
		ST846	CD	1	2001, Nth	Healthy human/faeces	TET, ERY, GEN, STR	tet(M), tet(L), erm(B), aadE
		ST848	SN223	1	2006, Sth	Piggery II/waste lagoon	TET, ERY, STR, NIT	tet(M), erm(B), aadE
		ST859	EU	1	2002, Nth	Sewage/water from Hospital B	TET, ERY, AMP, GEN, STR, NIT	tet(M), tet(L), erm(B), aadE, aac(6')-Ie-qph (2'')-Ia
		STNew	SN226.2	1	2006, Ctr	Piggery III/faeces	TET, ERY, STR	tet(M), tet(L), erm(B), aadE
		ST18	CA	1	2001, Nth	Healthy human/faeces	ERY, VAN, AMP, CIP, GEN	erm(B), vanA, aac(6')-Ie-qph (2'')-Ia
		ST125	126	1	2007, Nth	Hospital A/urine	ERY, AMP, VAN, TEC, CIP, NIT	erm(B), vanA
ST132	88.1, 88.2	2	2001-02, Nth	Sewage/water from Hospitals B and C	ERY, AMP, GEN, STR, CIP	erm(B), aadE, aac(6')-Ie-qph (2'')-Ia		
	ST132	88	1	1999, Ctr	Hospital B/blood	TET, ERY, VAN, TEC, AMP, Q/D, GEN, STR, CIP	tet(M), tet(L), erm(B), vanA, aadE, aac(6')-Ie-qph (2'')-Ia	
	ST132	Z	1	2001, Nth	Sewage/water from Hospital C	ERY, AMP, CIP, GEN, STR	erm(B), aadE, aac(6')-Ie-qph (2'')-Ia	

Continued

Table 2. Continued

Species	Cu tolerance genes ^a	ST ^b	PFGE ^c	No. of isolates	Isolation date, region	Origin/source	Antibiotic resistance profile	Antibiotic resistance genes
<i>E. faecalis</i>		ST280	150	1	2002, Ctr	Hospital E/exudate	TET, ERY, AMP, GEN, STR, CIP	tet(M), tet(L), aadE, aac(6')-Ie-aph (2'')-Ia
		ST280	100	1	2002, Nth,	Hospital B/urine	ERY, AMP, VAN, TEC, GEN, STR, CIP,	erm(B), vanA, aadE, aac(6')-Ie-aph (2'')-Ia
	tcrB	ST16	T6	1	2011, Nth	Aquaculture A/sediment from upstream river	TET, ERY, Q/D	tet(M), erm(B)
		ST49	44	2	2001, Nth	Butcher shop/poultry carcass	TET, GEN	tet(M), tet(L), aac(6')-Ie-aph (2'')-Ia
		ST53	41.2, 53	2	2001, Nth	Butcher shop/poultry carcass	TET, ERY, GEN	tet(M), tet(L), erm(B), aac(6')-Ie-aph (2'')-Ia
		ST260	52	1	2001, Nth	Butcher shop/poultry carcass	TET, ERY, STR, CIP	tet(M), erm(B), aadE
	tcrB + cueO	ST74	SN242	1	2006, Ctr	Piggery III/feed	TET, ERY, Q/D, STR	tet(M), tet(L), erm(B), aadE
		ST224	SN205.4, SN237	3	2006, Sth	Piggery II/manure, waste lagoon, dust	TET, ERY, Q/D, CHL, GEN, STR	tet(M), tet(L), erm(B), aadE
<i>E. hirae</i>		ST288	48	1	2001, Nth	Butcher shop/poultry carcass	TET, ERY, GEN, STR	tet(M), tet(L), erm(B), aac(6')-Ie-aph (2'')-Ia
		ST330	T5	1	2011, Nth	Aquaculture B/sediment	TET, ERY, Q/D, CHL, GEN, STR, CIP	tet(M), tet(L), erm(B), aadE
		ST445	SN242	2	2006, Ctr	Piggery III/solid manure, facilities	TET, ERY, Q/D, CHL, STR, NIT	tet(M), tet(L), tet(S), erm(B), aadE
	tcrB + cueO	T7		3	2011, Nth	Aquaculture A/water, water from upstream river	TET, ERY, NIT	tet(M), tet(L), erm(B)
<i>E. casseliflavus</i>			SN250	1	2006, Sth	Piggery I/waste lagoon	TET, ERY, AMP, CIP	tet(L), erm(B)
	tcrB		SN252	1	2006, Sth	Piggery II/dust	TET, ERY, STR	tet(M), erm(B), aadE
			SL3	1	2010, Nth	Supermarket B/salad	(-)	ND
	tcrB + cueO		T9	1	2010, Nth	Aquaculture B/feed	ERY	erm(B)
<i>E. gallinarum</i>	tcrB		SN251	1	2006, Ctr	Piggery III/antiseptic	TET, ERY, Q/D, CHL	tet(L), tet(S), erm(B)
			60	1	2001, Nth	Butcher shop/poultry carcass	CIP, Q/D	(-)
			61	1	2001, Nth	Butcher shop/poultry carcass	TET, ERY, CIP, Q/D	tet(M), tet(L), erm(B)

Sth, south; Ctr, centre; Nth, north; TET, tetracycline; ERY, erythromycin; Q/D, quinupristin/dalfopristin; CIP, ciprofloxacin; STR, streptomycin [high-level resistance (HLR)]; NIT, nitrofurantoin; CHL, chloramphenicol; GEN, gentamicin (HLR); AMP, ampicillin; VAN, vancomycin; TEC, teicoplanin; (-), susceptibility to all isolates tested or absence of all antibiotic resistance genes searched for; ND, not determined.

^bIsolates designated as 'unknown' resistance mechanism have an MIC of CuSO₄ of 16–24 mM.

^cMLST was performed for representative PFGE types from the different niches.

^dEach different PFGE number or letter represents a different clone.³⁰ The number after a dot of clones 88, SN208, SN226, 41 and SN205 represents the number of bands differing from the strain considered as the original clone within our collection. Here are represented 59*E. faecium* and *E. faecalis* isolates out of 95 with PFGE, which were included for MLST studies.

experimental design of such studies, with most of them confined to isolates recovered from specific hosts.^{8,10,15–17} The occurrence of *tcrB* in *Enterococcus* from humans may be due to the acquisition of strains from the food chain through different kinds of meals (besides pork, *tcrB* was also observed in poultry meat, trout and ready-to-eat salad isolates).

A correlation between the level of resistance and the occurrence of Cu tolerance genes was observed for most of the *E. faecium* isolates, which showed MIC₅₀ values of CuSO₄ similar to those in other studies (24 versus 22 mM).^{15,31} However, other Cu tolerance mechanisms might also be present in this and other species, as suggested by the increase (>1000 times) of the CuSO₄ MIC for *E. faecium* GE1 transconjugants that did not acquire *tcrB* and/or *cueO* genes and by the frequent occurrence of high CuSO₄ MICs (above the MIC for the control *E. faecium* BM4105RF) for non-*E. faecium* species lacking the genes searched for.

The distribution of the *tcrB/cueO* genes in different species and diverse clonal lineages, as well as successful transfer during conjugation assays, highlight the critical role of horizontal transfer in the adaptation of *Enterococcus* spp. to toxic concentrations of Cu. The prevalence of the *cueO* gene, first described in this study, was similar to that of *tcrB* in isolates recovered from different sources, as both genes were coidentified in most isolates. Their co-transfer during mating assays, as well as the previous identification of a plasmid carrying both genes (GenBank accession no. HM565231.1), suggest they could often occur in the same genetic elements. The identification of *cueO* in species other than *E. faecium* is described here for the first time. Besides contributing to detoxification processes when high concentrations of Cu are present in the cell, multicopper oxidases also seem to be involved in the systemic virulence of some bacterial genera^{32–34} and therefore specific studies are needed to clarify the potential roles of *cueO* among *Enterococcus* spp.

The use of high amounts of Cu is a current concern,³⁵ particularly after the demonstration of the selection of the *tcrB* gene in piglets <20 kg.^{10,15} This concern increases when *tcrB* is co-transferred with antibiotic resistance genes, as previously demonstrated by the occurrence of 175–194 kb plasmids carrying this gene along with *erm(B)*, encoding erythromycin resistance.^{14,15,31} Our results not only confirm the co-transfer of Cu tolerance with *erm(B)*, but also describe the co-transfer of *tcrB/cueO* with genes encoding resistance to other antibiotics, such as tetracycline or high concentrations of streptomycin. Interestingly, we also documented the frequent co-transfer of Cu tolerance with resistance to first-line antibiotics used to treat severe *Enterococcus* human infections, such as vancomycin, gentamicin or ampicillin. For the first time, we demonstrated the transfer of resistance to ampicillin along with *tcrB*, *cueO* and, particularly, an unknown Cu tolerance mechanism in *E. faecium* recovered from different sources (e.g. piggyeries and clinical isolates) and belonging to important clonal lineages associated with the nosocomial environment (e.g. ST18 clonal lineage).³ The several antibiotic resistance and Cu tolerance genotypes observed in different transconjugants highlight the involvement of diverse genetic elements in the maintenance of MDR strains in different settings, which could be selected by both antibiotics and Cu. Clonal studies on different *Enterococcus* species revealed that *tcrB/cueO* are widespread in several PFGE types, including STs associated with human infections (e.g. *E. faecium* ST18 and ST78 clonal lineages and *E. faecalis* CC2) and community sources.^{3,36}

In summary, the use of Cu compounds as antiseptics or animal feed additives might contribute to the selection and maintenance of Cu-tolerant MDR isolates in hospitals or farms. Of particular concern is the co-selection of resistance to Cu and first-line antibiotics used for the treatment of severe enterococcal infections, such as glycopeptides, ampicillin and high concentrations of aminoglycosides. More studies are needed to evaluate the critical concentrations of Cu for the selection/maintenance of MDR strains in different environments and to better understand the role of some genes in the adaptation of *Enterococcus* spp. to different hosts.

Acknowledgements

We would like to thank Dr Ziniu Yu (State Key Laboratory of Agricultural Microbiology and National Engineering Research Center for Microbial Pesticides, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, Hubei Province, PR China) for the control strain *B. thuringiensis* BMB171, Dr Christopher Peterson (Public Health Agency of Canada; National Microbiology Laboratory, Winnipeg, Manitoba) for the *L. monocytogenes* isolate 08-5578 and Dr Jan Martinussen (Center for Systems Microbiology, Department of Systems Biology, Technical University of Denmark) for the *L. lactis* subsp. *cremoris* MB301 with pDBORO plasmid. We would also like to thank Francisca Seco and Ricardo Silva (Universidade Fernando Pessoa) for their technical support.

Funding

This project was funded by research grants from the Fundação para a Ciência e Tecnologia (PTDC/AAC-AMB/103386/2008; grant no. PEst-C/ EQB/LA0006/2011) and Investigação Jovem da Universidade do Porto/Santander Totta (PP_IJUP2010-107). Work in the laboratory of T. M. C. is supported by grants from the European Union (7FPEU-HEALTH-F3-2011-282004_EvoTAR), the Ministry of Economy and Competitiveness of Spain (PI12/01581, PI10-02588) and the regional government of Madrid (S2010/BMD2414_PROMPT-CM). E. S. is supported by a PhD fellowship of the Fundação para a Ciência e Tecnologia (grant number SFRH/BD/63955/2009; POPH-QREN).

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

References

- 1 Cattoir V, Leclercq R. Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce? *J Antimicrob Chemother* 2012; **68**: 731–42.
- 2 Regt MJA, van Schaik W, van Luit-Asbroek M *et al.* Hospital and community ampicillin-resistant *Enterococcus faecium* are evolutionarily closely linked but have diversified through niche adaptation. *PLoS One* 2012; **7**: e30319.
- 3 Willems RJ, Top J, van Schaik Wet *et al.* Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *mBio* 2012; **3**: e00151-12.

- 4 Rademacher C, Masepohl B. Copper-responsive gene regulation in bacteria. *Microbiology* 2012; **158**: 2451–64.
- 5 Somanovic MI, Ding C, Thiele DJ et al. Copper in microbial pathogenesis: meddling with the metal. *Cell Host Microbe* 2012; **11**: 106–15.
- 6 European Commission. SCAN—Scientific Committee for Animal Nutrition. *Opinion of the Scientific Committee for Animal Nutrition on the Use of Copper in Feedingstuffs*. 2003. http://ec.europa.eu/food/fs/sc/scan/out115_en.pdf (13 June 2013, date last accessed).
- 7 Salgado CD, Sepkowitz KA, John JF et al. Copper surfaces reduce the rate of healthcare-acquired infections in the intensive care unit. *Infect Control Hosp Epidemiol* 2013; **34**: 479–86.
- 8 Aarestrup FM, Hasman H, Jensen LB et al. Antimicrobial resistance among enterococci from pigs in three European countries. *Appl Environ Microbiol* 2002; **68**: 4127–9.
- 9 Hasman H, Aarestrup FM. The *tcrB* gene is part of the *tcrYAZB* operon conferring copper resistance in *Enterococcus faecium* and *Enterococcus faecalis*. *Microbiology* 2005; **151**: 3019–25.
- 10 Hasman H, Kempf I, Chidaïne B et al. Copper resistance in *Enterococcus faecium*, mediated by the *tcrB* gene, is selected by supplementation of pig feed with copper sulfate. *Appl Environ Microbiol* 2006; **72**: 5784–9.
- 11 Solioz M, Abicht HK, Mermod M et al. Response of gram-positive bacteria to copper stress. *J Biol Inorg Chem* 2010; **15**: 3–14.
- 12 Hodgkinson V, Petris MJ. Copper homeostasis at the host–pathogen interface. *J Biol Chem* 2012; **287**: 13549–55.
- 13 Latorre M, Olivares F, Reyes-Jara A et al. CutC is induced late during copper exposure and can modify intracellular copper content in *Enterococcus faecalis*. *Biochem Biophys Res Commun* 2011; **406**: 633–7.
- 14 Hasman H, Aarestrup FM. *tcrB*, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. *Antimicrob Agents Chemother* 2002; **46**: 1410–6.
- 15 Amachawadi RG, Shelton NW, Vinasco J et al. Selection of fecal enterococci exhibiting *tcrB*-mediated copper resistance in pigs fed diets supplemented with copper. *Appl Environ Microbiol* 2011; **77**: 5597–603.
- 16 Fard RM, Heuzenroeder MW, Barton MD. Antimicrobial and heavy metal resistance in commensal enterococci isolated from pigs. *Vet Microbiol* 2011; **148**: 276–82.
- 17 Kim J, Lee S, Choi S. Copper resistance and its relationship to erythromycin resistance in *Enterococcus* isolates from bovine milk samples in Korea. *J Microbiol* 2012; **50**: 540–3.
- 18 van Schaik W, Top J, Riley DR et al. Pyrosequencing-based comparative genome analysis of the nosocomial pathogen *Enterococcus faecium* and identification of a large transferable pathogenicity island. *BMC Genomics* 2010; **11**: 239.
- 19 Official Journal of the European Union. List of the authorised additives in feedingstuffs (situation as 15 July 2003) published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs. 25.2.2004, C 50/1-144. <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2004:050:0001:0144:EN:PDF> (25 November 2013, date last accessed).
- 20 Novais C, Coque TM, Costa MJ et al. High occurrence and persistence of antibiotic-resistant enterococci in poultry food samples in Portugal. *J Antimicrob Chemother* 2005; **56**: 1139–43.
- 21 Arias CA, Robredo B, Singh KV et al. Rapid identification of *Enterococcus hirae* and *Enterococcus durans* by PCR and detection of a homologue of the *E. hirae* *mur-2* gene in *E. durans*. *J Clin Microbiol* 2006; **44**: 1567–70.
- 22 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement M100-S21*. CLSI, Wayne, PA, USA, 2011.
- 23 Aarestrup FM, Agerso Y, Gerner-Smidt P et al. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn Microbiol Infect Dis* 2000; **37**: 127–37.
- 24 Dahl KH, Lundblad EW, Rokenes TP et al. Genetic linkage of the *vanB2* gene cluster to Tn5382 in vancomycin-resistant enterococci and characterization of two novel insertion sequences. *Microbiology* 2000; **146**: 1469–79.
- 25 Novais C, Coque TM, Ferreira H et al. Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. *Appl Environ Microbiol* 2005; **71**: 3364–8.
- 26 Novais C, Sousa JC, Coque TM et al. Molecular characterization of glycopeptide-resistant *Enterococcus faecium* isolates from Portuguese hospitals. *Antimicrob Agents Chemother* 2005; **49**: 3073–9.
- 27 Novais C, Coque TM, Sousa JC et al. Antimicrobial resistance among faecal enterococci from healthy individuals in Portugal. *Clin Microbiol Infect* 2006; **12**: 1131–4.
- 28 Novais C, Freitas AR, Silveira E et al. Spread of multidrug-resistant *Enterococcus* to animals and humans: an underestimated role for the pig farm environment. *J Antimicrob Chemother* 2013; **68**: 2746–54.
- 29 Cavaco LM, Hasman H, Aarestrup FM. Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. *Vet Microbiol* 2011; **150**: 344–8.
- 30 Tenover FC, Arbeit RD, Goering RV et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; **33**: 2233–9.
- 31 Amachawadi RG, Scott HM, Alvarado CA et al. Occurrence of the transferable copper resistance gene *tcrB* among fecal enterococci of U.S. feedlot cattle fed copper-supplemented diets. *Appl Environ Microbiol* 2013; **79**: 4369–75.
- 32 Grass G, Rensing C. CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. *Biochem Biophys Res Commun* 2001; **286**: 902–8.
- 33 Sitthisak S, Howieson K, Amezola C et al. Characterization of a multicopper oxidase gene from *Staphylococcus aureus*. *Appl Environ Microbiol* 2005; **71**: 5650–3.
- 34 Achard ME, Tree JJ, Holden JA et al. The multi-copper-ion oxidase CueO of *Salmonella enterica* serovar Typhimurium is required for systemic virulence. *Infect Immun* 2010; **78**: 2312–9.
- 35 EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Scientific opinion on the safety and efficacy of copper compounds (E4) as feed additives for all animal species: cupric sulphate pentahydrate based on a dossier submitted by Manica S.p.A. *EFSA Journal* 2012; **10**: 2969.
- 36 Kuch A, Willems RJ, Werner G et al. Insight into antimicrobial susceptibility and population structure of contemporary human *Enterococcus faecalis* isolates from Europe. *J Antimicrob Chemother* 2012; **67**: 551–8.

Supplementary data

Table S1. Primers used to search copper resistance genes described in different Gram positive genus

Gene coding for:	Primers	Sequence (5'-3')	Nucleotide position (GenBank accession no.)	Amplicon size (bp)	Reference
Multicopper oxidase ^a	cueOF	CCCTAGGCGGTGTTTTCATA	7253-7233	996	This study
	cueOR	TCAATTGTCAAGGCAACCAAA (HM565232.1)	6258-6277		
Multicopper oxidase ^b	mco_pBMB171/P0245F	TTTAATTGCCGCATGTTCTG	268109-268088	1520	This study
	mco_pBMB171/P0245R	CGTGACAGTGGAACATCCAT	266590-266609 (CP001904.1)		
Multicopper oxidase ^c	mco_p5578F	AAGCGCAGAAAGGAAAGACA	2288-2307	207	This study
	mco_p5578R	TAACTTGAGAAAGGCCTCCA	2494-2475 (CP001603.1)		
ATPase-copper export ^d	trcBF	CATCACGGTAGCTTTAAGGAGATT	4954-4978	663	10
	trcBR	ATAGAGGACTCCGCCACCATG	5616-5595 (AY048044.2)		
ATPase-copper export ^e	copBF	GATTGAGCAATCGGGTGAGT	12786-12805	1219	This study
	copBR	TGATGGATATGGAGCACGAA	14004-13985 (DQ089807.2)		

^aGene found in *Enterococcus faecium* (GenBank accession no. HM565232.1) and *Listeria monocytogenes* (U15554.3).

^bGene found in different *Bacillus* species (Genbank accession no. CP001904.1; DQ889677.1).

^cGene found in different *Listeria* species (Genbank accession no. CP001904.1; NZ_JH556645.1) and *Staphylococcus aureus* (GQ900477.1).

^dGene found in different *Enterococcus* species (Genbank accession no. AY048044.2; NZ_AEBY01000000), *Weissella thailandensis* (HE575152.1) and *Escherichia coli* (U16658.1).

^eGene found in different *Lactococcus* species and subspecies (Genbank accession no. DQ089807.2; CP003133.1; CP002368.1; HE651325.1), *Streptococcus thermophilus* (CP003499.1) and *Lactobacillus delbrueckii* (CP002341.1), *Weissella koreensis* (CP002900.1) and *Leuconostoc citreum* (DQ489739.1).

Table S2. Co-transfer of copper and antibiotic resistance genes and transconjugants recovered from plates supplemented with different compounds.

Compounds used as selective agent	Positive mating assays (%) and species	% of wild strains transferring Cu ^T genes ^{a, b, h, k}	Transconjugants		
			Cu ^T genes ^k	CuSO ₄ MIC	AB ^R phenotype/genotype ^{l, j}
Erythromycin (8mg/L)	<i>E. faecium</i>	25/25 (100%)			
		SN522, SN534, SN545, SN592	<i>tcrB</i> + <i>cueO</i>	24	ERY [<i>erm</i> (B)] , FA, RIF
		SN180, SN341, SN427 , SN559, SN594	<i>tcrB</i> + <i>cueO</i>	20, 16	ERY [<i>erm</i> (B)], TET [n=4, <i>tet</i> (M), <i>tet</i> (L); n=1, <i>tet</i> (L)], FA, RIF
		F57 ^e , C618 , E273	<i>tcrB</i> (n=1), <i>tcrB</i> + <i>cueO</i> (n=2)	24	ERY [<i>erm</i> (B)], TET [n=3, <i>tet</i> (M), <i>tet</i> (L)], STR (<i>aadE</i>), FA, RIF
		C384	<i>tcrB</i> + <i>cueO</i>		ERY [<i>erm</i> (B)], CHL, TET [<i>tet</i> (M), <i>tet</i> (L)], STR (<i>aadE</i>), FA, RIF
		E289	<i>tcrB</i> + <i>cueO</i>	28	ERY[<i>erm</i> (B)], GEN, TET [<i>tet</i> (M), <i>tet</i> (L)], STR (<i>aadE</i>), FA, RIF
		E1 , E15^c , E64	<i>tcrB</i> + <i>cueO</i>	28	ERY [<i>erm</i> (B)], VAN (<i>vanA</i>), STR (<i>aadE</i>)
		C75 , C402	<i>tcrB</i> + <i>cueO</i>	24	ERY [<i>erm</i> (B)], VAN (<i>vanA</i>), FA, RIF
		SN573, SN558	<i>tcrB</i> + <i>cueO</i> , <i>tcrB</i>		ERY [<i>erm</i> (B)], VAN (<i>vanA</i>), TET [<i>tet</i> (M)], STR (<i>aadE</i>) (n=1)
	<i>E. faecalis</i>	F812	<i>tcrB</i> + <i>cueO</i>		ERY [<i>erm</i> (B)], TET [(<i>tet</i> (M), <i>tet</i> (L)], STR (<i>aadE</i>) , FA, RIF
		F742	<i>tcrB</i> + <i>cueO</i>		ERY [<i>erm</i> (B)], STR (<i>aadE</i>), FA, RIF
	<i>E. gallinarum</i>	SN694	<i>tcrB</i>		ERY [<i>erm</i> (B)], GEN, TET [<i>tet</i> (M), <i>tet</i> (L)], FA, RIF
		F392	<i>tcrB</i>		ERY [<i>erm</i> (B)], TET [(<i>tet</i> (M), <i>tet</i> (L)], FA, RIF
Tetracycline (8mg/L)	<i>E. faecium</i>	20/23 (87%)			
		18/20 (90%)			
		F577	<i>tcrB</i> + <i>cueO</i>	28	TET [<i>tet</i> (M), <i>tet</i> (L)], FA, RIF
		SN71 , SN341, SN534, SN594, MED43C1	<i>tcrB</i> + <i>cueO</i>	28	TET [n=2, <i>tet</i> (M); n=3, <i>tet</i> (M), <i>tet</i> (L)], ERY [<i>erm</i> (B)], FA, RIF
		SN573	<i>tcrB</i> + <i>cueO</i>		TET [<i>tet</i> (M)], STR (<i>aadE</i>), FA, RIF
		C384^f	<i>tcrB</i> + <i>cueO</i>		TET [<i>tet</i> (M), <i>tet</i> (L)], ERY [<i>erm</i> (B)], CHL, FA, RIF
		E86, E273	<i>tcrB</i> + <i>cueO</i>		TET [<i>tet</i> (M), <i>tet</i> (L)], ERY [<i>erm</i> (B)], STR , FA, RIF
		SN558	<i>tcrB</i>	20	TET [<i>tet</i> (M)], ERY [<i>erm</i> (B)], VAN (<i>vanA</i>), FA, RIF
	<i>E. faecalis</i>	F651	<i>tcrB</i> + <i>cueO</i>		TET [<i>tet</i> (M), <i>tet</i> (L)], FA, RIF
		F812^f	<i>tcrB</i> + <i>cueO</i>	28 ^m	TET [<i>tet</i> (M), <i>tet</i> (L)], ERY [<i>erm</i> (B)]
		F663	<i>tcrB</i> + <i>cueO</i>		TET [<i>tet</i> (M), <i>tet</i> (L)], GEN [<i>aac</i> (6')- <i>Ie-aph</i> (2'')- <i>Ia</i>], FA, RIF
		F742 ^f	<i>tcrB</i> + <i>cueO</i>		TET [<i>tet</i> (M), <i>tet</i> (L)], ERY [<i>erm</i> (B)], STR (<i>aadE</i>), FA, RIF
		F806^f	<i>tcrB</i> + <i>cueO</i>		TET [<i>tet</i> (M), <i>tet</i> (L)], ERY [<i>erm</i> (B)], GEN, STR (<i>aadE</i>), FA, RIF
	<i>E. gallinarum</i>	F392	<i>tcrB</i>		TET [<i>tet</i> (M), <i>tet</i> (L)], ERY [<i>erm</i> (B)], FA, RIF
	<i>Enterococcus spp</i>	SN150 ^c	<i>tcrB</i> + <i>cueO</i>		TET [<i>tet</i> (M)], ERY [<i>erm</i> (B)], GEN [<i>aac</i> (6')- <i>Ie-aph</i> (2'')- <i>Ia</i>], FA, RIF

Compounds used as selective agent	Positive mating assays (%) and species	% of wild strains transferring Cu ^T genes ^{a, b, h, k}	Transconjugants		
			Cu ^T genes ^k	CuSO ₄ MIC	AB ^R phenotype/genotype ^{i, j}
Ampicillin (16mg/L)	<i>E. faecium</i>	11/12 (92%)			
		SN71^g , SN148 ^g	<i>tcrB</i> + <i>cueO</i>	28	AMP (<i>pbp5</i>), FA, RIF
		H28798^{g, l}	Unknown	20	AMP (<i>pbp5</i>), ERY [<i>erm</i> (B)], STR (<i>aadE</i>), VAN (<i>vanA</i>), FA, RIF
		E233 ^{g, l} , E169^{g, l} , E4^{g, l} , H207^{g, l}	Unknown	16, 20	AMP (<i>pbp5</i>), FA, RIF
		E49^g	<i>tcrB</i>	32	AMP (<i>pbp5</i>), VAN (<i>vanA</i>), FA, RIF
		VD79C1^{g, l}	Unknown	20	AMP (<i>pbp5</i>), ERY [<i>erm</i> (B)], GENT[<i>aac</i> (6')-Ie-aph (2'')-Ia], VAN (<i>vanA</i>), FA, RIF
Gentamicin (125mg/L)	<i>E. faecalis</i>	2/2 (100%)			
		F812^f	<i>tcrB</i> + <i>cueO</i>		GEN, TET [<i>tet</i> (M), <i>tet</i> (L)], FA, RIF
		SN122 ^{d, f}	<i>tcrB</i> + <i>cueO</i>		GEN, ERY [<i>erm</i> (B)], FA, RIF
Vancomycin (6mg/L)	<i>E. faecium</i>	4/7 (57%)			
		SN573	<i>tcrB</i> + <i>cueO</i>		VAN (<i>vanA</i>), TET [<i>tet</i> (M)], FA, RIF
		C75	<i>tcrB</i> + <i>cueO</i>		VAN (<i>vanA</i>), ERY [<i>erm</i> (B)], GEN [<i>aac</i> (6')-Ie-aph (2'')-Ia], FA, RIF
		C402	<i>tcrB</i> + <i>cueO</i>	24	VAN (<i>vanA</i>), ERY [<i>erm</i> (B)], STR (<i>aadE</i>), FA, RIF
Copper sulphate (8-16 mM)	<i>E. faecium</i>	8/8 (100%)			
		SN71	<i>tcrB</i> + <i>cueO</i>	28	(-)
		MED43C1 , SN427 , E289	<i>tcrB</i> + <i>cueO</i>	24, 28	ERY [<i>erm</i> (B)], GEN (n=1) [(<i>aac</i> (6')-Ie-aph (2'')-Ia), TET [n=1, <i>tet</i> (M); n=2, <i>tet</i> (L)], STR (<i>aadE</i>), FA, RIF
		SN558	<i>tcrB</i>	28	VAN (<i>vanA</i>), FA, RIF
		H305	<i>tcrB</i> + <i>cueO</i>	24	ERY [<i>erm</i> (B)], VAN (<i>vanA</i>), FA, RIF
		C402	<i>tcrB</i> + <i>cueO</i>	24	ERY [<i>erm</i> (B)], STR (<i>aadE</i>), VAN (<i>vanA</i>), FA, RIF
		H70411^{e, g}	Unknown	12	AMP, ERY [<i>erm</i> (B)], VAN (<i>vanA</i>), FA, RIF

^a Strains marked in bold originate transconjugates to which CuSO₄ MICs were determined.

^b Underline wild strains were used in mating assays with more than one compound.

^c Negative conjugation in mating assays with gentamicin.

^d Negative conjugation in mating assays with tetracycline.

^e Negative transfer of *tcrB* and/or *cueO* to recipient strains.

^f Positive conjugation with recipient *E. faecalis* JH2-2.

^g Positive conjugation with recipient *E. faecium* GE1.

^h All strains without the sign "f" or "g" had a positive conjugation with recipient *E. faecium* BM4105RF.

ⁱ AB^R, antibiotic resistance, VAN, vancomycin; AMP, ampicillin; ERY, erythromycin; ; TET, tetracycline; CHL, chloramphenicol; GEN, high level of resistance (HLR) to gentamicin; STR, HLR to streptomycin; FA, fusidic acid; RIF, rifampicin.

^j Only acquired resistances.

^k Cu^T, copper tolerance; Isolates designated as "SN" are from swine/piggeries, as "F" from poultry, as "C" or "MED" from healthy humans, as "E" from hospital sewage, as "H" from hospitalized patients.

^l Isolates without *tcrB* and/or *cueO* but with a CuSO₄ MIC $\geq$ 16mM

^m The MIC of the recipient *E. faecalis* JH2-2 was also 28mM.

***Enterococcus* spp. from different origins and clonal lineages carry two *merA* genes found in several *Firmicutes* members and are co-transferred with antibiotic resistance and copper tolerance**

Eduarda Silveira¹, Mariana Barros¹, Ana R. Freitas¹, Patrícia Antunes^{1,2}, Teresa M.

Coque^{3,4,5}, Luísa Peixe¹, Carla Novais^{1*}

¹REQUIMTE. Laboratório de Microbiologia. Faculdade de Farmácia. Universidade do Porto, Portugal;

²Faculdade de Ciências da Nutrição e Alimentação. Universidade do Porto, Portugal; ³Servicio de Microbiología. Hospital Ramón y Cajal. Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain; ⁴Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBER-ESP), Madrid, Spain; ⁵Unidad de Resistencia a Antibióticos y Virulencia Bacteriana asociada al Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain.

Running Title: Spread of *merA* genes in *Enterococcus*

Keywords: mercury, *merA*, antibiotic resistance

Corresponding author: Carla Novais, Ph.D. Laboratório de Microbiologia. Faculdade de Farmácia, Universidade do Porto. Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal Phone: +351 220428588; FAX: +351 226 093 390; E-mail: casilva@ff.up.pt

Parte deste trabalho foi apresentado no 22nd *European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)*, Londres, Reino Unido em Abril de 2012. Parte do trabalho foi também publicado como suplemento da revista científica *Clinical Microbiology and Infection* (vol.18, suplemento S3:447, P1631).

Artigo em preparação

ABSTRACT

This study provides a snapshot of the occurrence of different *merA* (mercury) genes among *Enterococcus* spp. from several sources and of their ability to be spread with antibiotic resistance and copper tolerance genes. *Enterococcus* (n=918) from different species and human, animal, food and environmental sources from Portugal (1996-2012) were included. Antimicrobial susceptibility/conjugation assays were performed by standard procedures, screening of mercury, copper and antibiotic resistance genes by PCR, and clonality by PFGE/multilocus-sequence-typing. *Enterococcus* carried two types of *merA* genes (3%-*merA2*, mainly *E. faecium*; 2%-*merA3*, mainly *E. faecium* and *E. faecalis*; $p < 0.05$) also found in other members of *Firmicutes* phylum. Similar rates were observed in human, animal, food and environmental isolates ($p > 0.05$). They were detected in diverse clonal lineages including those associated with human infections (e.g., *E. faecium*- ST18; ST78). Isolates carrying *merA* were more resistant ($p < 0.05$) to antibiotics [*merA2*-tetracycline-*tet(M)/tet(L)*, streptomycin-*aadE*, ampicillin, nitrofurantoin; *merA3*-erythromycin-*erm(B)*, ciprofloxacin, chloramphenicol, gentamicin- *aac(6')-Ie-aph-(2'')-Ia*] than other isolates. Copper tolerance genes (*tcrB*, *cueO*) were also often detected with *merA2*. Transfer of *merA2*, *tcrB*, *cueO* and antibiotic resistance was detected in 75% of isolates carrying all these genes. This is the first comprehensive analysis showing the prevalence of four types of *merA* genes within enterococcal populations from diverse ecological niches and clonal lineages. Among multiple chemical challenges faced by microorganisms, pollution by mercury compounds, could also contribute to the maintenance of MDR strains in different environments.

INTRODUCTION

Over the last two decades *Enterococcus faecium* and *Enterococcus faecalis* became increasingly important nosocomial pathogens worldwide, mostly including particular multidrug resistant (MDR) clonal lineages (Willems *et al.*, 2012). In order to understand their increasing success, studies have mainly focused on the impact of antibiotic use in the selection of MDR strains, remaining the influence of other eco-challenges (e.g., metals and other biocides) less explored. Mercury (Hg) exists naturally in small amounts in the environment, but its levels have been rising worldwide by anthropogenic activities (e.g., pharmaceutical industry, pesticides) (Nascimento *et al.*, 2003; EFSA, 2012). Once released, Hg undergoes a series of chemical and physical transformations as it cycles between atmosphere, land, and water. This phenomenon regularly exposes humans, plants, animals and their microorganisms to Hg by different routes (e.g., food chain) (EFSA, 2012).

The most common bacterial tolerance mechanism is the enzymatic reduction in Hg^{2+} to Hg^0 by the mercuric reductase (MerA) encoded by the *merA* gene. It is part of the *mer* operon encoding a group of proteins involved in the detection, scavenging, transport and reduction of Hg (Nascimento *et al.*, 2003; Mathema *et al.*, 2011). Variation in structure and organization of *mer* operon as well as in sequences of individual genes (e.g., *merA*) were reported among different bacteria, pointing to their mosaic nature (Osborn *et al.*, 1997, Moller *et al.*, 2013). Two types of *mer* operons were mainly described including those conferring tolerance only to inorganic Hg salts (narrow type) or both to organomercurials and inorganic Hg salts (broad type) (Osborn *et al.*, 1997, Nascimento *et al.*, 2003; Mathema *et al.*, 2011). Horizontal transfer among exchange bacterial communities seems to have contributed to the spread and evolution of *mer* operons (Osborn *et al.*, 1997; Mindlin *et al.*, 2002, with descriptions of different bacterial genera sharing the same *mer* genetic platforms (Mindlin *et al.*, 2002) and bacteria of the same genus carrying different *mer* variants (Bogdanova *et al.*, 1998; Stapleton *et al.*, 2004).

Among *Enterococcus* the distribution of *mer* operons or *merA* genes (often used to screen *mer* operons in several works) (Zscheck *et al.*, 1990; Ojo *et al.*, 2004; Skurnik *et al.*, 2010; Moller *et al.*, 2013;) was scarcely studied. They were described in isolates recovered from a primate (Davies *et al.*, 2005), clinical (Zscheck *et al.*, 1990; Soge *et al.*, 2008) and oral human samples (Soge *et al.*, 2008). However, most of these works did not provide a comprehensive analysis of the prevalence of *merA* genes within the enterococcal populations from diverse ecological niches or of the clones involved in their spread along with antibiotic resistance or

other features. With this study we intended to have a snapshot of the prevalence of four types of *merA* genes spread in different bacterial genera among *Enterococcus* from several sources as well as their ability to be disseminated with antibiotic resistance and copper tolerance genes.

METHODS

Epidemiological background, species identification and study of antibiotic susceptibility. Nine hundred and eighteen isolates were obtained from different sources (hospitalized and healthy humans, animals, foods, sewage, and environment) of the North, Centre and South of Portugal during 1996-2012. Samples were collected from i) hospitalized humans (108 clinical isolates from different patients at 5 hospitals), ii) healthy humans (125 from feces of 79 individuals); iii) environment (49 isolates from 16 water samples from hospital sewage, 3 isolates from 2 water samples of an urban river) (iii) food for human consumption (155 isolates from 75 poultry carcass lots of 11 brands sold across Portugal, 27 isolates from 7 lots of muscle/viscera of rainbow trout sold in 4 supermarkets and 2 samples from an aquaculture), 21 isolates from 18 ready to eat salads of 7 brands; and iv) the animal production setting (232 isolates from 71 samples of 6 piggeries animal feces/skin, animal residues, feed/drinking water, air, facilities walls/floors/equipment/dust; and 198 isolates from 41 samples of 2 trout aquacultures water/sediment, feed samples).

Enterococcus were identified by standard methods (Gram, catalase, esculin hydrolysis, growth in NaCl 6,5%) and PCR (*E. faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*, *E. durans*, *E. hirae*) (Novais *et al.*, 2013). Susceptibility to vancomycin, ampicillin, tetracycline, erythromycin, ciprofloxacin, chloramphenicol, nitrofurantoin and high concentrations of gentamicin and streptomycin was determined by agar dilution or disk diffusion methods following CLSI guidelines (CLSI, 2011). The search of genes coding for resistance to glycopeptides (*vanA*, *vanB*, *vanC1*, *vanC2*), macrolides [*erm*(A), *erm*(B), *erm*(C)], tetracyclines [*tet*(M), *tet*(L), *tet*(O), *tet*(K), *tet*(S)], aminoglycosides [*aac*(6')-Ie-aph (2'')-Ia, *aadE*] and tolerance to copper (*trcB*, *cueO*) was performed by PCR as described (Novais *et al.*, 2013, Silveira *et al.*, 2013). Part of the isolates analyzed in this work corresponds to *Enterococcus* from previous surveillance studies and were selected from each source based in the following features: different samples, species and antibiotic resistance profile (Novais *et al.*, 2004, Novais *et al.*, 2005a; Novais *et al.*, 2005b; Novais *et al.*, 2005c, Novais *et al.*, 2006; Novais *et al.*, 2013).

Search of mercury tolerance genes. Four *merA* genes types, to which we randomly attributed the numbers 1, 2, 3 and 4 for distinction, were searched by PCR. The *merA* types (*merA1*, *merA2* and *merA3*) selection was based on their occurrence in the genomes of different *Enterococcus* species as well as in other bacteria genera available at GenBank database, pointing to their mobilization among microorganisms potentially sharing the same ecosystems. The *merA4* is extensively spread among Gram-negative bacteria, which also share the same ecosystems with *Enterococcus* (e.g., *Enterobacteriaceae*). Other criterion for this *merA* types selection was their inclusion in *mer* operons representing narrow (*merA2*, *merA3*) and broad (*merA1*, *merA4*) spectrum variants (Mathema *et al.*, 2011; Nascimento *et al.*, 2013). The *merA1* allele has 64-68% of amino acid identity with *merA2* and *merA3* and the latter of 76% among each other. Primers, Genbank sequences used for primer design and bacterial genus where they were previously described are indicated in Table 1. Positive amplicons from each PCR were sequenced and compared with known GenBank sequences to validate the method.

Clonal relatedness. Clonality was established by Pulsed-Field-Gel Electrophoresis (PFGE; *Sma*I enzyme) in *merA* positive *E. faecium* and *E. faecalis* representative of different sources (Tenover *et al.*, 1995; Novais *et al.*, 2005a). PFGE was not applied when only a single isolate of a species was available or when the species could not be determined. Multilocus Sequence Typing (MLST) was also performed for *E. faecium* and *E. faecalis* representative of different PFGE types (<http://www.mlst.net>).

Conjugation assays. Transfer of different *merA* alleles was analyzed for isolates representative of different sources and included 5 *E. faecalis* and 9 *E. faecium*. It was performed by the filter mating method at a 1:1 donor-recipient ratio using *E. faecium* strain BM4105RF, *E. faecium* GE1 or *E. faecalis* JH2-2 as recipients strains (all resistant to rifampicin and fusidic acid; *E. faecium* GE1 also resistant to tetracycline; all *merA* negative). Transconjugants were recovered after incubation at 37°C for 24-48h from plates of Brain Heart Infusion (BHI) agar supplemented with 30mg/L of rifampicin, 25mg/L of fusidic acid plus other antibiotics (8mg/L of tetracycline, 8mg/L of erythromycin, 6mg/L of vancomycin, 10mg/L of ampicillin) or 16mM copper sulphate. Most of transconjugants were recovered in previous studies (Novais *et al.*, 2005a,b,c; Novais *et al.*, 2013; Silveira *et al.*, 2013;). They were confirmed by the presence of *merA* genes by PCR and resistance to rifampicin and fusidic acid by disk diffusion (CLSI, 2011). Co-transfer of antibiotic resistance was confirmed at

phenotypical and genotypical level, as described above.

Statistical analysis. Differences in the prevalence of *merA* genes among species or ecological niches as well as the prevalence of antibiotic resistance among isolates with and without *merA* were analyzed by Fisher exact test ($\alpha = 0.05$) using the GraphPad Prism software, version 6.0a.

RESULTS AND DISCUSSION

Two *merA* alleles are spread among *Enterococcus* from several species, clonal lineages and sources. Only *merA2* and *merA3* genes were detected and with low prevalence rates (3%-n=29/918; 2%-19/918, respectively) (Table 2). With exception of one clinical *E. faecalis* carrying both genes, they were observed in different isolates. Amplicons of *merA2* and a *merA3* showed 100% and 99% of nucleotide identity with *Enterococcus faecium* strain E0680 (Genbank accession no. AHWN01000031.1) and *Enterococcus faecalis* 664.1H1 (previously identified as *E. faecium*) (Genbank accession no. AY351675.1), respectively.

With exception of poultry and ready-to-eats salads, where none of the *merA* genes were found, and of *merA3* in piggeries, the distribution of *merA2* (piggeries-9%, trout for human consumption-4%, healthy human-3%, hospital sewage-2% aquaculture environment-1%, clinical isolates-1%) or *merA3* (trout for human consumption-11%, clinical isolates-9%, hospital sewage-6%, healthy human-2%, piggeries-0.4%) among other environments/hosts was similar ($p>0.05$).

The *merA2* (n=29 isolates) was more frequently found among *E. faecium* (83% versus 7% in *E. faecalis* and 11% in other species-*E. hirae*, *Enterococcus* spp.) ($p<0.05$). The *merA3* (n=19) presented similar rates between *E. faecium* and *E. faecalis* (45% and 40%; $p>0.05$) but lower in other species-*E. gallinarum*, *E. casseliflavus*, *Enterococcus* sp (15%; $p<0.05$). Clonality studies revealed 17 PFGE types among *E. faecium* (n=18) studied and 5 among *E. faecalis* (n=5) (Table 2). *E. faecium* belonged to ST18 (ST18, ST132, ST431), ST78 (ST393) clonal lineages, CC5 (ST185), ST430 and the singletons ST846, ST859. *E. faecalis* studied (n=5) were identified also in different ST.

Two *merA* variants were spread among *Enterococcus* from different settings, species and clonal lineages, including those associated with human infections. This wide distribution was

not surprising as several studies have been showing a remarkable spread of *merA* genes across multiple microorganisms from different environments (including pristine ones), which have been suggested to be continuously selected/maintained by worldwide spread of mercury compounds (Stapleton *et al.*, 2004; Soge *et al.*, 2008; Rydberg *et al.*, 2010; Skurnik *et al.*, 2010; Mathema *et al.*, 2011; Boyd *et al.*, 2012; Kim *et al.*, 2012; Moller *et al.*, 2013). The analysis of gene databases spotted both *merA2* and *merA3* in several Gram-positive and Gram-negative belonging to *Firmicutes* phylum (*merA2*-*Streptococcus*, *Dialister*; *merA3*-*Aerococcus*) (Table 1). Although we did not found *merA1*, Soge *et al.* (2008) also identified this gene in *Enterococcus*, *Streptococcus*, *Enterobacteriaceae* and non-fermenter bacilli. The ubiquitous nature of *Enterococcus*, their ability for genetic exchange with microorganisms sharing the same ecosystems and adaptation to environmental challenges (Zscheck *et al.*, 1990; Silveira *et al.*, 2013; Werner *et al.*, 2013) might explain the *merA* types found and their spread by different sources. Considering the mosaic nature of *mer* operons previously described as well as the possibility of the occurrence of vestigial *merA* genes (Osborn *et al.*, 1997), more comprehensive molecular studies are needed to understand the functionality of the operons carrying the *merA* observed as well as the co-evolution of the genetic platforms carrying them among several bacterial hosts. The low prevalence of the *merA* genes detected (2-3%) was similar to a previous work reporting 4% of *merA* genes among *Firmicutes* (Moller *et al.*, 2013). This result could be related to the occurrence of other *merA* variants not searched, considering the existence of more than 200 MerA homologs encoding for the mercuric reductases subunits described (Boyd *et al.*, 2012) or to a limited selective pressure imposed by mercury in the environments studied.

The detection of *Enterococcus* carrying *merA* from aquatic to terrestrial environments, among human and animals and within diverse sample types per setting (Table 2) stresses the potential dispersion routes of isolates carrying these genes between different hosts, namely through food chain and/or environmental contamination. For example, *Enterococcus* carrying *merA2* was detected in i) feed and trout from the same aquaculture, ii) feed, manure and dust of the same piggery, iii) hospitalized human and hospital sewage. Also, *Enterococcus* carrying *merA3* were observed in trout from aquaculture, trout sold in supermarkets and healthy human faeces (Table 2). Depending on the exposure of each host/environment to different mercury concentrations across the transmission pathway (e.g., animal production setting-food-human), these strains could be more or less selected (environments are enriched in Hg tolerant microorganisms when Hg concentrations are increased) (Summers *et al.*, 1993; Skurnik *et al.*, 2010) and help to maintain other carrying adaptive features (e.g.,

antibiotic resistance) in particular settings.

The dispersion of *merA* genes among *Enterococcus* is linked to horizontal transfer and to antibiotic resistance. As a polyclonal population was associated with the dispersion of *merA* genes, horizontal transfer was studied for isolates representative of different species and *merA* alleles. Only *merA2* was transferred (n=7 wild strains out of 11; 63%), which was detected in transconjugants along with resistance to tetracycline (n=7 transconjugants) or erythromycin (n=6) as well as with the clinical relevant antibiotics used for the treatment of enterococcal infections streptomycin (n=4), gentamicin (n=3), vancomycin (n=1) or ampicillin (n=1) (Table 2). The absence of *merA3* in transconjugants could be explained by their occurrence in non-transferable genetic platforms or, alternatively, on genetic platforms without genes coding for resistance to the antibiotics or copper sulphate used in selection media, thus preventing their recover.

In order to evaluate if all wild enterococcal population with *merA* genes reflected the results found in transconjugants, antibiotic resistance patterns were compared between wild strains carrying *merA2* (n=29) or *merA3* (n=19) with those without *merA* (n=871) genes. Interestingly, *Enterococcus* carrying *merA2* were also more resistant to tetracycline [*tet*(M), *tet*(L)], high concentrations of streptomycin (*aadE*), ampicillin (not associated with *blaZ*) or nitrofurantoin than *merA* negative isolates ($p<0.05$) (Figure 1; Table 2). Similar antibiotic resistance patterns to this study were detected by Summers *et al.* (1993) for mercury tolerant *Enterococcus* recovered from oral and intestinal samples of primates after the use of dental amalgams with mercury. On the other hand *Enterococcus* carrying *merA3* were more resistance to other antibiotics as erythromycin [*erm*(B)], ciprofloxacin, chloramphenicol or high concentrations of gentamicin [*aac*(6')-*le-aph* (2'')-*la*] ($p<0.05$) (Figure 1; Table 2). The often association of tetracycline, erythromycin, beta-lactams or aminoglycosides resistance genes with *merA* in Gram-positive and Gram-negative bacteria (Summers *et al.*, 1993; Wireman *et al.*, 1997; Ready *et al.*, 2007), could be the result of a genetic “convergence” within different microorganisms of the primordial widespread *merA* platforms with others constantly selected by the vast used of these antibiotics in human and animal settings for several decades (Wiedenbeck *et al.*, 2011; Baquero *et al.*, 2013; Meyer *et al.*, 2013).

Our data also suggest that the co-dissemination of antibiotic resistance with *merA* genes is supported by variable genetic platforms as transconjugants present diverse antibiotic resistance phenotypes. Among those genetic platforms carrying *merA* genes, some seem to be potentially more widespread/stable in *E. faecium* (*merA2*) or both in *E. faecium* and *E.*

faecalis (*merA3*). Among *Enterococcus*, *merA3* was previously described to be located in the same conjugative plasmid as *aadK* (streptomycin resistance) (Davies *et al.*, 2005) and *merA1* within Tn5385, transporting genes coding for resistance to erythromycin (*ermAM*), gentamicin (*aac6'-Ie-aph2''-Ia*), tetracycline [*tet(M)*] and penicillin (*bla*) (Rice *et al.*, 1998) or Tn6009 carrying *tet(M)* (Soge *et al.*, 2008). However, most of our phenotypic/genotypic data related to *merA* alleles and antibiotic resistance seem to point to the occurrence of *merA* in other genetic elements/modules because *merA1* was not found and half of the isolates with *merA3* are not resistant to streptomycin.

Almost all *merA2* carrying *Enterococcus* (mostly *E. faecium*) also harbour and transferred the genes *tcrB* and *cueO* coding for copper tolerance, recently evaluated in another study of our group (Table 2) (Silveira *et al.*, 2013). This data along with the co-detection of *tcrB/cueO/merA2* genes in few available GenBank sequences (e.g. AHWN01000031.1; AIUL01000023.1) suggests that they might be often located in the same genetic elements in *E. faecium*. Those *merA2* carrying *Enterococcus* without *tcrB* and *cueO* were *E. faecalis* or *E. hirae*, potentially pointing for recombination events and/or different evolution of *merA2* platforms in different species. The *merA2* type in *E. hirae* was not described at Genbank database, being this its first report.

CONCLUDING REMARKS

In summary, *Enterococcus* from different species and clonal lineages, including those often associated with human infections (e.g. *E. faecium* ST18/ST78) are reservoirs of two *merA* alleles. The contamination of different environments with mercury and/other metals (e.g., copper) as well as antibiotics might promote the selection of more fit strains to multiple challenges. More studies are essential to determine the minimum concentration of mercury and other metals able to select MDR strains and to understand the evolution and persistence of mobile genetic platforms potentially co-transporting genes coding for different adaptive features enabling bacteria to survive within different environments and hosts.

Acknowledgments

This project was funded by research grants from the Fundação para a Ciência e Tecnologia (PTDC/AAC-AMB/103386/2008; grant no. PEst-C/EQB/LA0006/2011) and Investigação Jovem da Universidade do Porto/Santander Totta (PP_IJUP2011-71). Eduarda Silveira is supported by a Ph.D. fellowship of the Fundação para a Ciência e Tecnologia (grant number SFRH/BD/63955/2009; POPH-QREN). Work in Teresa M. Coque lab is supported by grants

from the European Union (7FPEU-HEALTH-F3-2011-282004_EvoTAR), Ministry of Economy and Competitiveness of Spain-Instituto de Salud Carlos III (PI12/01581). We want to acknowledge to Helena Pinto (Faculdade de Farmácia. Universidade Porto) for technical support.

REFERENCES

1. **Baquero F, Tedim AP, Coque TM.** 2013. Antibiotic resistance shaping multi-level population biology of bacteria. *Front Microbiol.***4**:15.
2. **Bogdanova ES, Bass IA, Minakhin LS, Petrova MA, Mindlin SZ, Volodin AA, Kalyaeva ES, Tiedje JM, Hobman JL, Brown NL, Nikiforov VG.** 1998. Horizontal spread of mer operons among Gram-positive bacteria in natural environments. *Microbiology.* **144**:609-620.
3. **Boyd ES, Barkay T.** 2012. The mercury resistance operon: from an origin in a geothermal environment to an efficient detoxification machine. *Front Microbiol.***3**:349
4. **Clinical Laboratory Standards Institute.** Performance standards for antimicrobial susceptibility testing; Twenty first informational supplement M100-S21, CLSI, Wayne, PA, USA, 2011.
5. **Davis IJ, Roberts AP, Ready D, Richards H, Wilson M, Mullany P.** 2005. Linkage of a novel mercury resistance operon with streptomycin resistance on a conjugative plasmid in *Enterococcus faecium*. *Plasmid.* **54**:26-38.
6. **EFSA Panel on Contaminants in the Food Chain (CONTAM).** 2012. Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. *EFSA Journal* **10**:2985.
7. **Kim MK, Zoh KD.** 2012. Fate and transport of mercury in environmental media and human exposure. *J Prev Med Public Health.* **45**:335-343.
8. **Mathema VB, Thakuri BC, Sillanpää M.** 2011. Bacterial mer operon-mediated detoxification of mercurial compounds: a short review. *Arch Microbiol.* **193**:837-844.
9. **Meyer E, Gastmeier P, Deja M, Schwab F.** 2013. Antibiotic consumption and resistance: data from Europe and Germany. *Int J Med Microbiol.* **303**:388-395.
10. **Mindlin S Z, Bass I A, Bogdanova E S, Gorlenko Z M, Kalyaeva E S, Petrova M A, Nikiforov V. G.** 2002. Horizontal Transfer of Mercury Resistance Genes in Environmental Bacterial Populations. *Molecular Biology.* **36**:160-170.

11. **Møller AK, Barkay T, Hansen MA, Norman A, Hansen LH, Sørensen SJ, Boyd ES, Kroer N.** 2013. Mercuric reductase genes (*merA*) and mercury resistance plasmids in High Arctic snow, freshwater and sea-ice brine. *FEMS Microbiol Ecol.* In press
 12. **Nascimento AM, Chartone-Souza E.** 2003. Operon *mer*: bacterial resistance to mercury and potential for bioremediation of contaminated environments. *Genet Mol Res.* **2**:92-101.
 13. **Novais C, Coque TM, Costa MJ, Sousa JC, Baquero F, Peixe LV.** 2005a. High occurrence and persistence of antibiotic-resistant enterococci in poultry food samples in Portugal. *J Antimicrob Chemother.* **56**:1139–1143.
 14. **Novais C, Coque TM, Ferreira H, Sousa JC, Peixe L.** 2005b. Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. *Appl Environ Microbiol.* **71**:3364–3368.
 15. **Novais C, Coque TM, Sousa JC, Baquero F, Peixe L.** 2004. Local genetic patterns within a vancomycin-resistant *Enterococcus faecalis* clone isolated in three hospitals in Portugal. *Antimicrob Agents Chemother.* **48**:3613-3617
 16. **Novais C, Coque TM, Sousa JC, Peixe LV.** 2006. Antimicrobial resistance among faecal enterococci from healthy individuals in Portugal. *Clin Microbiol Infect.* **12**:1131-1134.
 17. **Novais C, Freitas AR, Silveira E, Antunes P, Silva R, Coque TM, Peixe L.** 2013. Spread of multidrug-resistant *Enterococcus* to animals and humans: an underestimated role for the pig farm environment. *J Antimicrob Chemother.* In press
 18. **Novais C, Sousa JC, Coque TM, Peixe LV.** 2005c. Molecular characterization of glycopeptide-resistant *Enterococcus faecium* isolates from Portuguese hospitals. *Antimicrob Agents Chemother.* **49**:3073-3079.
 19. **Ojo KK, Tung D, Luis H, Bernardo M, Leitao J, Roberts MC.** 2004. Gram-positive *merA* gene in gram-negative oral and urine bacteria. *FEMS Microbiol Lett.* **238**:411-416.
 20. **Osborn AM, Bruce KD, Strike P, Ritchie DA.** 1997. Distribution, diversity and evolution of the bacterial mercury resistance (*mer*) operon. *FEMS Microbiol Rev.* **19**:239-262.
 21. **Ready D, Pratten J, Mordan N, Watts E, Wilson M.** 2007. The effect of amalgam exposure on mercury- and antibiotic-resistant bacteria. *Int J Antimicrob Agents.* **30**:34-39
-

22. **Rice LB, Carias LL.** 1998. Transfer of Tn5385, a composite, multiresistance chromosomal element from *Enterococcus faecalis*. J Bacteriol. **180**:714-721
 23. **Rydberg J, Klaminder J, Rosén P, Bindler R.** 2010. Climate driven release of carbon and mercury from permafrost mires increases mercury loading to sub-arctic lakes. Sci Total Environ. **408**:4778-4483.
 24. **Silveira E, Freitas Ana R, Antunes P, Barros M, Campos J, Coque T M, Peixe L, Novais C.** 2013. Co-transfer of resistance to high concentrations of copper and first line antibiotics among *Enterococcus* from different origins (human, animal, environment and food) and clonal lineages. J Antimicrob Chemother. In Press
 25. **Skurnik D, Ruimy R, Ready D, Ruppe E, Bernède-Bauduin C, Djossou F, Guillemot D, Pier GB, Andremont A.** 2010. Is exposure to mercury a driving force for the carriage of antibiotic resistance genes? J Med Microbiol. **59**:804-807.
 26. **Soge OO, Beck NK, White TM, No DB, Roberts MC.** 2008. A novel transposon, Tn6009, composed of a Tn916 element linked with a *Staphylococcus aureus* mer operon. J Antimicrob Chemother. **62**:674-80.
 27. **Stapleton P, Pike R, Mullany P, Lucas V, Roberts G, Rowbury R, Wilson M, Richards H.** 2004. Mercuric resistance genes in gram-positive oral bacteria. FEMS Microbiol Lett. **236**:213-220.
 28. **Summers AO, Wireman J, Vimy MJ, Lorscheider FL, Marshall B, Levy SB, Bennett S, Billard L.** 1993. Mercury released from dental "silver" fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. Antimicrob Agents Chemother. **37**:825-834.
 29. **Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. **33**:2233–2239.
 30. **Werner G, Coque TM, Franz CM, Grohmann E, Hegstad K, Jensen L, van Schaik W, Weaver K.** 2013. Antibiotic resistant enterococci-tales of a drug resistance gene trafficker. Int J Med Microbiol. **303**:360-79.
 31. **Wiedenbeck J, Cohan FM.** 2011. Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. FEMS Microbiol Rev. **35**:957-976.
-

32. **Willems RJ, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J.** 2012 .Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *MBio* **3**:e00151-12.
33. **Wireman J, Liebert CA, Smith T, Summers AO.** 1997. Association of mercury resistance with antibiotic resistance in the gram-negative fecal bacteria of primates. *Appl Environ Microbiol.* **63**:4494-4503.
34. **Zscheck KK, Murray BE.** 1990. Evidence for a staphylococcal-like mercury resistance gene in *Enterococcus faecalis*. *Antimicrob Agents Chemother.* **34**:1287-1289.

Table 1. Primers used for *merA* genes amplification.

Primer name ^{a,b}	Sequence (5'-3')	Nucleotide position (Genbank accession no.)	Amplicon size (bp)	Reference
merA1_F ^c merA1_R ^c	ATGACTCAAAATTCATATAAAATAC TTAGCCTGCACAACAAGATAA	2893-2917 (AECE01000068.1) 1274-1294 (AECE01000068.1)	1644	Ojo et al
merA2_F merA2_R	GTCGGATGCGTTCCTTCTAA GACGCTCGCTTCTTTGAATC	33146-33165 bp (AHWN01000031.1) 32688-32707 bp (AHWN01000031.1)	478	This study
merA3_F merA3_R	GGTTGGTTTAACAGAAGAACAAG GCCATTGTTAAATATGGTGC	7494-7516 (AY351675.1) 7744-7763 (AY351675.1)	270	This study
merA4_F merA4_R	ACCATCGGCGGCACCTGCGT ACCATCGTCAGGTAGGGGAACAA	64877-64896 (AB591424.1) 63671-63693 (AB591424.1)	1226	Liebert et al 1997

^aThe numeration 1, 2, 3 and 4 was attributed to distinguish the primers used to amplify different variants of *merA* genes. ^bExamples of bacterial genera carrying *merA* genes variants amplified by each primer pair (the examples only include sequences with 98-100% of amino acid identity with the sequence used to design the primers): **merA1**-*Enterococcus faecalis* (EFU05581.1), *Staphylococcus aureus* (YP_006938295.1), *Staphylococcus epidermidis* (AAO03682.1), *Staphylococcus hominis* (WP_002487968.1), *Klebsiella pneumoniae* (ABY75610.1), *Escherichia coli* (AAU45402.1), *Enterobacter cloacae* (AAU45404); *Acinetobacter junii* (AAU45403.1); **merA2**-*Streptococcus gordonii* (YP_001449687), *Streptococcus mitis* (WP_001031941), *Streptococcus sanguinis* (WP_002933267), *Streptococcus parasanguinis* (WP_003009663), *Dialister microaerophilus* (WP_007556353), *Enterococcus faecium* (AHWN01000031.1); **merA3**-*Enterococcus faecium* (AAR10425.1), *Enterococcus faecalis* (EOL80227.1), *Aerococcus viridans* (EFG48778); **merA4**-*Salmonella enterica* (YP_001102037.1), *Escherichia coli* (YP_002891186.1), *Aeromonas hydrophila* (YP_006966054.1), *Pseudomonas aeruginosa* (EFQ39102.1), *Pseudomonas putida* (YP_007232234.1), *Achromobacter xylosoxidans* (AFU56929.1), *Acidovorax* sp (WP_008902870.1), *Klebsiella pneumoniae* (EKB62293.1), *Acinetobacter* sp (CAC80891.1). ^cThese primers also amplify *merA2* gene with an amplicon 1063bp. As they present several nucleotide failures in the homology with *merA2* sequence (ex: *E. faecium* EnGen0019 strain E0680 GenBank accession no. AHWN01000031.1) we chose to search *merA2* gene with *merA2* primers pair. With exception of two isolates only amplifying with *merA2* primer pair, both combinations amplified the *merA2* gene.

Table 2. Epidemiological background, antibiotic resistance and copper tolerance genes of *Enterococcus* carrying *merA* genes.

<i>merA</i> genes	Species	ST ^a	PFGE (subtypes) ^b	No. Isolates	Source/sample	Date	Antibiotic resistance ^c	Antibiotic resistance genes ^c	Cu tolerance genes ^c	Transfer of <i>merA</i> genes ^d
<i>merA2+merA3</i>	<i>E. faecalis</i>	159	N	1	Hospital A/blood	1999	TET, ERY, GEN, VAN, CIP	<i>tet(M)</i> , <i>erm(B)</i> , <i>aac(6')-Ie-aph(2'')-Ia</i> , <i>vanA</i>	-	(-) ^{e,f}
	<i>E. faecium</i>	108	SN248	1	Piggery II/Manure	2006	TET, ERY, STR, GEN, CHL, NIT	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aac(6')-Ie-aph(2'')-Ia</i> , <i>aadE</i>	<i>tcrB + cueO</i>	(+) ⁱ
		185	A5	3	Piggery IV/Soil	2007	TET, VAN, [NIT]	<i>tet(M)</i> , <i>vanA</i>	<i>tcrB + [cueO]</i>	(+) ⁱ
		393	SN208	3	Piggery II/Manure, waste lagoon	2006	TET ⁴ , ERY ² , STR ³ , [GEN], AMP ⁴ , [CIP], NIT	<i>tet(M)</i> ³ , <i>tet(L)</i> , <i>erm(B)</i> , <i>bbp5</i> ⁶	<i>tcrB + cueO</i>	(+) ^{gh}
		430	SN211	1	Piggery II/Dust	2007	TET, ERY, STR, AMP, CIP, NIT	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aadE</i>	<i>tcrB + cueO</i>	(+) ^{gh}
		431	SN208 (1)	1	Piggery II/Dust	2006	TET, ERY, STR, AMP, NIT	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aadE</i>	<i>tcrB + cueO</i>	(+) ^{gh}
		<i>purK9</i> [*]	SN226 (2)	3	Piggery III/faeces, antiseptic, dust	2006	TET, ERY, STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aadE</i>	<i>tcrB + [cueO]</i>	(+) ^{gh}
		846	CD	1	Healthy human/faeces	2001	TET, ERY, STR, CIP	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>	<i>tcrB + cueO</i>	(-)
		<i>purK12</i> [*]	ND	1	Healthy human/faeces	2001	TET, ERY, STR, NIT	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aadE</i>	<i>tcrB + cueO</i>	(+) ⁱ
		859	EU	1	Sewage hospital B/water	2002	TET, ERY, STR, AMP, GEN	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>aac(6')-Ie-aph(2'')-Ia</i> , <i>aadE</i>	<i>tcrB + cueO</i>	(+) ⁱ
		<i>purK17</i> [*]	T56	2	Aquaculture B/feed	2012	TET, ERY	<i>tet(M)</i>	<i>tcrB + cueO</i>	(-)
<i>merA3</i>	<i>E. faecium</i>	<i>purK17</i> [*]	T57	1	Aquaculture B/trout	2012	-	-	<i>tcrB + cueO</i>	(+) ^h
		<i>purK9</i> [*]	CJ	1	Healthy human/faeces	2001	TET, ERY, STR	<i>tet(M)</i> , <i>erm(B)</i> , <i>aadE</i>	<i>tcrB + cueO</i>	(+) ^h
		ND	SN232	1	Piggery II/Dust, water environment	2007	TET, ERY, STR, CIP, GEN	<i>tet(L)</i> , <i>aac(6')-Ie-aph(2'')-Ia</i>	<i>tcrB + cueO</i>	(-)
		ND	ND	2	Piggery II/Medicine, feed	2006-2007	TET, ERY, STR, [CIP], AMP, [CIP], NIT	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , [aac(6')-Ie-aph(2'')-Ia]	<i>tcrB + cueO</i>	(-)
		ND	ND	2	Piggery III/faeces, manure	2006	TET, ERY, STR, [GEN], [NIT]	[<i>tet(M)</i>], <i>tet(L)</i> , [<i>erm(B)</i>], [aac(6')-Ie-aph(2'')-Ia]	<i>tcrB + cueO</i>	(-)
		X [*]	CH	1	Healthy human/faeces	2001	TET, ERY, STR, CHL	<i>tet(M)</i> , <i>aadE</i>	-	(-)
		ND	ND	1	Piggery I/Waste lagoon	2006	TET, ERY, AMP, CIP	<i>tet(L)</i> , <i>erm(B)</i>	<i>tcrB + cueO</i>	(-)
		ND	ND	1	Piggery II/Waste lagoon	2006	TET	<i>tet(M)</i> , <i>tet(L)</i>	-	(-)
		Enterococcus sp	ND	1	Piggery II/Waste lagoon	2006	TET, ERY, STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>aadE</i>	<i>tcrB + cueO</i>	(-)
		<i>purK_new</i>	CK	1	Healthy human/faeces	2001	TET, ERY, STR, CHL	<i>erm(B)</i>	-	(-) ^d
<i>merA3</i>	<i>E. faecium</i>	18	78	1	Hospital A/catheter	2001	ERY, VAN, AMP, CIP, CHL, NIT	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>vanA</i>	-	(-) ^d
		132	103 (1)	1	Hospital A/exudate	2003	ERY, GEN, VAN, AMP, CIP	<i>erm(B)</i> , <i>vanA</i> , <i>aac(6')-Ie-aph(2'')-Ia</i>	-	(-) ^d
		<i>purK2</i> [*]	T53	1	Aquaculture B/trout	2012	TET, ERY	<i>erm(B)</i> , <i>tet(L)</i>	-	(-)
		<i>purK17</i> [*]	T54	2	Supermarket IV/trout	2012	TET, ERY, [CIP]	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>	<i>cueO</i>	(-)
		<i>purK8</i> [*]	CL	1	Healthy human/faeces	2001	ERY	<i>erm(B)</i>	<i>cueO</i>	(-)
		X [*]	EX	1	Sewage Hospital C/water	2001	ERY, GEN, CIP	<i>aac(6')-Ie-aph(2'')-Ia</i>	-	(-) ^{gh}
		X [*]	HU	1	Hospital E/urine	2001	TET, ERY, STR, GEN, CHL, CIP	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , [aac(6')-Ie-aph(2'')-Ia], <i>aadE</i>	-	(-) ^{gh}
		X [*]	HY	1	Hospital E/urine	2001	TET, ERY, STR	<i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i> , [aac(6')-Ie-aph(2'')-Ia], <i>aadE</i>	-	(-) ^{gh}
		ND	HZ	1	Hospital E/urine	2001	ERY, GEN	<i>tet(M)</i> , <i>erm(B)</i> , [aac(6')-Ie-aph(2'')-Ia]	-	(-)
		ND	ND	1	Hospital E/ blood	2001	TET, ERY, STR, GEN, CHL, CIP	<i>tet(L)</i> , <i>erm(B)</i> , [aac(6')-Ie-aph(2'')-Ia], <i>aadE</i>	-	(-) ^{gh}
<i>E. gallinarum</i>	<i>E. casseliflavus</i>	ND	ND	3	Hospital E/urine, unknown	2001	ERY, STR, [GEN], CHL, CIP	[<i>tet(M)</i>], [<i>tet(L)</i>], [<i>erm(B)</i>], [aac(6')-Ie-aph(2'')-Ia], <i>aadE</i>	-	(-)
		ND	ND	1	Healthy human/faeces	2001	TET, ERY, GEN, CHL, CIP	<i>tet(M)</i> , <i>erm(B)</i> , [aac(6')-Ie-aph(2'')-Ia], <i>vanC1</i>	-	(-)
		ND	ND	1	Piggery II/Manure	2006	TET, ERY, STR	<i>tet(M)</i> , <i>varC2</i>	<i>tcrB</i>	(-)
		Enterococcus spp	ND	1	Sewage hospital B/water	2001	ERY, GEN	<i>erm(B)</i> , [aac(6')-Ie-aph(2'')-Ia]	-	(-)

^a Abbreviations: ST, sequence type; ND-not determined; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; STR, high level of resistance (HLR) to streptomycin; NIT, nitrofurantoin; CHL, chloramphenicol; GEN, HLR to gentamicin; AMP, ampicillin; VAN, vancomycin. ^bEach different PFGE number or letter represent a different clone. ^cUnderlined antibiotic resistance phenotypes and antibiotic resistance or copper tolerance genotypes were also detected in transconjugants. ^d(+) Positive transfer of *merA* gene; (-) negative transfer of *merA* gene. ^enegative transfer of *merA* gene, although transconjugants carrying antibiotic resistance genes were obtained. ^fTranconjugants were recovered from Brain Heart Infusion (BHI) supplemented with rifampicin (RIF), fusidic acid (FA) and vancomycin. ^gTranconjugants were recovered from BHI supplemented with RIF, FA and ampicillin. ^hTranconjugants were recovered from BHI supplemented with RIF, FA and tetracycline. ⁱTranconjugants were recovered from BHI supplemented with RIF, FA and erythromycin. ^jTranconjugants were recovered from BHI supplemented with RIF, FA and CuSO₄. ^kThe ST of these isolates could not be concluded. For the *E. faecium* we included the result of *purK* allele, showing that these isolates do not belong to high-risk clones, usually presenting the allele 1 of this gene. For the *E. faecalis* the combination of the alleles types of *gdh*, *gyd*, *xpt*, *yqil* e *aroE* was different among them and thus different numbers of ST will be attributed.

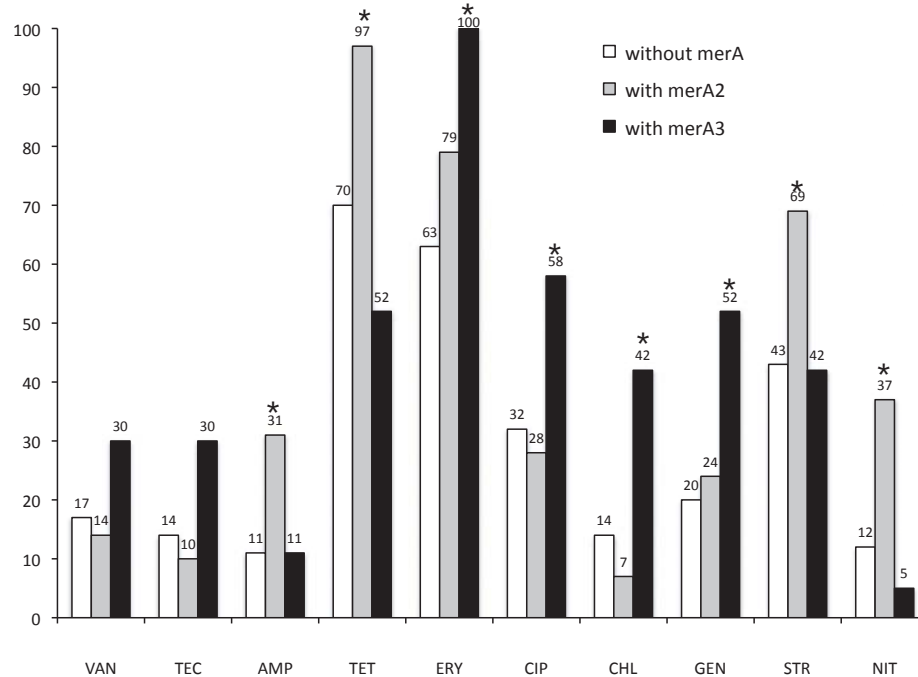


Figure 1. Distribution of antibiotic resistance among *Enterococcus* with *merA2* (n=29), *merA3* (n=19) and without *merA* genes (n=871). * p<0.05-Fisher exact test was used to compare antibiotic prevalence rates of *Enterococcus* carrying a specific *merA* with those without these genes.

Abbreviations: VAN, vancomycin; TEC, teicoplanin; AMP, ampicillin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; CHL, chloramphenicol; GEN, high concentrations of gentamicin; STR, high concentrations of streptomycin; NIT, nitrofurantoin.

3.3. Modularização de plataformas genéticas móveis portadoras de genes que codificam para tolerância a metais, compostos de amônio quaternário e/ou resistência a antibióticos

Publicações, artigos submetidos para publicação e em preparação:

3.3.1. *Antibiotic resistance, copper and mercury tolerance genes are carried by RepA_N pLG1-like megaplasms among Enterococcus from several origins and clones.*

3.3.2. *First description of a multidrug-resistant ST17 Enterococcus faecium carrying a transferable Inc18-like::qacZ plasmid*

3.3.3. *Different genetic supports for the tet(S) gene in Enterococci.*

Este capítulo engloba três estudos relacionados com a caracterização molecular de plataformas genéticas envolvidas na transferência horizontal de genes que codificam para tolerância a metais, compostos de amônio quaternário e/ou resistência a antibióticos. Nos três estudos é evidenciada a plasticidade genética de *Enterococcus* spp., portador de plataformas (plasmídeos e elementos de integração cromossomal) frequentemente modulares e envolvendo diversas sequências de inserção, nomeadamente *IS1216*. Os isolados integrados no estudo do ponto 3.3.1 foram selecionados dos trabalhos incluídos na secção anterior (3.2). Neste estudo foi efetuada a caracterização de plataformas portadoras de genes de resistência a antibióticos e de tolerância a vários metais, nomeadamente ao cobre e mercúrio. O estudo do ponto 3.3.2 avalia não só a dispersão de diferentes genes que codificam para tolerância a compostos de amônio quaternário, como caracteriza o elemento genético portador de *qacZ*. No estudo do ponto 3.3.3 foi efetuada a caracterização de elementos genéticos portadores de genes que codificam para a resistência à tetraciclina [*tet(S)*], sendo que o plasmídeo de um dos isolados deste estudo [portador de plataforma *tet(S)* tipo G] tem co-localizado o gene *qacZ*, descrito no estudo do ponto 3.3.2. Neste último estudo, Eduarda Silveira participou na identificação da localização cromossomal ou plasmídica de *tet(S)* e na avaliação da transferência horizontal de Tn6000.

Antibiotic resistance, copper and mercury tolerance genes are carried by RepA_N pLG1-like megaplasms among *Enterococcus* from several origins and clones

Eduarda Silveira¹, Mariana Barros¹, Ana R. Freitas¹, Patrícia Antunes², Teresa M.

Coque^{3,4,5}, Luísa Peixe¹, Carla Novais^{1*}

¹REQUIMTE. Laboratório de Microbiologia. Faculdade de Farmácia. Universidade do Porto, Portugal;

²Faculdade de Ciências da Nutrição e Alimentação. Universidade do Porto, Portugal; ³Servicio de Microbiologia. Hospital Ramón y Cajal. Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain; ⁴Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBER-ESP), Madrid, Spain; ⁵Unidad de Resistencia a Antibióticos y Virulencia Bacteriana asociada al Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain.

Running Title: Large plasmids carry copper tolerance genes in enterococci.

Keywords: Copper, Mercury, megaplasms, modular platforms, insertion sequences

Corresponding author: Carla Novais, Ph.D. Laboratório de Microbiologia. Faculdade de Farmácia, Universidade do Porto. Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal. Phone: +351 220428588; FAX: +351 226 093 390; E-mail: casilva@ff.up.pt

Parte deste trabalho foi apresentado no *22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)*, Londres, Reino Unido em Abril de 2012. Parte do trabalho foi também publicado como suplemento da revista científica *Clinical Microbiology and Infection* (vol.18, suplemento S3:447, P1631).

Artigo em preparação

ABSTRACT

Objectives: Widespread *Enterococcus* clones are associated with cumulative acquisition of genes encoding adaptive traits as resistance to antibiotics (AB^R) and tolerance to metals (T). Copper (Cu) and mercury (Hg) are widespread in nature, mostly by anthropogenic activities, and could contribute for maintenance of AB^R in different niches. We characterized genetic elements carrying Cu^T, Hg^T and AB^R genes in strains from several origins.

Methods: *E. faecium*-Efm (n=21; CC5, CC9, ST18 and ST78 clonal lineages as well as ST108, ST434, ST846, ST859) and *E. faecalis*-Efls (n=5; CC2, ST288, ST445, ST159) from humans (n=10), animal (n=12), hospital sewage (n=2) and food (n=2) were analyzed (1997-2010). They carried genes coding for reduced susceptibility to Cu (*tcrB* and/or *cueO*-n=23; MIC_{CuSO₄}=24-28mM), Hg (*merA*-n=9) and AB [*tet(M)*-n=12; *erm(B)*-n=19; *vanA*-n=6]. Wild type strains and/or transconjugants carrying *tcrB*, *cueO* or *merA* were further analyzed. PCR, S1/I-CeuI-PFGE and southern blotting hybridization were used to search AB^R, Cu/Hg^T and plasmid replicase/relaxases genes and determine their location. Cu/Hg^T genetic environment was studied by Long-PCR, RFLP and sequencing. *In silico* analysis of *Enterococcus* genomes available at the GenBank database carrying *tcrB/cueO/merA* genes was also performed.

Results: All *tcrB* and/or *cueO* genes were located in plasmids of Efm (120-300kb) and Efls (75-120kb), with a variable conjugation frequency (10⁻³-10⁻⁶). In strains carrying both genes (n=19/23) they were co-located in the same plasmid (Efm and Efls), which in six isolates, also carried *merA* (all Efm). A single Efm carried a *merA* in the chromosome and an Efls two different *merA* alleles (76% amino acid identity). Co-localized with *tcrB/cueO/merA* genes in the same plasmid were *erm(B)* (n=14), *tet(M)* (n=8), *tet(L)* (n=6), *vanA* (n=3), rep_{20/pLG1} (n=17, all Efm), rep_{1/pIP501} (n=3), rep_{2/pRE25/pEF1} (n=2) and/or rel_{pEF1} (n=9, all Efm). The region containing *tcrB*, *cueO* and/or *merA* was flanked by IS (e.g. ISL3 family, ISEfm2, IS1216) in available sequences (this study and genomes in gene databases), occasionally containing cadmium or arsenic operons. Moreover, the *tcrB/cueO/merA* platforms were more commonly found among Efm (32%, n=85/264 available genomes) than among other species (2%, n=7/363; Efls, *Enterococcus spp.*). The rep_{pLG1} was observed in 82% of these genomes (73 Efm, 2 *E. durans*), although a direct link (same contig) could not be established with *tcrB/cueO/merA* genes.

Conclusions: Clonally unrelated *Enterococcus* of different niches often carry transferable megaplasmids (rep_{20/pLG1}) containing genes encoding resistance to AB^R and metals. The intensive use of AB (e.g., tetracyclines, macrolides) and Cu in animal production, and the environmental pollution by Hg might facilitate persistence and spread of multiresistant *Enterococcus* in community and, indirectly, the evolution of genetic elements by recombinatorial events, involving modules of genes potentially abundant in local metagenomes.

INTRODUCTION

Enterococcus spp. are widely spread in nature and colonize different hosts (e.g., human, animal) (Werner *et al.*, 2013). In the last decades, they were recognized as important hospital-acquired pathogens at global scale, particularly multidrug resistant *E. faecium* and *E. faecalis* clonal lineages (e.g. *E. faecium*-ST17/ST18/ST78; *E. faecalis*-CC2) (Willems *et al.*, 2009; Willems *et al.*, 2012). Genomic comparative studies on both species revealed the occurrence of diverse genetic content (e.g., related to antibiotic resistance, virulence, colonization) among specific groups of strains, illustrating the relevance of lateral gene transfer (LGT) to this diversity and enhanced survival to ecological differences (McBride *et al.*, 2007; Wiedenbeck & Cohan, 2011; Coque *et al.*, 2012; Lebreton *et al.*, 2013; Werner *et al.*, 2013). LGT among *Enterococcus* (within members of this genus or with other genera) is favoured by their inclusion in specialized exchange communities (GEC; *dynamic interplay between groups of bacterial species, genetic elements and environments*) frequent occurring in ecosystems (e.g. human/animal gut, food, clinical and animal production environment) under diverse selective pressures (Coque *et al.*, 2012). Thus, each *Enterococcus* species are 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 (Wiedenbeck & Cohan, 2011; Coque *et al.*, 2012).

The characterization of mobile genetic elements among *Enterococcus* have been mainly focused on those carrying relevant antibiotic resistance genes (Freitas *et al.*, 2013, Werner *et al.*, 2013), but other transferable adaptive features potentially contributing to endurance of specific clonal lineages or antibiotic resistance patterns (e.g., in clinical or animal production environment) still remain understudied (Hasman *et al.*, 2002; Oho *et al.*, 2004; Laverde *et al.*, 2011). Previous works from our and other groups unveiled the presence of diverse genes coding for copper (Cu; *tcpB*-efflux pump; *cueO*-multicopper oxidase) and mercury (Hg; diverse *merA* alleles-mercuric reductase) tolerance among *Enterococcus* from different origins (Hasman *et al.*, 2002; Ojo *et al.*, 2004; Silveira *et al.*, 2013; Silveira *et al.*, manuscript in preparation). However, further characterization of the genetic elements associated with their often mobilization along with antibiotic resistance was not achieved. Cu and Hg are widely spread in nature, mainly due to anthropogenic activities (e.g., Cu-animal feed; Hg-industrial processes) and the selective pressure associated with their presence could have an impact in the maintenance of multidrug resistant *Enterococcus*, and indirectly, in their genomic modulation and evolution. In this context, we aimed to characterize the diversity and genetic plasticity of transferable elements carrying genes coding for antibiotic resistance and/or tolerance to Cu (*tcpB*, *cueO*) and Hg (two *merA* alleles) in *Enterococcus* from different origins. The analysis of

available genomes of *Enterococcus* and other bacterial genera of the same exchange communities was also performed to evaluate the occurrence and the variability of genetic regions containing *tcrB*, *cueO* and/or *merA* genes in a wider range of genetic backgrounds.

METHODS

Bacterial isolates and epidemiological background. Twenty-one *E. faecium* and 5 *E. faecalis* were selected from a large collection previously screened for the presence of different Cu and Hg tolerance genes (Silveira *et al.*, 2013; Silveira *et al.*, manuscript in preparation). Isolates were selected based on the presence of *tcrB* (efflux pump ATPase), *cueO* (multicopper oxidase) and *merA* (mercuric reductase) genes, origin, antibiotic resistance profile and clonality (Table 1). They were recovered from different biological products of hospitalized patients (n=6), hospital sewage water (n=2), healthy human faeces (n=4), poultry carcasses (n=3), swine and piggeries environment (n=9) and ready-to-eat salads (n=2), during 1997-2010. They corresponded to different PFGE types (19 of *E. faecium*, 4 in *E. faecalis*) and sequence types (ST, 14 of *E. faecium* and 4 of *E. faecalis*), representing widespread hospital clonal lineages fitting in major BAPS groups [*E. faecium* clonal lineage 18 (ST18, ST132, ST640), 78 (ST393); *E. faecalis* CC2 (ST49)], widespread in the animal setting [*E. faecium* CC5 (ST5, ST6, ST150)], or described occasionally [*E. faecium* CC9 (ST421), CC22 (ST32), ST108, ST434, ST846, ST859; and *E. faecalis* ST159, ST288, ST445] (Willems *et al.*, 2009; Willems *et al.*, 2012). Eighteen transconjugants carrying *tcrB*, *cueO* and/or *merA* were obtained from the 26 *Enterococcus* [recipient strains *E. faecium* BM4105RF (n=17) or *E. faecalis* JH2-2 (n=1); plasmid free] in previous studies (Table 1) (Silveira *et al.*, 2013; Silveira *et al.*, manuscript in preparation). In these cases, the study of mobile genetic elements was performed in the transconjugants instead of wild strains to facilitate genetic analysis. Transconjugants expressed tolerance to CuSO₄ (24-28mM) comparing with the recipient *E. faecium* BM4105RF (8-12mM) (Table 1). Acquired tolerance to CuSO₄ was not possible to evaluate for the transconjugant obtained with the recipient *E. faecalis* JH2-2, as this strain has already high levels of CuSO₄ tolerance (28mM). Conjugation frequency was calculated per *E. faecium* BM4105RF or *E. faecalis* JH2-2 recipient cells. The remaining 8 wild strains not able to transfer any metal gene were also included (Table 1).

Localization of metal tolerance and antibiotic resistance genes. Plasmid or chromosomal localization of genes coding for copper (*tcrB*, *cueO*) or mercury (*merA2* and

merA3; the numbers 2 and 3 were previously designated to differentiate two *merA* alleles with 76% of amino acid identity; Silveira *et al.*, manuscript in preparation) tolerance as well as for resistance to glycopeptides (*vanA*), tetracyclines [*tet(M)*, *tet(L)*] or macrolides [*erm(B)*] was studied by hybridization of PFGE S1 or I-CeuI digested DNA using standard procedures (Sambrook *et al.*, 1989; Barton *et al.*, 1995). PFGE was performed as described (switch time of 5s to 25s for 6h followed for 30s to 45s for 18h for S1-PFGE and 5s to 35s for 22h for I-CeuI-PFGE) (Kaufman *et al.*, 1998). The *tcrB*, *cueO*, *merA2*, *merA3* and antibiotic resistance gene probes included in hybridization assays were generated by PCR using positive controls (strains from our collection which genes were sequenced) as template DNA. Labelling and detection were carried out using the Gene Images AlkPhos Direct Labelling system kit, following the manufacturers' instructions (Amersham GN/GE Healthcare Life Sciences UK Limited). Plasmid size was estimated using the DNA marker Low Range PFGE Marker (New England Biolabs, Inc., Ipswich, USA) and the Image Lab (TM) Software (BIO-RAD).

Plasmid analysis.

Classification of plasmids was based on the presence of specific modules for replication (rep-initiator proteins) and mobilization (relaxases). Relaxases (rel) were sought by a multiplex-PCR typing method which differentiates relaxases of the MOBQ, MOBP, MOBC and MOBV families related to 27 known plasmids (Garcillan-Barcia *et al.*, 2009; Clewell *et al.*, 2013, Freitas *et al.*, 2013; Francia MV, unpublished data). Replication initiator proteins (rep) were investigated by amplification of 24 replicons, which allows discriminating among DNA sequences from more than 100 published Gram-positive plasmids (Jensen *et al.*, 2010; Clewell *et al.*, 2013, Freitas *et al.*, 2013). Designation of rep and rel sequences pointed out the plasmid type in which they were initially identified, as well as the numeric nomenclature originally used by Jensen *et al.* (2010) or Freitas *et al.* (2013). Genomic location of the more prevalent rel/rep sequences found by PCR was determined by hybridization with specific probes in the same membranes used to evaluate metal tolerance and antibiotic resistance genes localization. Specific probes were created by PCR from DNA of reference plasmids.

Characterization of *tcrB*, *cueO* and *merA* genetic environment. The characterization of *tcrB*, *cueO* or *merA* genetic environment was performed in isolates (n=9) representative of different sources, species, clones, carrying metal tolerance genes in different locations (plasmids with variable sizes, 75-300kb; chromosome) and with different plasmid contents (antibiotic resistance, metal tolerance, rep and rel genes) (Table 1, Table 2). A PCR mapping was performed using primers described in Table 2, which design was based in

genetic platforms carrying *tcxB*, *cueO* and/or *merA* available at the GenBank database (accession numbers described in Table 2). PCR conditions were 1mM of each primer, 2.5mM MgCl₂, 1x of reaction buffer, 0.4 mM of each deoxynucleoside triphosphate, 2.5U Takara LA Taq polymerase (Takara™ Bio Inc., Shiga, Japan). The amplification conditions were 25 cycles of 30s at 96°C, 1 min at 55-60°C, 1-5 min at 72°C; 1 cycle 10 min at 72°C. The diversity of amplicons was established through comparison of PCR amplicons sizes, RFLP patterns (Taq I, AluI) and further sequencing of representative fragments.

Bioinformatic analysis

A comparative analysis of sequences and genetic sytheny detected in this study with those available in the Genbank dababase was performed using the searching basic local sequence alignment (BLAST) tool and the CLUSTALW algorithm for multiple sequence alignment provided by the National Center for Biotechnology Information (NCBI) and the European Bioinformatic Centre (EBI), respectively.

RESULTS AND DISCUSSION

***Enterococcus* from different sources and clonal lineages carry large conjugative plasmids with genes coding for antibiotic resistance, copper or mercury tolerance.**

The *tcxB* or *cueO* genes were located in plasmids in all cases, with size range of 120-300bp for *E. faecium* and 90-120bp for *E. faecalis* (Table 1). In strains carrying both genes (n=19/26) they were co-located in the same plasmid (*E. faecium* and *E. faecalis*), which in six isolates, also harboured *merA2* (all *E. faecium*). The *merA3* gene was detected in an *E. faecium* chromosome or, along with a *merA2*, in an *E. faecalis* 75kb plasmid. Despite *tcxB*, *cueO* and/or *merA2*, plasmids of most *E. faecium* and *E. faecalis* studied also carried variable antibiotic resistance genes, which included *erm(B)* in 13 isolates, *tet(M)* in 8, *tet(L)* in 6 and *vanA* in 2. The *merA2+merA3* were co-localized with *erm(B)* and *van(A)*. In six isolates no antibiotic resistance genes were observed within the same plasmid as *tcxB*, *cueO* or *merA* genes. Plasmid analysis revealed the occurrence of several replicases and relaxases, with a higher prevalence for the rep_{20/pLG1} (n=19) followed by rep_{1/pIP501} (n=9) and/or rep_{2/pRE25/pEF1} (n=8). Other rep genes were occasionally observed: rep_{9/pAD1} (n=6), rep_{7/pK214/pT181/pUSA02} (n=4), rep_{18a/pEF418} (n=3), rep_{14a/pRI1-like} (n=3), rep_{18b/pB82} (n=1), rep_{17/pRUM} (n=1), rep_{18c/pCIZ2} (n=2) or rep_{22/pHTbeta} (n=1). The rel_{6/pEF1} was the most detected (n=13 *E. faecium*), followed by rel_{2/pCIZ2} (n=3), rep_{14a/pRI1-like} (n=1), rel_{10/pLG1} (n=1), rel_{5/pAD1} (n=1) or rel_{8/pHTbeta} (n=1). The hybridization with probes of the more

prevalent replicases (rep_{20/pLG1}, rep_{1/pIP501}, rep_{2/pRE25/pEF1}) and relaxases (rel_{6/pEF1}) obtained by PCR, showed that plasmids carrying *tcrB/cueO/merA2* genes have, in most cases, rep_{20/pLG1} (n=17; all *E. faecium*, all sources with exception of ready-to-eat salads) and rel_{6/pEF1} (n=9; all *E. faecium*). Few strains also carried rep_{1/pIP501} (n=3) and/or rep_{2/pRE25/pEF1} (n=2), and include an *E. faecalis* with *merA2+merA3* but not *tcrB/cueO*. Conjugation frequency of these plasmids carrying Cu/Hg tolerance genes ranged between 10⁻³-10⁻⁶ per recipient cell. Plasmids carrying the different metal tolerance genes were observed in *E. faecium* and *E. faecalis* from several clonal lineages, including those associated with human infections (*E. faecium*-ST18/ST78; *E. faecalis* CC2), and recovered from diverse ecological niches (human, animal, environment, food). Also, isolates from the same source carried plasmids with different contents (genes coding for resistance to antibiotics, metal tolerance, replicases and relaxases) (Table 1).

The co-localization of *tcrB* and antibiotic resistance genes [e.g. *vanA*, *erm(B)*] was previously described in plasmids of 175-194kb recovered from pig strains (Hasman *et al.*, 2002; Amachawadii *et al.*, 2011; Amachawadii *et al.*, 2013), although in these studies very few isolates were included and replicase and relaxases characterization was not performed. We showed that beside pigs, plasmids carrying Cu and antibiotic resistance genes were spread in diverse ecological niches and, occasionally, also carry *merA2* allele involved in Hg tolerance. The occurrence/transfer of metal tolerance genes with *tet* and *erm(B)* (this and other studies) (Hasman *et al.*, 2002; Amachawadii *et al.*, 2011; Silveira *et al.*, 2013) could be explained by the extensively spread of the latter by animal and human hosts (Aarestrup *et al.*, 2000; Novais *et al.*, 2013), due to the high consumption of tetracyclines and macrolides, and by their integration on transposable elements frequently involved in recombination events (Cochetti *et al.*, 2008; Roberts *et al.*, 2009). The *vanA+tcrB+cueO* occurrence in hospital sewage and nosocomial isolates could also be linked with the high rates of vancomycin resistant *Enterococcus* and, consequently, availability of *vanA* in local metagenomes within Portuguese clinical environment (ECDC, 2013; Freitas *et al.*, 2013).

E. faecium seems to be the main reservoir of *tcrB*, *cueO* and *merA2* genes (Silveira *et al.*, 2013; Silveira *et al.*, manuscript in preparation) and this specificity might be favoured by the spread of these genes by narrow host range pLG1 plasmids. The rep_{pLG1} has been associated with large plasmids of variable size carried by *E. faecium* nosocomial strains (Laverde-Gomez *et al.*, 2011; Rosvoll *et al.*, 2012; Freitas *et al.*, 2013) as well as by the globally spread ST5/CC5 *E. faecium* clone recovered from piggeries (Freitas *et al.*, 2011). Their inconsistent size, detected in this and other studies was suggested to be related to genetic rearrangements (Freitas *et al.*, 2011; Rosvoll *et al.*, 2012), justifying the variable content found, including other replicases (rep1 and/or rep2 in two isolates). These

plasmids can enclose genes coding for virulence (Laverde-Gomez *et al.*, 2011), antibiotic resistance (Laverde-Gomez *et al.*, 2011; Freitas *et al.*, 2011; Rosvoll *et al.*, 2012) carbohydrate metabolism (Laverde-Gomez *et al.*, 2011), pili (Laverde-Gomez *et al.*, 2011) and/or metal Cu tolerance genes (Cu and Cadmium-Cd) (Laverde-Gomez *et al.*, 2011; Freitas *et al.*, 2011). These features, associated with their ability to transfer, might favour survival, selection and persistence of MDR bacteria in environments with selective pressures associated with diverse compounds (e.g., in animal production setting, sewage) as well as the colonization of different hosts, both human and animal. Of remark, the few *E. faecalis* with Cu/Hg tolerance genes included in this study did not carry pLG1 replicase as well as most of isolates from other species of our collection (e.g., *E. gallinarum*, *E. casseliflavus*) (data not shown), species with lower prevalence of *tcrB*, *cueO* or *merA2* tolerance genes (Silveira *et al.*, 2013; Silveira *et al.*, manuscript in preparation).

In an attempt to evaluate the spread of *tcrB*, *cueO*, *merA2* and rep_{pLG1} in more than 500 enterococcal genomes, a GenBank search of these genes was performed. This analysis revealed that *tcrB*, *cueO* and/or *merA2* were are mainly carried by *E. faecium* (32%, n=85/264 available genomes) than in other species (2%, n=7/363; *E. faecalis*, *E. durans*, *E. italicus*, *E. casseliflavus*, *E. hirae*, *Enterococcus* sp.) corroborating previous local epidemiological results (Silveira *et al.*, 2013; Silveira *et al.*, manuscript in preparation). The rep_{pLG1} was observed in 82% of these genomes (73 *E. faecium*, 2 *E. durans*). Although in most of these isolates it was not possible to establish a direct link between Cu/Hg tolerance genes and rep_{pLG1} in the same contig, their high association rate along with our hybridization data, suggest that pLG1 plasmids could be the main spreaders of *tcrB*, *cueO* and/or *merA2* genes among *E. faecium* and contribute for dissemination of antibiotic resistance in different ecological niches.

Copper and mercury tolerance genes are inserted in modular platforms mediated by insertion sequences (IS) containing diverse genes coding for tolerance to different metals and shared by several members of the *Firmicutes* phylum. Variable genetic regions containing metal tolerance genes were detected among the 9 *Enterococcus* studied. In order to facilitate the description and discussion of our results and sequence data found in genetic databases we will divide the genetic platforms in three parts, accordingly with Figure 1 and 2. Part 1 corresponds to the right side and part 2 to the left side of *cueO* gene (Figure 1). The part 3 is dedicated to *merA3* genetic regions, detected in different isolates than those carrying *tcrB* and *cueO* (Figure 2). The sequences available at the Genbank database chosen for the *in silico* analysis were selected based on the possibility to connect Part1 to Part2, or Part 3 to arsenic operon.

a) Part 1:

On isolates of this study carrying *tcrB* and *cueO* (n=6) a region of ~11.4 kb connecting both genes was amplified in all cases, although four RFLP types were observed (Table 2). The CuSO₄ tolerance phenotype demonstrated by these bacteria (24-28mM) suggests that one or both genes are inserted in functional operons, thus constituting a possible adaptive feature of these microorganisms to the surrounding environment. The sequencing of this amplicon (1 isolate) and the analysis of available genomes in gene databases showed that operons containing *cueO* or *tcrB* genes are often adjacent and flanked by different ISs, suggestive of composite transposons (Figure 1). The *tcrYAZB* is usually flanked in both sides by IS6 family transposases, including IS1216 in the right side (adjacent to *tcrB* gene or after a cluster of genes; our study and available sequences) and ISS1 or other transposases in the left side, closely to the *tcrY* gene. The gene cluster where *cueO* is inserted also contains a two-component system response regulator, a two-component system histidine kinase (a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions) (Stock *et al.*, 2000; Thorsten *et al.*, 2006) and a lipoprotein of unknown function. It is flanked by transposases of ISL3 family (e.g., as IS1251) in one or both sides, ISEfm2 (mutator family; left side of *cueO*) or none (left side of *cueO*). The two first options were observed both in isolates from this study as well as gene databases. In one of the strains from this study, genes coding for death-on-curing protein (*doc* gene in figure; Fic Superfamily) and for an addiction module antidote (*ama* gene in figure; antitoxin MazE superfamily) were detected within an amplicon of ~6,4kb representing a cluster of genes positioned after *tcrB* and both genes were flanked by a resolvase and a IS1216 (Figure 1). The *doc* and *ama* genes are related to prophage maintenance system killer proteins, causing bacterial death when the plasmid is lost (Lehnher *et al.*, 1993). The search of these genes in the same enterococcal sequences carrying the *cueO* and *tcrYAZB* operons of gene databases selected for this study (Figure 1) revealed that proteins with 36 to 58% of identity and belonging to Fic Superfamily (identified as death-on-curing protein) and MazE superfamily (identified as hypothetical proteins) were present in most of them, although its distance to *tcrB* gene was variable (~10-30kb), in the cases that this was possible to determine (data not shown).

Partial sequences of *cueO* or *tcrYAZB* operons were also observed in other bacterial genera beside *Enterococcus*. The all module between the lipoprotein and *cueO* was detected in *Listeria monocytogenes* and was flanked on lipoprotein side by a IS from the ISL3 family (IS1167) (GenBank accession no. NC_022046.1; NC_022051.1), as in the case of *Enterococcus* (IS1251). Some of the genes of *tcrYAZB* operon (>90% of amino acid identity) were detected in bacteria such *Aerococcus viridans* (94-97% identity with *tcrY* and *tcrA*; ADNT01000090.1), *Streptococcus parauberis* (94% identity with *tcrB*;

ALYM01000001.1), *Streptococcus* spp. (98% identity with *tcrZ*; NC_016749.1, EDT47861.1) and *Weissella thailandensis* (93% identity with *tcrB* followed by partial *tcrA*-91% identity, *tcrY*-87% identity and a IS from IS3 family; HE575152.1).

b) Part 2:

The *cueO* left region also revealed that it is high recombinatorial with diverse IS (IS1485, IS6670, both of IS30 family, and DDE type transposases) or phage integrase. These elements seem to be related to insertion or variations of specific genetic modules (represented by coloured boxes in Figure 1), including genes coding for metal tolerance, as Hg or Cd. The module with *merA2* gene (white box of Figure 1, type A), related with Hg tolerance, was detected in our isolates (within 4,5kb amplicon) as well as available sequences. This *merA* gene had 99% of amino acid identity with different species of *Streptococcus* (GenBank accession no. EID31437.1, YP_001449687.1, WP_002933267.1, EPR93890.1, WP_003009663.1), *Dialister micraerophilus* (WP_007556353.1) and 96% with *Exiguobacterium* sp. (CAA67822.1), the latter described to have Tn5085 (Mindlin & Petrova, 2013). However, besides *merA2*, *Enterococcus*, *Streptococcus* and *Dialister* only have a regulator gene (99-100% of amino acid identity with each other and 67% with *Exiguobacterium* sp.), lacking the transport Hg genes of Tn5085. The *merA2* of all three genera sequences was flanked in the right side by a IS of ISL3 family and in the left side only by an Inverted Repeat of ISL3 (not annotated in the sequences of gene databases), suggesting a common acquisition of this genetic cluster. This organization was previously identified in *Streptococcus* (Stapleton *et al.*, 2004) and ISL3 was suggested to be part of a composite transposon involved in the transfer of this operon. In *Enterococcus merA2* negative sequences, a different *merA* (here designated as *merA5*; 68% of amino acidic identity with *merA2*) was observed along with a Cd operon, and was close to a phage integrase (BRE-C superfamily) (green box of Figure 1, types B, D and E). The module of green box (type B; Figure 1) also contained the transposon Tn1546 (glycopeptides resistance, VanA type) in some of the sequences. The *merA5* as well as its regulator were only detected in *Enterococcus*, although the Cd tolerance protein gene was also observed in *Carnobacterium* sp. flanked by IS1216 (YP_008714474.1). Furthermore, most of the modules (pink, green and blue boxes of Figure 1) contained heavy metals translocate ATPases of E1-E2 superfamily. These ATPases were suggested to be related to the mobilization of diverse metals ions including Cd and Cu (blue box of Figure 1), cadmium, cobalt, Hg, lead and zinc (green box), and potentially to zinc, Cd, Hg, lead (pink box). Two of them were also detected in other bacteria genera beside *Enterococcus*, as *Lactococcus* (WP_003706759.1), *Streptococcus* (WP_003109030.1), *Tetragenococcus* (YP_004887839.1), *Bavariicoccus*

(WP_022797163.1) (98-99% amino acid identity; ATPase of the blue box) or in *Lactobacillus* (WP_010620832.1) (99% amino acid identity; ATPase of the green box). The region between integral membrane protein and a type II restriction enzyme (grey box of Figure 1, A to F) was also present in *Listeria monocytogenes* (GenBank accession no. NC_022046.1).

c) Part 3:

In our study the gene *merA3* was observed in different isolates than those carrying the platforms described above, which was also corroborated by the sequences with this gene available at Genbank database (Figure 2). Although it has only 76% of amino acid identity with *merA2* their regulator is very alike (95% identity). The *merA3* found in a clinical *E. faecalis* from our study was integrated in a *mer* operon with a similar structure to the one previously identified in a *E. casseliflavus* (AY351675.1) from a primate isolate (Davis *et al.*, 2005) and they are both flanked by two IS of ISL3-family, one only residual. In the left side of this operon a mucin binding protein (improves gut bacterial colonization) (Huycke *et al.*, 2002) adjacent to an IS1062 (IS30 family) was identified. Other variants of this operon were observed in different sequences of *E. faecalis* (Figure 2), all flanked by the IS mentioned. Interestingly, our sequence as well as most of those analysed also had a variable region containing genes coding for arsenic (As) tolerance (Figure 2). The *merA3* reductase was further detected in *Aerococcus viridans* (99% amino acid identity; WP_003143766.1), although the other genes of the operon could not be analyzed. Also, the entire or partial *arsA*, *arsR*, *arsD* genes were detected in different *Firmicutes* (*Streptococcus* spp., *Bavariicoccus* spp., *Listeria* spp.) (WP_000781149.1; WP_022796166.1, YP_006674063.1; EPT44776.1, YP_006674061.1, WP_022796168.1; YP_006674060.1, WP_022796169.1). In two of *E. faecalis* included in this study the genetic environment of *merA3* could not be determined as PCR reactions connecting *merA3* to other expected adjacent genes were negative. In these cases *merA3* could be a residual gene of *merA* operon or be inserted in an operon with a different genetic organization.

Our experimental data and analysis of sequences available at the Genbank database suggest that *tcrB*, *cueO* and *merA* genes carried by *Enterococcus* and related to Cu and Hg tolerance, are located in genetic regions often carrying a diversity of further genetic determinants related to tolerance to other metals, as Cd and As. The detection of common modules (here considered common gene clusters) or at least genes (which themselves could be considered a module) in different *Enterococcus* strains as well as bacteria from diverse genera of *Firmicutes* phylum, points out for the importance of horizontal transfer among bacteria from the same genetic exchange communities, which can share similar lifestyles, e.g., face similar chemical challenges (e.g., metals). The possibility of long term

maintenance of pLG1 plasmids due to the presence of prophage maintenance killer systems associated with their potential to transfer, might increase their opportunity to recruit more available modules in local metagenomes, namely by homologous recombination associated with the large amount of IS present (Baquero, 2008). This increased recombination could also be favoured by the presence of diverse selective pressures, as some studies on *Cupriavidus metallidurans* and *Pseudomonas putida* have shown that exposure to heavy metals could increase the expression of transposases genes (Baquero, 2008; Haritha *et al.*, 2009; Mijndendonckx *et al.*, 2010).

However, the detection of common modules in different sequences associated with partial IS (not functional for transposition) could indicate stabilization or domestication of clusters of genes via the rearrangement of these mobile elements within the plasmid. To understand the functionality of the metal tolerance genes within these modules, beside those coding for Cu tolerance to which we have phenotype results, would be relevant to determine if they still constitute adaptive features of these bacteria to surrounding environments or just fallen into the genome under a variety of circumstances, namely by invading phages or transposons (Wiedenbeck *et al.*, 2011). For example *merA2* or *merA5* gene clusters do not carry the Hg transporter genes classical described for Hg operons (Mindlin *et al.*, 2013). Nevertheless, Stapleton *et al.* (2004) identified functional operons lacking these genes in *Streptococcus* (94% identity with *merA2*- EFN94808.1; 99% identity with *merR*-EFN94807.1), but only conferring 2- to 4- fold decrease to Hg susceptibility compared to *merA* negative isolates, which still would be beneficial under appropriated circumstances. Phenotype studies would also be necessary for cadmium and arsenic operons, the latter variable within the sequences analysed.

CONCLUDING REMARKS

In summary, pLG1-like plasmids seem to contribute to the spread of antibiotic resistance genes (e.g., tetracycline, erythromycin or vancomycin) in *E. faecium*, while in *E. faecalis* they are carried by other genetic platforms. Genes coding for tolerance to different metals are located in highly recombined genetic regions, within modular structures, as described for other bacterial genera both in plasmids and chromosome (Siezen *et al.*, 2005; Davies *et al.*, 2009; Houdt *et al.*, 2009; Gomez-Sanz *et al.*, 2013). If all the operons coding for metal tolerance are functional, the use or presence of different compounds (metals as Cu and Hg or antibiotics) could be contributing for the selection and maintenance of these genetic elements among *Enterococcus*, favouring their opportunity to recombine and capture clusters of genes involved in different adaptive features (e.g., antibiotic resistance, metal tolerance) to the surrounding environments or to the host (e.g., pili, virulence factors

as described in full pLG1 sequence) (Laverde-Gomez *et al.*, 2011). More studies are needed to characterize the content of pLG1 plasmids and their relevance in the ecology and pathogenicity of *Enterococcus*.

ACKNOWLEDGEMENTS

This project was funded by research grants from the Fundação para a Ciência e Tecnologia (PTDC/AAC-AMB/103386/2008; grant no. PEst-C/EQB/LA0006/2011) and Investigação Jovem da Universidade do Porto/Santander Totta (PP_IJUP2011-71). Eduarda Silveira is supported by a Ph.D. fellowship of the Fundação para a Ciência e Tecnologia (grant number SFRH/BD/63955/2009; POPH-QREN). Work in Teresa M. Coque lab is supported by grants from the European Union (7FPEU-HEALTH-F3-2011-282004_EvoTAR), Ministry of Economy and Competitiveness of Spain-Instituto de Salud Carlos III (PI12/01581).

REFERENCES

1. **Aarestrup FM, Agerso Y, Gerner-Smidt P, Madsen M, Jensen LB.** 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn Microbiol Infect Dis.* 37(2):127-137
2. **Amachawadi RG, Scott HM, Alvarado CA, Mainini TR, Vinasco J, Drouillard JS, Nagaraja TG.** 2013. Occurrence of the transferable copper resistance gene *tcrB* among fecal enterococci of U.S. feedlot cattle fed copper-supplemented diets. *Appl Environ Microbiol.* 79:4369-4375.
3. **Amachawadi RG, Shelton NW, Shi X, Vinasco J, Dritz SS, Tokach MD, Nelssen JL, Scott HM, Nagaraja TG.** 2011. Selection of fecal enterococci exhibiting *tcrB*-mediated copper resistance in pigs fed diets supplemented with copper. *Appl Environ Microbiol.* 77:5597-5603
4. **Baquero F.** 2008. Modularization and evolvability in antibiotic resistance. In: Baquero F., Nombela C., Cassell G.H., Gutierrez-Fuentes (ed.), *Evolutionary Biology of bacterial and Fungal Pathogens*. ASM Press, Whashington DC.
5. **Barton BM, Harding GP, Zuccarelli AJ.** 1995. A general method for detecting and sizing large plasmids. *Anal Biochem.* 226:235-240.
6. **Clewell DB, Weaver KE, Dunny GM, Coque TM, Francia MV, Hayes F.** 2013. Extrachromosomal and Mobile Elements in Enterococci. *Transmission, Maintenance, and*

Epidemiology. In: Clewell DB, Shankar N, Ike Y, and Gilmore M (ed). The *Enterococci*: Pathogenesis, Molecular Biology and Antibiotic Resistance. In revision.

7. **Cochetti I, Tili E, Mingoia M, Varaldo PE, Montanari MP.** 2008. *erm(B)*-carrying elements in tetracycline-resistant pneumococci and correspondence between Tn1545 and Tn6003. *Antimicrob Agents Chemother.* **52**:1285-90
8. **Coque T M, Freitas A R, Novais C, Peixe L, Baquero F.** 2012. Mobile Genetic Elements and Lateral Genetic Transfer in Enterococci. In: Teresa Semedo-Lemsaddek (ed.), *Enterococcus and Safety*. Nova Science Publishers, Incorporated.
9. **Davies MR, Shera J, Van Domselaar GH, Sriprakash KS, McMillan DJ.** 2009. A novel integrative conjugative element mediates genetic transfer from group G *Streptococcus* to other {beta}-hemolytic *Streptococci*. *J Bacteriol.* **191**:2257-2265.
10. **Davis IJ, Roberts AP, Ready D, Richards H, Wilson M, Mullany P.** 2005. Linkage of a novel mercury resistance operon with streptomycin resistance on a conjugative plasmid in *Enterococcus faecium*. *Plasmid.* **54**:26-38.
11. **European Centre for Disease Prevention and Control (ECDC).** 2013. Antimicrobial resistance surveillance in Europe 2012. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm: ECDC; 2013.
12. **Freitas AR, Coque TM, Novais C, Hammerum AM, Lester CH, Zervos MJ, Donabedian S, Jensen LB, Francia MV, Baquero F, Peixe L.** 2011. Human and swine hosts share vancomycin-resistant *Enterococcus faecium* CC17 and CC5 and *Enterococcus faecalis* CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. *J Clin Microbiol.* **49**:925-31.
13. **Freitas AR, Novais C, Tedim AP, Francia MV, Baquero F, Peixe L, Coque TM.** 2013. Microevolutionary events involving narrow host plasmids influences local fixation of vancomycin-resistance in *Enterococcus* populations. *PLoS One.* **8**(3):e60589.
14. **Garcillan-Barcia MP, Francia MV, de la Cruz F.** 2009. The diversity of conjugative relaxases and its application in plasmid classification. *FEMS Microbiol Rev* **33**: 657-687.
15. **Gómez-Sanz E, Kadlec K, Feßler AT, Zarazaga M, Torres C, Schwarz S.** 2013. Novel *erm(T)*-carrying multiresistance plasmids from porcine and human isolates of methicillin-resistant *Staphylococcus aureus* ST398 that also harbor cadmium and copper resistance determinants. *Antimicrob Agents Chemother.* **57**:3275-3282.
16. **Haritha A, Sagar KP, Tiwari A, Kiranmayi P, Rodrigues A, Mohan PM, Singh SS.** 2009. MrdH, a novel metal resistance determinant of *Pseudomonas putida* KT2440, is flanked by metal-inducible mobile genetic elements. *J Bacteriol.* **191**:5976-5987.
17. **Hasman H, Aarestrup FM.** 2002. *tcrB*, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. *Antimicrob Agents Chemother.* **46**:1410-1416.

18. **Hasman H, Kempf I, Chidaine B, Cariolet R, Ersbøll AK, Houe H, Bruun Hansen HC, Aarestrup FM.** 2006. Copper resistance in *Enterococcus faecium*, mediated by the *tcrB* gene, is selected by supplementation of pig feed with copper sulfate. *Appl Environ Microbiol.* **72**:5784-5789.
19. **Huycke M. M.** 2002. Physiology of *Enterococci*. In M. S. Gilmore, D. B. Clewell, P. Couvalin, G. M. Dunny, BE Murray, and L. B. Rice (ed.), *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. ASM Press, Washington, D. C.
20. **Jensen LB, Garcia-Migura L, Valenzuela AJ, Løhr M, Hasman H, Aarestrup FM.** 2010. A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods* **80**:25-43.
21. **Kaufmann ME.** 1998. Pulsed-Field Gel Electrophoresis. In: Woodford N and Johnson AP, editors. *Molecular Bacteriology: protocols and clinical applications*. Humana Press: Inc Totowa NJ.
- 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 *hylEfm* genomic island in hospital *Enterococcus faecium* isolates. *Int J Med Microbiol.* **301**:165-75.
22. **Lebreton F, van Schaik W, Manson McGuire A, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJ, Earl AM, Gilmore MS.** 2013. Emergence of Epidemic Multidrug-Resistant *Enterococcus faecium* from Animal and Commensal Strains. *MBio.* 4(4). pii: e00534-13.
23. **Lehnherr H, Maguin E, Jafri S, Yarmolinsky MB.** 1993. Plasmid addiction genes of bacteriophage P1: *doc*, which causes cell death on curing of prophage, and *phd*, which prevents host death when prophage is retained. *J Mol Biol.* **233**: 414–428.
24. **Mascher T, Helmann JD, Unden G.** 2006. Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev.* **70**:910-938.
25. **McBride SM, Fischetti VA, Leblanc DJ, Moellering RC Jr, Gilmore MS.** 2007. Genetic diversity among *Enterococcus faecalis*. *PLoS One.* **2**:e582.
26. **Mijnendonckx K, Provoost A, Monsieurs P, Leys N, Mergeay M, Mahillon J, Van Houdt R.** 2011. Insertion sequence elements in *Cupriavidus metallidurans* CH34: distribution and role in adaptation. *Plasmid.* **65**:193-203.
27. **Mindlin S, Petrova M.** 2013. Mercury Resistance Transposons. In Roberts A.P. and Mullany P (ed.), *Bacterial Integrative Mobile Genetic Elements*. Landes Bioscience.
28. **Novais C, Freitas AR, Silveira E, Antunes P, Silva R, Coque TM, Peixe L.** 2013. Spread of multidrug-resistant *Enterococcus* to animals and humans: an underestimated role for the pig farm environment. *J Antimicrob Chemother.* **68**:2746-54.
29. **Ojo KK, Tung D, Luis H, Bernardo M, Leitao J, Roberts MC.** 2004. Gram-positive

- merA* gene in gram-negative oral and urine bacteria. FEMS Microbiol Lett. **238**:411-416
30. **Roberts AP, Mullany P.** 2009. A modular master on the move: the Tn916 family of mobile genetic elements. Trends Microbiol. **17**:251-258.
31. **Rosvoll TC, Lindstad BL, Lunde TM, Hegstad K, Aasnaes B, Hammerum AM, Lester CH, Simonsen GS, Sundsfjord A, Pedersen T.** 2012. Increased high-level gentamicin resistance in invasive *Enterococcus faecium* is associated with *aac(6')-Ie-aph(2'')-Ia*-encoding transferable megaplasms hosted by major hospital-adapted lineages. FEMS Immunol Med Microbiol. **66**:166-176.
32. **Sambrook J, Frisch E, Maniatis T.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
33. **Siezen RJ, Renckens B, van Swam I, Peters S, van Kranenburg R, Kleerebezem M, de Vos WM.** 2005. Complete sequences of four plasmids of *Lactococcus lactis* subsp. cremoris SK11 reveal extensive adaptation to the dairy environment. Appl Environ Microbiol. **71**:8371-8382.
34. **Silveira E, Barros M, Freitas A R, Antunes P, Coque T M, Peixe L, Novais C.** *Enterococcus* spp. from different origins and clonal lineages carry two *merA* genes found in several *Firmicutes* members and are co-transferred with antibiotic resistance and copper tolerance genes. Manuscript in preparation.
35. **Silveira E, Freitas A R, Antunes P, Barros M, Campos J, Coque T M, Peixe L., Novais C.** Co-transfer of resistance to high concentrations of copper and first-line antibiotics among *Enterococcus* from different origins (humans, animals, the environment and foods) and clonal lineages. J Antimicrob Chemother, In Press (doi:10.1093/jac/dkt479).
36. **Stapleton P, Pike R, Mullany P, Lucas V, Roberts G, Rowbury R, Wilson M, Richards H.** 2004. Mercuric resistance genes in gram-positive oral bacteria. FEMS Microbiol Lett. **236**:213-220.
37. **Stock AM, Robinson VL, Goudreau PN.** 2000. Two-component signal transduction. Annu Rev Biochem. **69**:183–215.
38. **Van Houdt R, Monchy S, Leys N, Mergeay M.** 2009. New mobile genetic elements in *Cupriavidus metallidurans* CH34, their possible roles and occurrence in other bacteria. Antonie Van Leeuwenhoek. **96**:205-26.
39. **Werner G, Coque TM, Franz CM, Grohmann E, Hegstad K, Jensen L, van Schaik W, Weaver K.** 2013. Antibiotic resistant enterococci-tales of a drug resistance gene trafficker. Int J Med Microbiol. **303**:360-79.
40. **Wiedenbeck J, Cohan FM.** 2011. Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. FEMS Microbiol Rev. **35**:957-976.

41. **Willems RJ, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J.** 2012. Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *MBio* **3**:e00151-12.
42. **Willems RJ, van Schaik W.** 2009. Transition of *Enterococcus faecium* from commensal organism to nosocomial pathogen. *Future Microbiol.* **4**:1125-1135.

Table 1. Epidemiological background and plasmid content of *Enterococcus* carrying copper and mercury tolerance genes.

Species (WT)	Metal tolerance genes	Isolate type ^a	ABR pattern ^a	Transfer of metal tolerance genes (frequency) ^d	Size (kb) of plasmid carrying metal tolerance genes ^b	PLASMID CONTENT												ST/CC ^c	PFGE	Origin/Source	Date				
						ABR ⁺ genes within plasmid carrying Cu/Hg tolerance genes ^b				Replication module		Conjugation module		Prevalent rel by PCR								Other rep/rel			
						tet tet erm van				pLG1 pEF1		pEF18 pEF1		pEF18 pEF18								pEF18 pEF18			
						(M)	(L)	(B)	(A)	rep20	rep2	rep1	rel6	rep20	rep2	rep1	rel6					rep20	rep2	rep1	rel6
<i>E. faecium</i>	<i>tcrB</i>	TC ^c	-	+(10 ⁻⁴)	200														ND		SL2	Salads/supermarket A	2010		
	<i>cueO</i>	TC ^c	TET, ERY, VAN	+(10 ⁻⁶)	160														rep7		670/ST18 clonal lineage	Hospital A /unknown	1997		
	<i>tcrB+cueO</i>	WT	TET, ERY*	ND	280															32/CC22	90	SN10	Piggery III/antiseptic	2007	
		TC ^d	TET, ERY, CHL, STR	+(ND)	210														rep7, rep18 _{ST18AB}		6/CC5	CE	Healthy human - faeces	2001	
		TC ^d	TET*, ERY, STR	+(10 ⁻⁴)	300														rel10 _{pLG1}		ND	CB	Healthy human - faeces	2001	
		TC ^d	TET, ERY, VAN	+(10 ⁻³)	300														rep14 _{rep18-like} , rep18 _{rel2-like}		18/ST18 clonal lineage	EA	Sewage/ water from Hospital C	2001	
		TC ^c	TET, ERY, STR	+(10 ⁻⁴)	230														rep7		421/CC9	59	Poultry /carcass	1999	
		TC ^c	ERY, VAN	+(10 ⁻⁴)	150																5/CC5	A	Hospital B/pus	2002	
		TC ^c	VAN, STR	+(10 ⁻⁶)	150														rep17 _{pLUM} , rel2 _{pCIZ2}		132/ST18 clonal lineage	88	Hospital B/blood	1999	
		TC ^d	ERY	+(ND)	180																5/CC5	A3.1 ¹	Piggery IV/swine faeces	2007	
		TC ^d	ERY	+(ND)	200																434/CS	SN215	Piggery VI/air facilities	2006	
		TC ^d	TET, ERY	+(ND)	230																150/CC5	SN216	Piggery VI/swine faeces	2006	
		TC ^d	TET*, ERY	+(ND)	150																ND	SN231	Piggery III/ residual water	2006	
		WT	-	ND	200																ND	ND	Salads/supermarket A	2010	
		WT	TET, ERY, STR	+(ND)	120																ST846	CD	Healthy human - faeces	2001	
TC ^c		-	+(10 ⁻³)	120																	CJ	Healthy human - faeces	2001		
TC ^c		TET, ERY, GEN, STR	+(10 ⁻⁵)	250																	ST859	EU	Hospital sewage B/ water	2002	
	TC ^c	-	+(10 ⁻⁴)	180																	393/ST78 clonal lineage	SN208	Piggery I/manure, waste lagoon	2006	
	TC ^c	TET, ERY, STR	+(10 ⁻⁴)	300																	new	SN226.2	Piggery VI/swine faeces	2006	
	TC ^c	TET, ERY, GEN, STR	+(10 ⁻⁴)	300																	108/ST	SN211	Piggery III/ solid manure	2006	
	WT	ERY, VAN, AMP, CIP, GEN	-	chromosome																	132/ST18 clonal lineage	103	Hospital A/exsudate	2003	
	<i>E. faecalis</i>	<i>tcrB</i>	WT	TET, GEN, STR	-	90																49/CC2	44	Poultry /carcass/butcher shop	2001
<i>tcrB+cueO</i>		TC ^{ss}	ERY, STR	+(ND)	120																	288/CS	48	Poultry /carcass/butcher shop	2001
		WT	TET, ERY, CHL, STR, NIT	ND	120																	445/ST	SN242	Piggery III/ feed	2006
<i>merA3</i> <i>merA2+merA3</i>		WT	TET, ERY, CIP, CHL, GEN, STR	ND	ND																	ND	ND	Hospital D/ urine	2001
		WT	TET, ERY, VAN, CIP, GEN	-	75																	159/ST	N	Hospital A/blood	1999

Grey color indicates that a specific gene was identified by PCR but is not located in the same plasmid as metal *cueO*, *tcrB* or *merA*. The black color indicates that a specific gene is located in the plasmid carrying *cueO*, *tcrB* or *merA*. ^aAbbreviations: TC, transconjugant; WT, wild type; ABR, antibiotic resistance; TET-tetracycline; ERY, erythromycin; VAN, vancomycin; CHL, chloramphenicol; STR, high concentration of streptomycin; GEN, high concentration of gentamicin; AMP, ampicillin; CIP, ciprofloxacin; NIT, nitrofurantoin; ST, sequence type; CC, clonal complex; ND, not determined; “-”, susceptible to all antibiotics tested; * Intermediate resistance; ^bRecipient strain-*E. faecalis* JH2-2; ^cRecipient strain-*E. faecium* BM4105RF; ^dConjugation frequency was calculated per *E. faecium* BM4105RF or *E. faecalis* JH2-2 recipient cells; ^eTransconjugants recovered in media supplemented with 30mg/L of rifampicin (RIF), 25mg/L of fusidic acid (FA) and 8-16 mM of CuSO₄. ^fTransconjugants recovered in media supplemented with 30mg/L of rifampicin (RIF), 25mg/L of fusidic acid (FA) and 8mg/L of erythromycin; ^gTransconjugants recovered in media supplemented with 30mg/L of rifampicin (RIF), 25mg/L of fusidic acid (FA) and 8mg/L of tetracycline; ^hthe isolates carrying plasmids/chromosomal genetic elements highlighted in bold were included in further molecular studies for genetic environment characterization of metal tolerance genes, which are represented in Table 2. ⁱThe complete MLST scheme was not available for this isolate, but the allele 9 of the gene *purK* indicates that it does not belong to high risk clonal complexes.

Table 2. Data of Long-PCR obtained with different primer combination.

Metal tolerance genes	Species	Epidemiological background	Location (kb)	Long-PCR (kb) ^a						
				P1-P2 ^b	P3-P2	P4-P5 ^c	P6-P7 ^c	P8-P9	P10-P11	
<i>tcrB+cueO</i>	<i>E. faecium</i>	HSW (water); ST18/ST18 clonal lineage	PL (300)	na	~2,3	~11,4 (II)	-	na	na	na
<i>tcrB+cueO+merA2</i>	<i>E. faecium</i>	P (carcass); ST421/CCC9	PL (230)	na	-	~11,4 (IV)	-	na	na	na
		HSW (water); ST859	PL (250)	~4,5 (I)	-	~11,4 (II)	~2,7 (I)	na	na	na
		HV (faeces); <i>purK9</i>	PL (120)	~2,5	-	~11,4(III)	~6,4	na	na	na
		HV (faeces); ST846	PL (120)	~4,5 (I)	-	~11,4 (II)	~2,7 (I)	na	na	na
		PG (manure); ST393/ST78 clonal lineage	PL (180)	~4,5 (I)	-	~11,4 (I)	~2,7 (I)	na	na	na
<i>merA3</i>	<i>E. faecium</i>	HP (exsudate); ST132/ST18 clonal lineage	Chr	na	na	na	na	(-)	(-)	(-)
	<i>E. faecalis</i>	HP (urine);	ND	na	na	na	na	~5,5	~2,5	~2,5
		HP (blood); ST159	PL (75)	na	na	na	na	(-)	(-)	(-)

^aSchematic representation of primers (P) is showed in Figure 1. In bold are represented the sequenced fragments. Primers sequences (GenBank accession no. or Reference): **P1**, merA2F-TGCTCTTGAAATGCAGGTG (Silveira et al., submitted); **P2**, cueOR-TCATTGTCAAGGCAACCAA (Silveira et al., 2013); **P3**, ISEfm2R-AGTTCCCGTACTCGGTCTT (AHYG01000049.1); **P4**, cueOF-CCCTAGGCGGTGTTTCATA (Silveira et al., 2013); **P5**, tcrBR-ATAGAGGACTCCGCCACCATTTG (Hasman et al., 2006); **P6**, tcrBF-CATCACGGTAGCTTTAAGGAGATTTTC (Hasman et al., 2006); **P7**, IS1216R-TGGCTTCCATGCCCTTTAATC (AHYG01000049.1); **P8**, IS1062R-TTTCGCCGAGTTTCTTTGTG (AY351675.1); **P9**, merA3R-CCTGCACAACAAGATAATTTTCG (Silveira et al., submitted); **P10**, merA3F-TTGCAACGGTTGGTTTAACA (Silveira et al., submitted); **P11**, arsR-TATTGCCATTGGCCTTCCCTT (GG692853.1); ^bRFLP with TaqI; ^cRFLP with AluI; Abbreviations: ND-not determined; na- not applicable; PL-plasmid; Chr-chromosome.

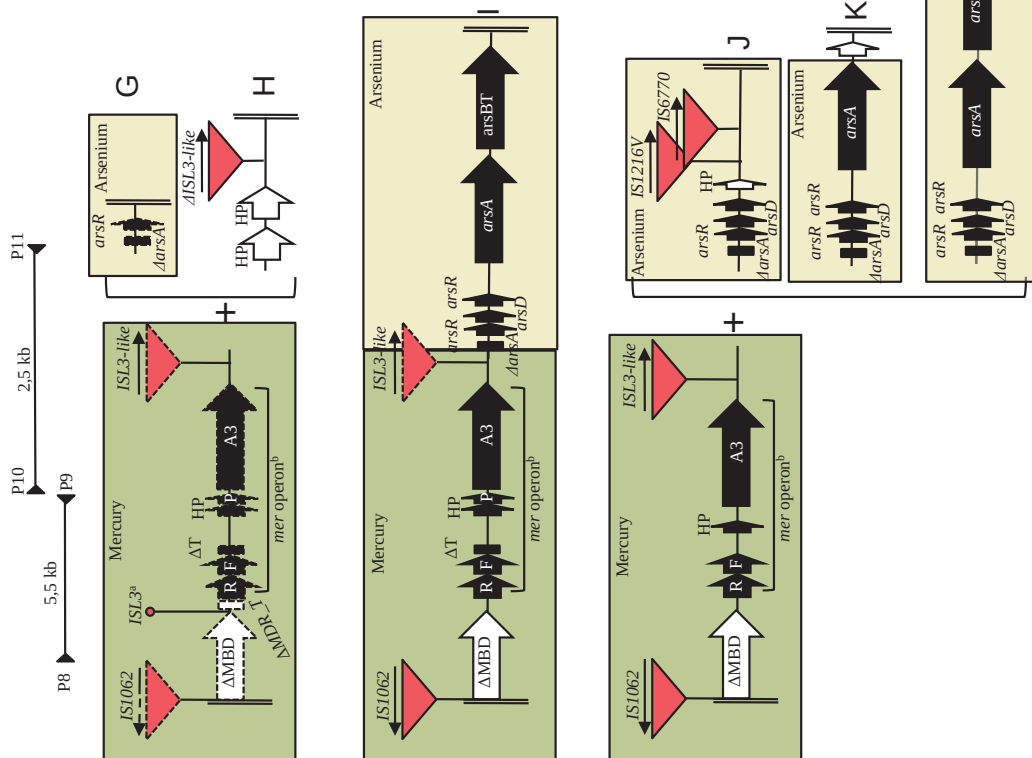


Figure 2. Schematic representation of *merA3* genetic environment. The sequences were detected in different species (type G: *E. faecalis*, this study; type H: *E. casseliflavus* Genbank accession no. AY351675.1; type I: *E. faecalis*-GG692853.1, ALOD01000061.1; type J: *E. faecalis*-AJED01000032.1; type K: *E. faecalis*-AJER01000024.1; type L: *E. faecalis*-ASCU01000007.1). Genes also sequenced in isolates from this study appear represented with dashed lines. Primer sequences (P8, P9, P10, P11) and amplicons sizes are represented in Table 2. Colours: black- genes related to tolerance to metals; red- insertion sequences; Boxes in different colours (dark and light green)- represent common modules (clusters of genes) shared by different isolates. Parallel vertical lines- sequence beyond the last gene was not included in the scheme. ^aInverted repeat of ISL3-like transposase; Abbreviations by alphabetical order: **arsA**-arsenical pump-driving ATPase; **arsB**-arsenite transducer membrane protein; **arsB-RP**-arsenite resistance protein; **arsD**-arsenical resistance trans-acting repressor; **arsR**-arsenical resistance repressor; **APMP**-arsenical pump membrane protein; **HP**-Hypothetical regulator; **merF**-transport, merP-transport, merT-transport and merA-reductase);

First description of a multidrug-resistant ST17 *Enterococcus faecium* carrying a transferable Inc18-like::*qacZ* plasmid

Silveira E.¹, Marques P.¹, Barros M.¹, Cardoso N.³, Freitas A. R.¹, Mourão J.¹, Coque T.M.⁴, Antunes P.^{1,2}, Peixe L.¹, Novais C.¹

¹REQUIMTE. Laboratório de Microbiologia. Faculdade de Farmácia. Universidade do Porto, Portugal; ²Faculdade de Ciências da Nutrição e Alimentação. Universidade do Porto, Portugal; ³Faculdade de Ciências da Saúde. Universidade Fernando Pessoa. Porto. Portugal. ⁴Servicio de Microbiologia. Hospital Ramón y Cajal. Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain; ⁴Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBER-ESP), Madrid, Spain; ⁵Unidad de Resistencia a Antibióticos y Virulencia Bacteriana asociada al Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain.

Running Title: A Inc18-like::*qacZ* plasmid in *Enterococcus*

Keywords: quaternary ammonium compounds, *qacZ*, antibiotic resistance, transferable plasmids

Corresponding author: Carla Novais, Ph.D. Laboratório de Microbiologia. Faculdade de Farmácia, Universidade do Porto. Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal
Phone: +351 220428588; FAX: +351 226 093 390; E-mail: casilva@ff.up.pt

Parte deste trabalho foi apresentado no 23rd *European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)*, Berlim, Alemanha em Abril de 2013. P1409.

Artigo submetido para publicação

ABSTRACT

A *qacZ* was found in a transferable ~40kb Inc18-like plasmid carried by a MDR-*E. faecium*-ST17 (hospital sewage) within a region containing *Tn4001* and *IS1216*. MICs to benzalkonium chloride were higher in transconjugants than recipient strain (10-11 versus 7,5mg/L). The data corroborates the genome plasticity of *E. faecium* to adapt to diverse environmental challenges and suggest that quaternary ammonium compounds residual concentrations could contribute to maintenance of specific MDR genetic platforms in *E. faecium*.

In the last decades *Enterococcus*, particularly specific multidrug resistant (MDR) clonal lineages (*E. faecium*-ST17, ST18, ST78; *E. faecalis*-ST6, ST40), became important nosocomial agents worldwide (Willems *et al.*, 2012). The great ability to adapt to diverse environmental challenges contributed to their dispersion in nature and thus to the spread of antibiotic resistance among several community settings beside hospital units (Werner *et al.*, 2013; Lebreton *et al.*, 2013). Along with the vast use of antibiotics, other compounds often present in human daily life, animal production setting and environment have been suggested to contribute for the selection of MDR strains (SCENIHR, 2009). Among them are the Quaternary Ammonium Compounds (QACs), a type of biocides widely applied in hospitals, industry, agriculture and household consumer products for different purposes (e.g., in disinfectants and cleaning products, food preservatives and cosmetic industry) (Buffet-Bataillon *et al.*, 2012). They are considered “hard antibacterial agents” due to their poor metabolization by organisms, retaining their biocidal properties when released in the environment (Kummerer *et al.*, 2004; Tezel *et al.*, 2011; Buffet-Bataillon *et al.*, 2012). Despite QACs wide use, the prevalence and location of genes coding for tolerance to these compounds among *Enterococcus*, specially in particular *Enterococcus* MDR clonal lineages associated with human infections, remains scarcely explored (Kazama *et al.*, 1998; Braga *et al.*, 2011; Bishoff *et al.*, 2012). The few studies available described *qacZ* in several *Enterococcus* species (Braga *et al.*, 2011), *small multidrug resistance* genes (*smr*; also named *qacC/D*) or *qacA/B* in *E. faecalis* (Bishoff *et al.*, 2012) and *qacEΔ* in an *Enterococcus* sp (Kazama *et al.*, 1998). In this study we analyzed the occurrence and distribution of different genes coding to QACs tolerance and, for the first time, characterized the genetic elements involved in their co-transfer with antibiotic resistance.

Enterococcus (n=918; 345 *E. faecium*, 321 *E. faecalis*, 75 *E. hirae*, 29 *E. gallinarum*, 19 *E. casseliflavus*, 6 *E. durans*, 123 *Enterococcus* spp) recovered between 1996-2012 from different Portuguese sources (125 from healthy humans, 108 human clinical isolates, 49 hospital sewage, 3 urban river, 232 piggeries, 198 aquacultures, 155 poultry, 27 rainbow trout, 21 ready-to-eat salads) and with variable antibiotic resistance patterns were included (Silveira *et al.*, 2013). Diverse clonal lineages of *E. faecium* (ST17, ST18, ST78, CC5, CC9, CC22, CC94 as well as diverse singletons) and *E. faecalis* (CC2, CC21 and diverse singletons) were represented. The most described *qac* genes coding for QACs tolerance among Gram-positive (two *smr* genes; *qacA/B*, *qacC/D*, *qacG*, *qacH*, *qacZ*) or Gram-negative bacteria (*qacEΔ*), and previously associated with different genera or species (indicating mobilization) were searched by several PCR reactions (Figure 1) (Nogushi *et al.*, 1999;

Smith *et al.*, 2008;). Only one *E. faecium* collected from the hospital sewage in 2002 carried the *qacZ* (coding only for QACs tolerance) previously identified among *Enterococcus* (Braga *et al.*, 2011) (100% amino acid identity; Genbank accession nos. AE016833.1, EO107701.1, EOM21028.1, EOM31912.1) and with 98% of amino acid identity with *qacH* (coding for QACs, ethidium bromide and proflavin tolerance) of *Staphylococcus saprophyticus* (Genbank accession no. CAA76544.1) (Heir *et al.*, 1998; Correa *et al.*, 2008). The *E. faecium* was identified as ST17, which belong to one of the main human adapted lineages within BAPS subgroup 3-3, and was resistant to ampicillin, tetracycline [*tet(S)*, *tet(M)*], erythromycin [*erm(B)*], to high concentrations of gentamicin (*aac6'-Ie-aph2''-Ia*) and streptomycin (*aadE*). These data contrast with the study of Braga *et al.* (2011), which found this gene in 33 out of 73 *Enterococcus* studied (14 clinical and 19 dairy products isolates). However, the significant higher number of isolates and diversity of sources included in our study, more representative of *Enterococcus* population, suggests they are not important reservoirs of *qacZ*.

The co-location of antibiotic resistance and QACs tolerance genes was determined by hybridization of I-Ceul and S1 nuclease-digested genomic DNA (Freitas *et al.*, 2013) with specific probes [*qacZ*, *tet(S)*, *tet(M)*, *erm(B)*, *aadE*, *aac(6')-Ie-aph(2'')-Ia*] (Silveira *et al.*, 2013). The presence of 11 replicase (*rep*) genes (*rep*_{1/pIP501}, *rep*_{2/pRE25/pEF1}, *rep*_{6/pAMx1}, *rep*_{8/pAM373}, *rep*_{14/pRI1-like}, *rep*_{17/pRUM}, *rep*_{18a/pEF418}, *rep*_{18c/pCIZ2}, *rep*_{20/pLG1}, *rep*_{21/pVEF1/2}, *rep*_{22/pHTbeta}), 6 relaxases (*rel*) genes (*rel*_{2/pCIZ2}, *rel*_{3/pRUM}, *rel*_{4/pLG1}, *rel*_{7/Inc18}, *rel*_{8/pHTbeta}, *rel*_{9/pCF10}) and 3 toxin-antitoxin systems (Axe-Txe from pRUM, ω - ϵ - ζ from Inc18 plasmids and *par* from pAD1), representing prevalent plasmid families, was assessed by PCR and hybridization (Jensen *et al.*, 2010; Freitas *et al.* 2013). The transfer of *qacZ* along with antibiotic resistance was evaluated by conjugation assays (Silveira *et al.*, 2013) using *E. faecium* BM4105RF, *E. faecium* GE1, *E. faecalis* JH2-2 and *Staphylococcus aureus* 8325 as recipients (all rifampicin-RIF/fusidic acid-FA resistant; *qacZ* negative). Transconjugants were recovered in BHI supplemented with RIF-30mg/L, FA-25mg/L, tetracycline-8mg/L, streptomycin-2000mg/L, gentamicin-125mg/L, erythromycin-8mg/L or ampicillin-10mg/L and confirmed by RIF/FA resistance and occurrence of *qacZ*. Co-transfer of antibiotic resistance was evaluated by phenotypic and genotypic assays (disk diffusion, PCR) (CLSI, 2011; Silveira *et al.*, 2013). Benzalkonium chloride (BAC), one of QACs most widely used, was evaluated by agar dilution method (CLSI, 2011), repeating experiments 3 times for the wild strains, recipient and transconjugants. MIC_{BAC} indicated represent the average of the 3 experiments. The concentration range of BAC used was 0.015-12mg/L, with increments of 0,5mg/L per plate between 4 and 12mg/L (16h of bacterial incubation before their use; plates supplemented

with BAC read at 24h). The wild *E. faecium* carried 7 plasmids (size range of ca. 10-150 kb) and the *qacZ* gene was located in a 40kb transferable plasmid along with *tet(S)*, *aac(6')-Ie-aph(2'')-Ia* and *aadE* genes, as well as a replicase of the Inc18 plasmid pRE25 (rep_{2/pRE25/pEF1}; accession no. X92945) (Figure 2). Resistance to erythromycin was codified by *erm(B)* located in other plasmids of 70 and 20kb, the latter also carrying *aadE* (streptomycin resistance) and a replicase of the RepA_N family (rep_{17/pRUM}). The plasmid of ~150kb carried rep_{20pLG1} (RepA_N family) and a small one of ~10 kb a rel_{2/pCIZ2}, both without antibiotic resistance genes searched. The *qacZ* carrying plasmid did not carry any toxin-antitoxin system. MIC_{BAC} was 10 mg/L for the wild strain and for the positive control strain *E. faecalis* V583, values similar to those previously described for this gene among *Enterococcus* (Braga *et al.*, 2011). Transconjugants were only obtained with the recipient *E. faecium* BM4105RF and beside *qacZ*, they were all resistant to tetracycline, to high concentrations of gentamicin and streptomycin, and two of them, also to erythromycin. MIC_{BAC} was of 10-11 mg/L, contrasting with 7.5 mg/L of the recipient *E. faecium* BM4105RF.

The *qacZ* genetic environment (~6kb) was characterized by long-PCR reactions including combination of primers for *qacZ* with others used to the search of antibiotic resistance genes or to the enterococcal genome wide spread *IS1216* (Figure 1). The larger amplicons flanking *qacZ* on both sides were sequenced. Sequencing revealed *qacZ* within a recombined region flanked by *IS1216* and adjacent to a partially sequenced Tn4001 (containing *aac6'-Ie-aph2''-Ia* coding for aminoglycoside resistance and flanked by *IS256*) (Daikos *et al.*, 2003). Also *E. faecalis* V583 (the only sequence with *qacZ* which genetic environment was available at Genbank database) was flanked by a copy *IS1216* in the left side of *qacZ*, as in our case, and another copy in the right side, after a replicase gene (AE016833.1).

This is the first report of a Inc18-like transferable plasmid carrying a *qacZ* gene in ST17-*E. faecium*. These broad host range plasmids have been described to contribute to the genetic plasticity of *Enterococcus* in different settings (Jensen *et al.*, 2010; Freitas *et al.*, 2013, Werner *et al.*, 2013) and to participate in the adaptation of this genus to environmental challenges, as antibiotics (Freitas *et al.*, 2013; Werner *et al.*, 2013). The great amount and diversity of nosocomial strains and mobile genetic elements within hospital sewage creates an opportunity for the occurrence of recombinatorial events, often mediated by IS, frequently resulting from genetic exchanges among organisms within bacterial communities (Baquero *et al.*, 2008) and thus, for the shaping and diversification of genetic platforms. MIC_{BAC} detected were much lower than concentrations applied in disinfectants (e.g., 400-1000ppm) (Tezel *et al.*, 2011), which can maintain their activity even in strains with *qac* genes. However, residual

concentrations of BAC previously found in the environment (e.g. hospital sewage-5mg/L) or food (0,01-14,4mg/kg) (Kummerer *et al.*, 2004, EFSA 2013), might contribute for the selection/maintenance of such genetic platforms carried by MDR strains.

ACKNOWLEDGMENTS

This project was funded by research grants from the Fundação para a Ciência e Tecnologia (PTDC/AAC-AMB/103386/2008; grant no. PEst-C/EQB/LA0006/2011). Eduarda Silveira is supported by a Ph.D. fellowship of the Fundação para a Ciência e Tecnologia (grant number SFRH/BD/63955/2009; POPH-QREN). Work in Teresa M. Coque lab is supported by grants from the European Union (7FPEU-HEALTH-F3-2011-282004_EvoTAR), Ministry of Economy and Competitiveness of Spain-Instituto de Salud Carlos III (PI12/01581). We would like to thanks to Dr. Paola Jeric (Microbiology, Parasitology and Immunology department, Medicine Faculty-UBA, Paraguay) and Joistein Bjorland (Norwegian Scholl of Veterinary Science) for control strains *Staphylococcus epidermidis* SE59 (*qacA/B* and *smr* genes), G (*qacG*:RN4220; pSK265); and H (*qacH*:RN4220; pSK265). We also would like to thanks to Vitoria Francia [Servicio de Microbiología, Hospital Universitario Marqués de Valdecilla e Instituto de Formación e Investigación Marqués de Valdecilla (IFIMAV), Santander, Spain] for the positive control *Enterococcus faecalis* V583 (*qacZ* positive).

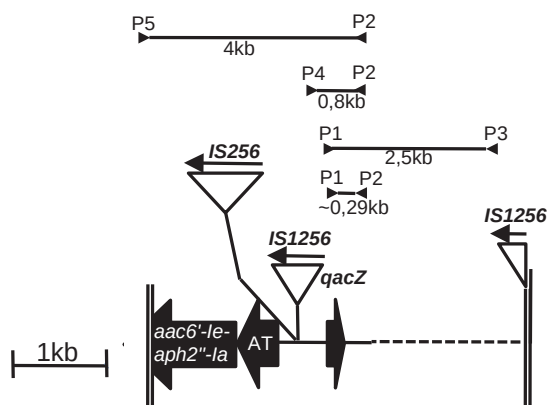
REFERENCES

1. **Aarestrup FM, Agerso Y, Gerner-Smidt P, Madsen M, Jensen LB.** 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn. Microbiol. Infect. Dis.* **37**(2):127–137.
2. **Baquero F, Martínez JL, Cantón R.** 2008. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* **19**:260-265.
3. **Bischoff M, Bauer J, Preikschat P, Schwaiger K, Mölle G, Hölzel C.** 2012. First detection of the antiseptic resistance gene *qacA/B* in *Enterococcus faecalis*. *Microb. Drug Resist.* **18**:7-12.
4. **Braga TM, Marujo PE, Pomba C, Lopes MF.** 2011. Involvement, and dissemination, of the enterococcal small multidrug resistance transporter QacZ in resistance to

- quaternary ammonium compounds. J. Antimicrob. Chemother. **66**:283-286.
5. **Buffet-Bataillon S, Tattevin P, Bonnaure-Mallet M, Jolivet-Gougeon A.** 2012. Emergence of resistance to antibacterial agents: the role of quaternary ammonium compounds-a critical review. Int. J. Antimicrob. Agents. **39**:381-389.
 6. **Clinical Laboratory Standards Institute.** Performance standards for antimicrobial susceptibility testing; Twenty first informational supplement M100-S21, CLSI, Wayne, PA,USA, 2011.
 7. **Correa JE, De Paulis A, Predari S, Sordelli DO, Jeric PE.** 2008. First report of *qacG*, *qacH* and *qacJ* genes in *Staphylococcus haemolyticus* human clinical isolates. J. Antimicrob. Chemother. **62**:956-960.
 8. **Daikos GL, Bamias G, Kattamis C, Zervos MJ, Chow JW, Christakis G, Petrikos G, Triantafyllopoulou P, Alexandrou H, Syriopoulou V.** 2003. Structures, locations, and transfer frequencies of genetic elements conferring high-level gentamicin resistance in *Enterococcus faecalis* isolates in Greece. Antimicrob Agents Chemother. **47**:3950-3953.
 9. **European Food Safety Authority.** 2013; Evaluation of monitoring data on residues of didecyldimethylammonium chloride (DDAC) and benzalkonium chloride (BAC). EFSA supporting publication 2013:EN- 483. 30 pp.
 10. **Freitas AR, Novais C, Tedim AP, Francia MV, Baquero F, Peixe L, Coque TM.** 2013. Microevolutionary events involving narrow host plasmids influences local fixation of vancomycin-resistance in *Enterococcus* populations. PLoS One. **8**:e60589.
 11. **Heir E, Sundheim G, Holck AL.** 1998. The *Staphylococcus qacH* gene product: a new member of the SMR family encoding multidrug resistance. FEMS Microbiol. Lett. **163**:49-56.
 12. **Huh J, Lee W G, Lee K, Shin W S, Yoo J H.** 2004. Distribution of insertion sequences associated with Tn1546-like elements among *Enterococcus faecium* isolates from patients in Korea. J. Clin. Microbiol. **42**:1897–1902.
 13. **Jensen LB, Garcia-Migura L, Valenzuela AJ, Løhr M, Hasman H, Aarestrup FM.** 2010. A classification system for plasmids from enterococci and other Gram-positive bacteria. J. Microbiol. Methods. **80**:25-43.
 14. **Kazama H, Hamashima H, Sasatsu M, Arai T.** 1998. Distribution of the antiseptic-resistance gene *qacE* delta 1 in gram-positive bacteria. FEMS Microbiol. Lett.; **165**:295-299.
 15. **Kümmerer K.** 2004. Resistance in the environment. J Antimicrob Chemother. **54**:311-
-

320.

16. **Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJ, Earl AM, Gilmore M.** 2013. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *MBio*. 4(4). pii: e00534-13.
 17. **Noguchi N, Hase M, Kitta M, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJ, Earl AM, Gilmore MS.** 1999. Antiseptic susceptibility and distribution of antiseptic-resistance genes in methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiol. Lett.*; **172**:247-253.
 18. **SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks).** 2009. Assessment of the Antibiotic Resistance Effects of Biocides. http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf (last date accessed 3 Novembro 2013).
 19. **Silveira E, Freitas Ana R, Antunes P, Barros M, Campos J, Coque TM, Peixe L, Novais C.** 2013. Co-transfer of resistance to high concentrations of copper and first line antibiotics among *Enterococcus* from different origins (human, animal, environment and food) and clonal lineages. *J. Antimicrob. Chemother.* In Press
 20. **Smith K, Gemmell CG, Hunter IS.** 2008. The association between biocide tolerance and the presence or absence of qac genes among hospital-acquired and community-acquired MRSA isolates. *J. Antimicrob. Chemother.* **61**:78-84.
 21. **Tezel U, Pavlostathis S G.** 2011. Role of quaternary ammonium compounds on antimicrobial resistance in the environment, p 349-387. *In* Patricia L. Keen and Mark H.M.M. Montforts (Ed), *Antimicrobial Resistance in the Environment*, 1st Edition. John Wiley & Sons, Inc Press.
 22. **Vakulenko S B, Donabedian S M, Voskresenskiy A M, Zervos M J, Lerner S A, Chow J W.** 2003. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrob. Agents Chemother.* **47**:1423–1426.
 23. **Werner G, Coque TM, Franz CM, Grohmann E, Hegstad K, Jensen L, van Schaik W, Weaver K.** 2013. Antibiotic resistant enterococci-tales of a drug resistance gene trafficker. *Int J Med Microbiol.* **303**:360-379.
 24. **Willems RJ, Top J, van Schaik W, Leavis H, Bonten M, Sirén J, Hanage WP, Corander J.** 2012. Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *MBio*. **3**:e00151-12.
-



Primer group and name	Primer no.	Sequence (5'-3')	GenbankK accession No. (nucleotide position)	Amplicon size (bp)	Reference
Search of <i>qac</i> genes					
<i>smr1_F</i> ^a		ATAAGTACTGAAGTTATTGGAAGT	GQ900434.1 (417-57-41780)	284	Smith et al., 2008
<i>smr1_R</i> ^a		TTCCGAAAATGTTTAACGAACTA	GQ900434.1 (42019-42042)		
<i>smr2_F</i> ^a		ACCGACAGCTGACCATGTTGCA	HQ663849.2 (10487-10508)	115	This study
<i>smr2_R</i> ^a		GCCTATTCCAACCGTTTCTGCCC	HQ663849.2 (1579-1603)		
<i>qacA/B_F</i>		CTATGGCAATAGGAGATATGGTGT	HE579074.1 (11256-11279)	417	Nogushi et al., 1999
<i>qacA/B_R</i>		CCACTACAGATTCTTCAGCTACATG	HE579074.1 (11648-11672)		
<i>qacG_F</i>		TTTCGTTTGAATTTGCTTT	Y16944.1 (1641-1660)	213	Smith et al., 2008
<i>qacG_R</i>		AATGGCTTTCTCCAAATACA	Y16944.1 (1834-1853)		
<i>qacH/Z_F</i>	1	CAATAGTCAGTGAAGTAATAGGCAGTG	Y16945.1 (1887-1913)	295	Smith et al., 2008
<i>qacH/Z_R</i>	2	TGTGATGATCCGAATGTGTTT	Y16945.1 (2161-2182)		
<i>qacEΔ_F</i>		ATCGCAATAGTTGGCGAAGT	KF056330.1 (18919-18938)	226	Nogushi et al., 1999
<i>qacEΔ_R</i>		CAAGCTTTTGCCCATGAAGC	KF056330.1 (19125-19145)		
PCR mapping^b					
<i>qacH/Z_F</i> + <i>IS1216_F</i>	1	CAATAGTCAGTGAAGTAATAGGCAGTG	Y16945.1 (1887-1913)		Smith et al., 2008
<i>IS1216_R</i>	3	AGGATTATATAAGAAAACCCG	AB699882.1 (645-663) AB699882.1 (125-145)	2000 2000, 2500	Hu et al., 2004 Hu et al., 2004
<i>qacH/Z_R</i> + <i>IS1216_F</i>	2	TGTGATGATCCGAATGTGTTT	Y16945.1 (2161-2182)		Smith et al., 2008
<i>aac6'-le-aph2''-la_R</i>	4	ACCTTCACGATAGCTAAGGTT	AB699882.1 (645-663)	860	Hu et al., 2004
	5	GTCTTAAAAAAGTGGCAATATCTCGTT	GQ900487.1 (7251-7277)	4000	Vakulenko et al., 2003

Figure 1. Primers used to search diverse genes coding for quaternary ammonium compounds tolerance and for the characterization of *qacZ* genetic environment. ^aThe numbers 1 and 2 were attributed to differentiate distinct sequences with the same name. ^bNo amplicons were detected in PCR reactions including the following combination of primers : *qacH/Z_F* plus *tet(S)_F*, *tet(S)_R*, *tet(M)_F*, *tet(M)_R*, *aac6'-le-aph2''-la_F*, *aac6'-le-aph2''-la_R*, *aadE_F* or *aadE_R*; *qacH/Z_R* plus *tet(M)_F*, *tet(M)_R*, *tet(S)_F*, *tet(S)_R*, *aac6'-le-aph2''-la_F*, *aadE_F*, *aadE_R* or *IS1216_R*. The vertical lines in figure represent the limits of the fragment sequenced. The dashed lines represent part DNA fragment not sequenced. Abbreviations: AT- acetyl transferase GNAT family.

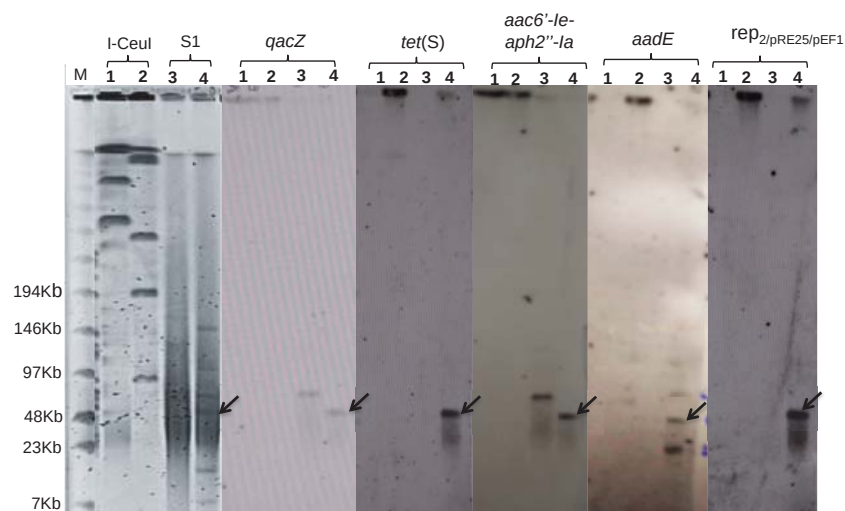


Figure 2. Hybridization assays of S1 and I-Ceu-I digested DNA of the *qacZ* carrying *E. faecium*. Co-hybridization of *qacZ*, antibiotic resistance and *rep2*_{pRE25/pEF1} genes are signed by black arrows. Numbers 1 and 2 correspond to I-CeuI PFGE hybridization and numbers 3 and 4 of S1 PFGE hybridization. 1, 3- *E. faecalis* V583 (positive control strain to *qacZ*; pTEF1 plasmid); 2, 4-Wild *E. faecium* strain (E241).

Different genetic supports for the *tet(S)* gene in Enterococci.

Carla Novais^{1,2}, Ana R. Freitas¹, Eduarda Silveira¹, Fernando Baquero^{3,4,5}, Luísa Peixe¹, Adam P. Roberts⁶, Teresa M. Coque^{3,4,5}.

¹REQUIMTE, Faculdade Farmácia. Universidade do Porto, Porto, Portugal; ²CEBIMED, Faculdade Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal; ³Servicio de Microbiología, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain; ⁴Unidad de Resistencia a Antibióticos y Virulencia Bacteriana del Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain; CIBER en Epidemiología y Salud Pública (CIBER-ESP), Madrid, Spain; and ⁶Division of Microbial Diseases, UCL Eastman Dental Institute, University College London, London, United Kingdom.

Antimicrobial Agents and Chemotherapy 2012; 56(11):6014-6018

ASM Press has authorized the reproduction of the final published PDF version of this article

Different Genetic Supports for the *tet(S)* Gene in Enterococci

Carla Novais, Ana R. Freitas, Eduarda Silveira, Fernando Baquero, Luísa Peixe, Adam P. Roberts and Teresa M. Coque

Antimicrob. Agents Chemother. 2012, 56(11):6014. DOI: 10.1128/AAC.00758-12.

Published Ahead of Print 20 August 2012.

Updated information and services can be found at:
<http://aac.asm.org/content/56/11/6014>

REFERENCES

These include:

This article cites 14 articles, 10 of which can be accessed free at: <http://aac.asm.org/content/56/11/6014#ref-list-1>

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), [more»](#)

Information about commercial reprint orders: <http://journals.asm.org/site/misc/reprints.xhtml>
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>

Journals.ASM.org

Different Genetic Supports for the *tet(S)* Gene in Enterococci

Carla Novais,^{a,b} Ana R. Freitas,^{a,c} Eduarda Silveira,^a Fernando Baquero,^{c,d,e} Luísa Peixe,^a Adam P. Roberts,^f and Teresa M. Coque^{c,d,e}

REQUIMTE, Faculdade Farmácia, Universidade do Porto, Porto, Portugal^a; CEBIMED, Faculdade Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal^b; Servicio de Microbiología, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain^c; Unidad de Resistencia a Antibióticos y Virulencia Bacteriana del Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain^d; CIBER en Epidemiología y Salud Pública (CIBER-ESP), Madrid, Spain^e; and Division of Microbial Diseases, UCL Eastman Dental Institute, University College London, London, United Kingdom^f

The diversity of *tet(S)* genetic contexts of 13 enterococci from human, animal, and environmental samples from different geographical areas is reported. The *tet(S)* gene was linked to either CTn6000 variants of chromosomal location or composite platforms flanked by IS1216 located on plasmids (~40 to 115 kb). The comparative analysis of all *tet(S)* genetic elements available in the GenBank databases suggests that CTn6000 might be the origin of a variety of *tet(S)*-carrying platforms that were mobilized to different plasmids.

The tetracycline resistome comprises over 1,189 genes identified in more than 84 Gram-positive and 354 Gram-negative species (17), which included 43 fully characterized *tet* genes coding for resistance to tetracycline by different mechanisms (<http://faculty.washington.edu/marilynr>). Genes involved in ribosomal protection, such as *tet(S)*, *tet(M)*, *tet(O)*, *tet(Q)*, and *tet(W)*, were identified in conjugative transposons (CTns), phage-derived structures, and/or conjugative plasmids (3, 4, 5, 11, 12, 16). The *tet(S)* gene has been found among *Firmicutes* and gammaproteobacteria from diverse ecological sources since the early 1950s (1, 2, 10, 11) (<http://faculty.washington.edu/marilynr/tetweb2.pdf>), and several genetic platforms carrying *tet(S)*, such as CTn6000, CTn916S, pKL0018, and pK214, have been described (5, 9, 11). Among enterococci, the genetic context of *tet(S)* has been characterized in only a single *Enterococcus casseliflavus* isolate from animal origin (5, 15). In this work, we described the genetic platforms of 13 *tet(S)*-carrying enterococci from different origins and geographical areas and compared them with sequences available in GenBank databases. This study gives for the first time a snapshot of the epidemiology and dynamics of the *tet(S)* gene by describing its location in different CTn6000 variants differing in insertions and deletions as well as in composite genetic platforms flanked by insertion sequences. Such diversity of genetic platforms seems to have facilitated its spread among diverse clonal backgrounds by different recombinatorial processes.

Antibiotic susceptibility testing was performed by standard methods (6), and characterization of other genes coding for resistance to tetracycline [*tet(M)*, *tet(L)*, *tet(K)*, and *tet(O)*] was accomplished by PCR (1). Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (<http://www.mlst.net>) (14). The 13 enterococci carrying *tet(S)* were identified as *Enterococcus faecalis* ($n = 5$), *Enterococcus faecium* ($n = 3$), *Enterococcus gallinarum* ($n = 2$), and other *Enterococcus* species ($n = 3$). Epidemiological details of the strains studied appear in Table 1. Isolates expressed resistance to tetracycline (100%; $n = 13$) and minocycline (92%; $n = 12$) and frequently also harbored *tet(M)* (54%; $n = 7$) or *tet(L)* (31%; $n = 4$).

Hybridization of I-CeuI/S1-digested genomic DNA with *tet(S)* and 23S rRNA probes demonstrated either a chromosomal ($n = 5$) or plasmid ($n = 8$; ~40 to 115 kb) location of the *tet(S)* gene (Table 1) (8). Screening of CTns included the identification of

integrases and excisionases of different CTns previously found to carry *tet(S)* as CTn6000 or CTn916 and further characterization of the transposon's backbone by PCR mapping based on available sequences (Table 2 and Fig. 1) (13). The *tet(S)* gene was located at a chromosome within platforms carrying *int/xis*_{CTn6000} sequences ($n = 5$) or on plasmids within platforms lacking *int/xis* sequences of any CTn tested ($n = 8$) (Fig. 1 and Table 1). The different *tet(S)* platforms were designated by capital letters. Characterization of chromosomal CTn6000 platforms was established by comparison of restriction fragment length polymorphism (RFLP) patterns obtained after digestion of PCR products with TaqI, AluI, DdeI, HindIII, or EcoRI and by the DNA sequences of representative fragment types (Fig. 1). Three variants, arbitrarily named types "B," "C," and "D" to differentiate them from the original CTn6000 (5), designated type "A" here, were identified (Table 1 and Fig. 1). Type C (GenBank accession no. JN208881) was found in Portugal in an ST55 (CC55) *E. faecalis* isolate identified in five different healthy humans and in one ST32/CC22 *E. faecium* strain and one *Enterococcus* species strain isolated from two different piggeries. Type C differs from type A by the lack of the 1,830-bp insertion within *orf14* consisting of a group II intron and an additional 17 bp. Type B (GenBank accession no. JN208880), isolated from an ST32/CC22 *E. faecium* strain from a fountain's water in Portugal, contains an IS_fm2 insertion with identifiable boundaries within the *orf13-tet(S)* region. Finally, type D, carried by an ST158 *E. faecalis* clone from Michigan, was able to be only partially identified. It carried *int*_{CTn6000}-*tet(S)* and the *orf23-orf29*_{CTn6000} region, although they were not able to be directly linked by using different PCR overlapping assays between different CTn6000 regions. Comparative analysis of the CTn6000 elements described in this study with sequences available at the GenBank database resulted in the detection of three unpublished genetic elements identical to CTn6000 variant C within the full-ge-

Received 12 April 2012 Returned for modification 16 June 2012

Accepted 14 August 2012

Published ahead of print 20 August 2012

Address correspondence to Carla Novais, canos2@gmail.com.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

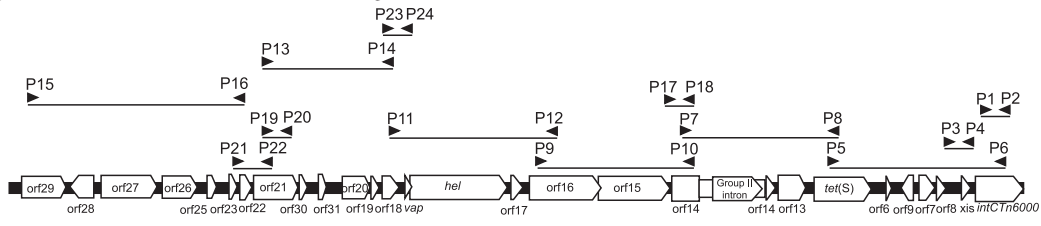
doi:10.1128/AAC.00758-12

TABLE 1 Epidemiological and genetic background of enterococci carrying *tet(S)*^d

Species	PFGE type	MLST clonal complex (sequence type)	Yr	Source	Sample	No. of isolates	Geographical location	Antibiotic resistance phenotype	Antibiotic resistance genotype	<i>tet(S)</i> location (size)	Genetic context of <i>tet(S)</i>
<i>E. casseliflavus</i> ^a	ND		1993	Monkey	Feces	1	Alberta, Canada	TET, STR	<i>tet(S)</i>	Chr	CTn6000 type A
<i>E. faecalis</i>	HV70	CC55 (ST55)	2001	Healthy human	Feces	1	North Portugal	TET, MIN, ERY, CHL, STR	<i>tet(S)</i> , <i>tet(M)</i> , <i>erm(B)</i>	Chr	CTn6000 type C
	I	(ST158)	2002	Hospitalized human	Wound, blood	1	Michigan, USA	TET, MIN, ERY, CIP, GEN, STR, VAN, TEC	<i>tet(S)</i> , <i>erm(B)</i> , <i>vanA</i>	Chr	ΔCTn6000 type D
	H	CC87 (ST28)	2002	Hospitalized human	Unknown	1	Belgrade, Serbia	TET, MIN, ERY, CIP, CHL, STR	<i>tet(S)</i> , <i>tet(M)</i> , <i>erm(B)</i>	PL (50 kb)	<i>tet(S)</i> platform type G
	SN242	(ST445)	2006	Piggery C ^b	Manure, residual water	2	Central Portugal	TET, MIN, ERY, CIP, CHL, STR	<i>tet(S)</i> , <i>tet(M)</i> , <i>tet(L)</i> , <i>erm(B)</i>	PL (115 kb)	<i>tet(S)</i> platform type H or I
<i>E. faecium</i>	A71	CC22 (ST32)	2008	Fountain	Drinking water	1	North Portugal	TET, MIN, ERY, CIP	<i>tet(S)</i>	Chr	CTn6000 type B
	SN210	CC22 (ST32)	2006	Piggery C ^b	Antiseptic ^c	1	Central Portugal	TET, MIN, ERY, CIP	<i>tet(S)</i> , <i>tet(M)</i> , <i>tet(L)</i>	Chr	CTn6000 type C
	Z	CC17 (ST17)	2002	Hospital sewage	Water	1	North Portugal	TET, MIN, ERY, CIP, GEN, STR, AMP	<i>tet(S)</i> , <i>tet(M)</i> , <i>erm(B)</i>	PL (40 kb)	<i>tet(S)</i> platform type G
<i>E. gallinarum</i>	F60		1999	Poultry	Carcass	1	North Portugal	TET, ERY, CIP, STR, VAN	<i>tet(S)</i> , <i>tet(M)</i> , <i>erm(B)</i> , <i>vanC1</i>	PL (70 kb)	<i>tet(S)</i> platform type E
	F61		1999	Poultry	Carcass	1	North Portugal	TET, MIN, ERY, VAN	<i>tet(S)</i> , <i>tet(L)</i> , <i>erm(B)</i> , <i>vanC1</i>	PL (45 kb)	<i>tet(S)</i> platform type E
<i>Enterococcus</i> spp.	SN244		2006	Piggery A ^b	Waste lagoon	1	South Portugal	TET, MIN	<i>tet(S)</i>	Chr	CTn6000 type C
	SN243		2006	Piggery C ^b	Food for animals	1	Central Portugal	TET, MIN, ERY, CIP, CHL, STR	<i>tet(S)</i> , <i>erm(B)</i>	PL (115 kb)	<i>tet(S)</i> platform type H
	SN245		2006	Piggery B ^b	Waste lagoon	1	South Portugal	TET, MIN, ERY, CIP	<i>tet(S)</i> , <i>erm(B)</i>	PL (70 kb)	<i>tet(S)</i> platform type F

^a This strain was previously characterized by Brouwer et al. (5), but it was included in Table 1 for comparison with enterococci carrying CTn6000 that were analyzed in this study.^b Different piggeries are identified with letters A, B, and C.^c Antiseptic solution used in piglets' skin after birth.^d Abbreviations: TET, tetracycline; MIN, minocycline; ERY, erythromycin; CIP, ciprofloxacin; CHL, chloramphenicol; STR, high level of resistance (HLR) to streptomycin; GEN, HLR to gentamicin; VAN, vancomycin; AMP, ampicillin; ND, not determined; Chr, chromosome; PL, plasmid.

TABLE 2 Strategies and conditions used for characterizing CTn6000



Primer group and name	Primer no.	Sequence (5'-3')	Position in CTn6000 (bp)	Amplicon size (bp)	Reference
Search of CTn6000					
<i>int</i> _{CTn6000} -F	1	CAT CGA GTC TAA CCG ATT GT	32158–32177	869	This study
<i>int</i> _{CTn6000} -R	2	GAC CCA AAC GAA CTT GTA CT	33007–33026		This study
<i>xis</i> _{CTn6000} -F	3	CGA AGT ATT AAC CGA ACA GA	31645–31664	203	This study
<i>xis</i> _{CTn6000} -R	4	TAT CAT CGC GAT CAA AAC GA	31828–31847		This study
PCR mapping, >3 kb					
<i>tetS</i> -F	5	TGG AAC GCC AGA GAG GTA TT	26732–26751	6,295	1
<i>int</i> _{CTn6000} -R	6	GAC CCA AAC GAA CTT GTA CT	33007–33026		This study
<i>orf14</i> _{CTn6000} -F	7	AGA CAG CTT CGC AAA AGT T	21766–21784	5,627	This study
<i>tetS</i> -R	8	ACA TAG ACA AGC CGT TGA CC	27373–27392		1
<i>orf16</i> _{CTn6000} -F	9	CAG GAA ACA ACG TTT TG	17408–17424	4,336	This study
<i>orf14</i> -R	10	CAC ATA AAA CAT ATT GCC ATA	21723–21743		This study
<i>orf18</i> _{CTn6000} -F	11	AAC AGG CCA ACT TGG TGA AG	11818–11837	6,161	This study
<i>orf16</i> -R	12	TCT GGC AAC GGT TCT TTC CA	17959–17978		This study
<i>orf21</i> -F	13	GGC TTG TAC TGT GAG C	8027–8042	3,811	This study
<i>orf18</i> _{CTn6000} -R	14	CTT CAC CAA GTT GGC CTG TT	11818–11837		This study
<i>orf29</i> -F	15	CGT CTT TGA GAC CGT ATT GG	1–20	7,013	This study
<i>orf23</i> _{CTn6000} -R	16	TTT CTC CGG CTG TAT GAG GT	6994–7013		This study
PCR mapping, <3 kb					
<i>orf14</i> II-F	17	CTG CGG AAT CGT TAG GTG TT	21520–21539	370	This study
<i>orf14</i> II-R	18	GCC GCT ACA GTC AAA AGA GG	21871–21890		This study
<i>orf21</i> II-F	19	AAC CCA TCG TCA AAA ACT CG	7795–7814	316	This study
<i>orf21</i> II-R	20	CTC GAT TGG CAA TCG TG	8095–8111		This study
<i>orf23</i> _{CTn6000} -F	21	TTG AAC AAC GAC GCA TCA AT	6897–6916	1,146	This study
<i>orf21</i> -R	22	AGC TCA CAG TAC AAG CC	8027–8043		This study
<i>orf18</i> FIII	23	TTG CCT TTT GAA ATT GGT GA	11600–11619	5 or 8	This study
VAPRII	24	CGA AAA CGG TCA GAT CAT CA	12195–12176		This study
IS1216F		ACC TTC ACG ATA GCT AAG GTT		Variable	14
<i>tetSR</i>	8			Variable	
IS1216R		AGG ATT ATA TAA GAA AAC CCG		Variable	14
<i>tetSR</i>	8				

nome sequences of three clinical American isolates, two *E. caseliflavus* strains and an ST9/CC9 *E. faecalis* β -lactamase-producing strain causing a large outbreak in the United States in 1986 (GenBank accession numbers NZ_GG692817.1, NZ_GG670387.1, and NZ_AEBZ01000002.1) (http://www.broadinstitute.org/annotation/genome/enterococcus_faecalis/GenomeDescriptions.html). Conversely, variants A and B were not detected in the GenBank databases. The analysis of the chromosomal integration sites of CTn6000 variants was performed as described previously (15) and revealed that types B and C use the gene coding for the L31 ribosomal protein as the hot spot for integration, as reported for the original CTn6000 type A (15). However, the L31-encoding gene was not the integration site for type D.

The *tet(S)* platforms located on plasmids corresponded to three *E. faecalis* isolates (2 PFGE types), one *E. faecium* isolate, two

E. gallinarum isolates, and two *Enterococcus* species isolates obtained from different hosts and arbitrarily designated types “E,” “F,” “G,” “H,” and “I” (Table 1 and Fig. 1). They contained IS1216 copies targeting different positions within *orf13*_{CTn6000} (types E and F), the *orf13*_{CTn6000}-*tet(S)* intergenic region (types G and H), and downstream *orf6*_{CTn6000} (type F). Type I comprises a copy of IS*Enta1* previously described in an *E. faecalis* isolate (AY884205.1) and a partial sequence of *cadE*, a gene linked to cadmium resistance which was also identified in type H. All sequences corresponding to *orf13*, the *orf13*-*tet(S)* intergenic region, and *orf6* showed a high degree of identity ($\geq 99\%$) with the original CTn6000 sequence.

Conjugative transfer of the CTn6000 variants A, B, C, and D was tested as previously described (15). Wild-type and recipient strains (*E. faecalis* JH2-2, *E. faecium* BM4105RF, and *Staphylococcus aureus* 8325) were used in a ratio of 1:1. Transconjugants were

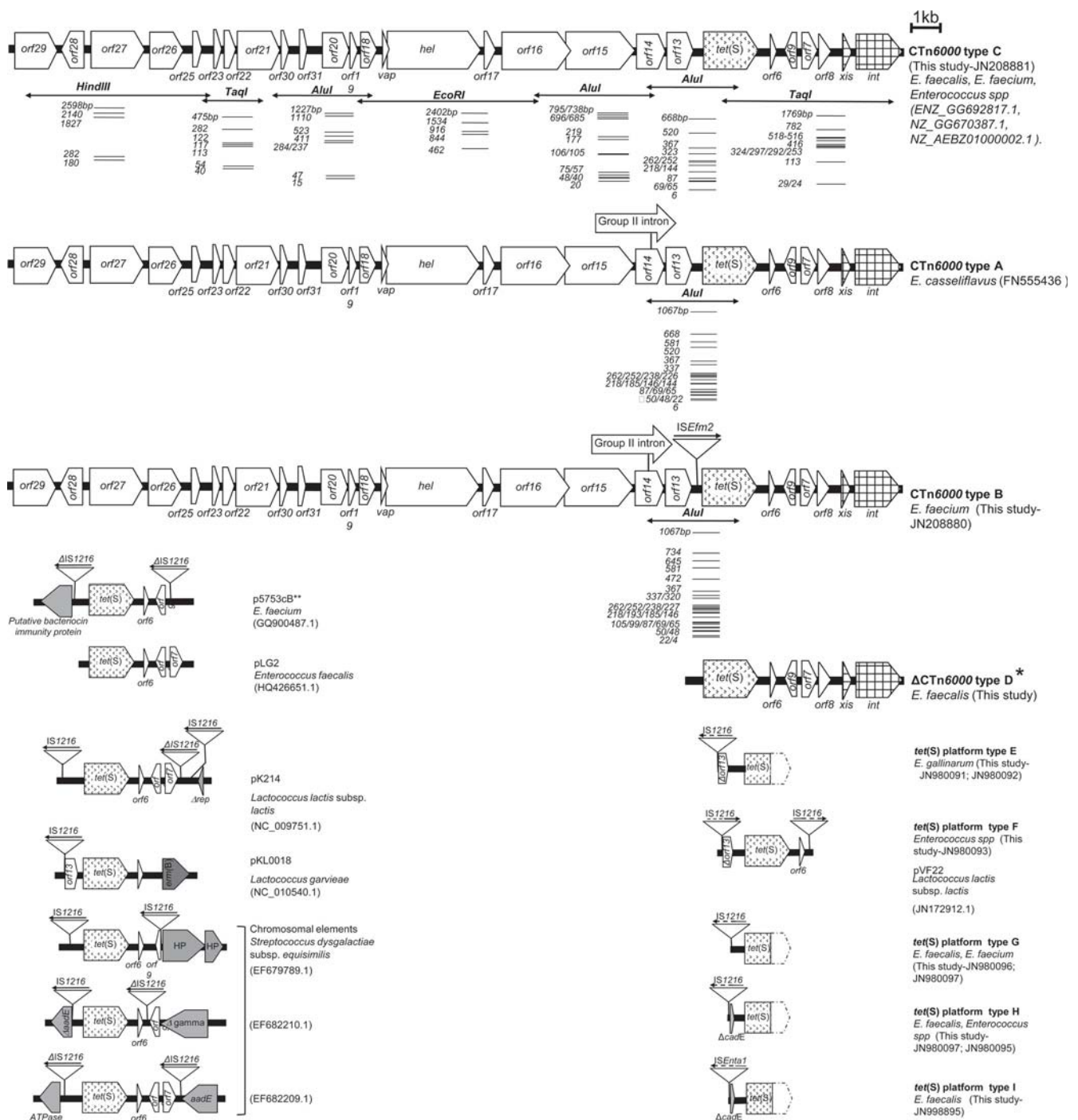


FIG 1 Diversity of complete and partial CTn6000 backbones and other genetic platforms carrying *tet(S)*. Boxes in gray indicate genes absent in CTn6000. Sequencing was accomplished for CTn6000 types C (fully), B, and D (partially). Amplicons of CTn6000 types B and D with RFLP patterns common to types A and C were not sequenced. *, this strain also carried the *orf23-orf29* CTn6000 region, although it was not able to be directly linked to the *int*_{CTn6000}-*tet(S)*. The boundaries of *tet(S)* were sequenced in *tet(S)* platform types E, F, G, H, and I. Dotted lines in *IS1216* and *tet(S)* gene schemes indicate that they were partially sequenced. **, the *tet(S)* gene is annotated as *tet(M)* in the sequence in GenBank under accession number [GQ900487.1](https://www.ncbi.nlm.nih.gov/nuccore/GQ900487.1).

selected in BHI agar supplemented with tetracycline (10 mg/liter), rifampin (25 mg/liter), and fusidic acid (5 mg/liter) and confirmed by resistance to the three antibiotics (disk diffusion) and by PCR amplification of CTn6000 *int1*/*xis* and/or *tet(S)*. We did not find any isolate fulfilling all these features, possibly due to the low

conjugation frequency (10^{-9}) of CTn6000 (15). Transfer of plasmids carrying *tet(S)* within each type of platform characterized was also not achieved.

This study documents the spread of *tet(S)* among enterococcal species from different origins mediated by CTn6000 or other ge-

netic platforms formed by composite structures often located on plasmids. Comparative analysis of the sequences corresponding to all *tet(S)* elements available at GenBank to date reflects the dynamics of *tet(S)* platforms by hosting different transposable elements involved in further arrangements mediated by insertion elements, often resulting in *IS1216* composite platforms.

Further analysis of enterococcal genomes and extrachromosomal genetic elements is necessary to fully understand the evolutionary “transferability” of *tet(S)* genetic contexts, that is, the ability to be successfully transferred and retained in different replicons and the resulting impact on the diversification and evolution of bacterial population structure (7).

ACKNOWLEDGMENTS

This project was funded by research grants from the European Commission (ACE-LSHM-2007-037410, EVOTAR-LSHM-2011-282004), the Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III, Spanish Ministry of Science and Innovation (PS09/02381), and the Fundação para a Ciência e Tecnologia (PTDC/AAC-AMB/103386/2008 and through grant no. PEst-C/EQB/LA0006/2011). Eduarda Silveira is supported by a Ph.D. fellowship of the Fundação para a Ciência e Tecnologia (grant number SFRH/BD/63955/2009; POPH-QREN). Work in the laboratory of A.P.R. is supported by grants of the European Commission (LSHM-2007-241446).

REFERENCES

1. Aarestrup FM, Agero Y, Gerner-Smidt P, Madsen M, Jensen LB. 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn. Microbiol. Infect. Dis.* 37(2):127–137.
2. Atkinson BA, Abu-Al-Jaibat A, LeBlanc DJ. 1997. Antibiotic resistance among enterococci isolated from clinical specimens between 1953 and 1954. *Antimicrob. Agent. Chemother.* 41:1598–1600.
3. Bacic M, et al. 2005. Genetic and structural analysis of the *Bacteroides* conjugative transposon CTn341. *J. Bacteriol.* 187:2858–2869.
4. Brenciani A, et al. 2010. *Phim46.1*, the main *Streptococcus pyogenes* element carrying *mef(A)* and *tet(O)* genes. *Antimicrob. Agents Chemother.* 54:221–229.
5. Brouwer MS, Mullany PP, Roberts AP. 2010. Characterization of the conjugative transposon Tn6000 from *Enterococcus casseliflavus* 664.1H1 (formerly *Enterococcus faecium* 664.1H1). *FEMS Microbiol. Lett.* 309:71–76.
6. Clinical and Laboratory Standards Institute. 2010. Performance standards for antimicrobial susceptibility testing; 20th informational supplement. CLSI document M100-S20. Clinical and Laboratory Standards Institute, Wayne, PA.
7. Coque TM, Freitas AR, Novais C, Peixe L, Baquero F. 2011. Mobile genetic elements and lateral genetic transfer in enterococci, p 59–100. In Smedo-Lemsaddek T, Barreto-Crespo MT, Tenreiro R (ed), *Enterococcus and safety. Advances in food safety and food microbiology*. Nova Science Publishers, Hauppauge, NY.
8. Freitas AR, et al. 2010. Global spread of the *hyl(Efm)* colonization-virulence gene in megaplasmids of the *Enterococcus faecium* CC17 polyclonal subcluster. *Antimicrob. Agents Chemother.* 54:2660–2665.
9. Lancaster H, Roberts AP, Bedi R, Wilson M, Mullany P. 2004. Characterization of Tn916S, a Tn916-like element containing the tetracycline resistance determinant *tet(S)*. *J. Bacteriol.* 186:4395–4398.
10. Liu LC, et al. 2008. Identification of *tet(S)* gene area in tetracycline-resistant *Streptococcus dysgalactiae* subsp. *equisimilis* clinical isolates. *J. Antimicrob. Chemother.* 61:453–455.
11. Maki T, Santos MD, Kondo H, Hirono I, Aoki T. 2009. A transferable 20-kilobase multiple drug resistance-conferring R plasmid (pKL0018) from a fish pathogen (*Lactococcus garvieae*) is highly homologous to a conjugative multiple drug resistance-conferring enterococcal plasmid. *Appl. Environ. Microbiol.* 75:3370–3372.
12. Melville CM, Brunel R, Flint HJ, Scott KP. 2004. The *Butyrivibrio fibrisolvens tet(W)* gene is carried on the novel conjugative transposon TnB1230, which contains duplicated nitroreductase coding sequences. *J. Bacteriol.* 186:3656–3659.
13. Novais C, et al. 2009. Characterization of Gram-positive conjugative transposons among international epidemic vancomycin-resistant isolates of *Enterococcus faecalis* and *Enterococcus faecium*, abstr P949, p S247. Abstr. 19th Eur. Congr. Clin. Microbiol. Infect. Dis., Helsinki, Finland.
14. Novais C, et al. 2008. Diversity of Tn1546 and its role in the dissemination of vancomycin-resistant enterococci in Portugal. *Antimicrob. Agents Chemother.* 52:1001–1008.
15. Roberts AP, Davis IJ, Seville L, Villedieu A, Mullany P. 2006. Characterization of the ends and target site of a novel tetracycline resistance-encoding conjugative transposon from *Enterococcus faecium* 664.1H1. *J. Bacteriol.* 188:4356–4361.
16. Roberts AP, Mullany P. 2009. A modular master on the move: the Tn916 family of mobile genetic elements. *Trends Microbiol.* 17:251–258.
17. Thaker M, Spanogiannopoulos P, Wright GD. 2010. The tetracycline resistome. *Cell. Mol. Life Sci.* 67:419–431.

CAPÍTULO 4

Conclusões gerais

Como **CONCLUSÃO GERAL**, ressalta deste trabalho que:

*A aquisição de genes de tolerância a biocidas por *Enterococcus* spp., residentes em diferentes comunidades ecológicas, pode favorecer a seleção, manutenção e evolução genómica de estirpes resistentes a antibióticos pertencentes a diversas linhagens clonais.*

Esta conclusão geral é suportada nas ilações obtidas nas seguintes conclusões parcelares:

4.1. EPIDEMIOLOGIA DA TOLERÂNCIA A BIOCIDAS EM *Enterococcus* spp.

Os genes de tolerância aos biocidas estudados não possuem uma especificidade de nicho ecológico, de espécie ou clonal.

4.1.1. ECOLOGIA

4.1.1.1 *Distintas comunidades ecológicas são reservatório de genes de tolerância ao cobre e mercúrio.*

- A deteção dos mesmos genes em *Enterococcus* spp. do nicho humano, animal, ambiental e em alimentos, indica o seu fluxo entre vários ambientes e hospedeiros.
- Pressões seletivas locais podem determinar o seu grau de abundância através de fenómenos de seleção e manutenção. Destaca-se, por exemplo, uma maior prevalência de genes de tolerância ao cobre (*tcrB* e *cueO*) em suiniculturas, produção intensiva animal em que se usam maiores concentrações deste metal como aditivo alimentar.

4.1.1.2. *Condições ambientais propícias podem determinar o aparecimento de novas características adaptativas: tolerância a compostos de amónio quaternário (QACs).*

- Genes de tolerância a QACs, frequentemente descritos em bactérias de Gram positivo e negativo, são raros em *Enterococcus* spp.
- Sendo os QACs largamente utilizados, outros determinantes genéticos desconhecidos podem estar a promover a adaptação de *Enterococcus* spp. a estes biocidas.
- A deteção de *qacZ* num isolado obtido de um esgoto hospitalar sugere que, em condições propícias (v.g, elevada pressão seletiva, disponibilidade no metagenoma local ou associação a elementos móveis e de recombinação), estes genes podem ser

captados por *Enterococcus* spp., contribuindo para a sua adaptação aos desafios locais.

4.1.2. ESTRUTURA POPULACIONAL

4.1.2.1. Desafios ambientais comuns determinam genótipos comuns em espécies distintas.

- Genes de tolerância a biocidas estão distribuídos em diferentes espécies de *Enterococcus* de vários nichos ecológicos. Estes dados sugerem um fluxo e manutenção dos genes estudados entre os membros de comunidades ecológicas, potencialmente, sujeitas a desafios ambientais similares.
- Apesar desta distribuição, os dados indicam que *E. faecium* é o reservatório mais relevante dos genes como *tcrB*, *cueO*, *merA*₂ e *qacZ*, corroborando a sua elevada plasticidade genética, associada a uma elevada adaptação a diferentes ecossistemas e hospedeiros.
- A ocorrência de genes diferentes (*tcrB*, *cueO* ou *merA*) nos mesmos isolados reforça a capacidade de adaptação de *Enterococcus* spp. a múltiplos desafios ambientais. Adicionalmente, o aumento da tolerância ao CuSO₄ de *Enterococcus* spp. portadores de *tcrB* e/ou *cueO*, ou a cloreto de benzalcónio naqueles portadores de *qacZ*, reforça a relevância da aquisição destes genes na adaptação a desafios químicos ambientais.
- A tolerância ao sulfato de cobre em isolados não portadores de genes *tcrB* e/ou *cueO*, apontam para a existência de outros determinantes genéticos associados à tolerância ao cobre em alguns *E. faecium* e, sobretudo, em outras espécies de *Enterococcus*. Mais estudos serão necessários para compreender o *biocidoma* associado a *Enterococcus* spp.

4.1.2.2. Múltiplas linhagens clonais de *E. faecium* e *E. faecalis* são reservatórios de genes que codificam para a tolerância a biocidas.

- Genes de tolerância ao cobre, mercúrio, ou compostos de amónio quaternário contribuem para a sobrevivência, adaptação e persistência de diversas linhagens clonais de *E. faecium* e *E. faecalis* em comunidades ecológicas distintas. Destaca-se um clone de *E. faecium*-CC5 (PFGE A) disperso no ambiente de suiniculturas portuguesas, assim como em suínos de outros países (Freitas *et al.*, 2011) e

persistente durante anos (este estudo e Novais *et al.*, 2005). Isolados pertencentes a este clone são portadores dos genes *tcrB* e *cueO*, sugerindo que o uso intensivo de cobre na alimentação animal contribui para a sua seleção e sucesso. Destacam-se também linhagens clonais associadas a infecção humana (*E. faecium* ST17, ST18, ST78; *E. faecalis* CC2) observadas em distintos nichos ecológicos e com genes de tolerância ao cobre, mercúrio, ou cloreto de benzalcónio, corroborando a hipótese de que o sucesso destas estirpes está associado à acumulação de diferentes características adaptativas, incluindo a tolerância a biocidas, como mostrado neste estudo. Esta acumulação de genes pode também contribuir para a invasão de novas comunidades ecológicas por estas linhagens clonais, incrementando a sua dispersão, recombinação genética e, potencialmente, a colonização de novos hospedeiros.

4.2. TOLERÂNCIA A BOCIDAS E RESISTÊNCIA A ANTIBIÓTICOS: TRANSMISSÃO HORIZONTAL DE GENES E CARACTERIZAÇÃO DE ELEMENTOS GENÉTICOS.

A transferência horizontal de genes aliada a fenómenos de recombinação genética contribui para a evolução adaptativa de Enterococcus spp. a diferentes desafios ambientais e manutenção da resistência a antibióticos.

4.2.1. O ambiente de suiniculturas é um reservatório subestimado de Enterococcus spp. com resistência múltipla a antibióticos que podem ser disseminados facilmente entre animais e potencialmente aos humanos.

- Genes que codificam para resistência a diversos antibióticos (por exemplo, tetraciclina, macrólidos, aminoglicosídeos, ampicilina ou glicopéptidos) são frequentemente co-disseminados, em eventos de transmissão horizontal, conduzindo à manutenção de estirpes com resistência múltipla.
- O contacto contínuo dos suínos com estas estirpes por diferentes vias (por exemplo, alimentos, pó, ar, pavimento e paredes) pode diminuir o impacto de medidas restritivas do uso de antibióticos e reforça a necessidade de intervenções preliminares ao nível da gestão pecuária.

4.2.2. Diferentes compostos podem contribuir para a seleção e manutenção de bactérias resistentes a antibióticos.

- Eventos de transferência horizontal de genes têm um papel relevante na co-dispersão da tolerância ao cobre, mercúrio e cloreto de benzalcônio, juntamente com a resistência a antibióticos.
- A expressão de tolerância/resistência a todos estes compostos em bactérias transconjugantes, aliada à sua co-localização nos mesmos plasmídeos, sugere que diferentes pressões seletivas podem favorecer a manutenção da resistência a antibióticos em diversas comunidades ecológicas (humanos, animais e ambiente).

4.2.3. Plasmídeos de diferentes famílias estão associados à co-dispersão da tolerância a biocidas e resistência a antibióticos.

Genes de tolerância ao cobre e mercúrio estão frequentemente associados a megaplasmídeos móveis, que transportam habitualmente genes de resistência à tetraciclina e/ou eritromicina.

- A maior prevalência de *tcrB*, *cueO* e *merA₂* em *E. faecium* parece estar relacionada com a localização destes genes em plasmídeos tipo pLG1, característicos desta espécie e com reduzido espectro de hospedeiros bacterianos, limitando a disseminação mais ampla destes genes. Uma maior caracterização destes plasmídeos é crítica para compreender o seu papel na adaptação ecológica e patogenicidade de *E. faecium*, tendo em conta que num deles ocorriam genes que codificam para fatores de virulência e metabolismo de carboidratos (Laverde *et al.*, 2011). Estudos moleculares mais detalhados são necessários para identificar o tipo de plasmídeos envolvidos em outras espécies.
- A associação de *qacZ* com um plasmídeo do tipo Inc18 corrobora a contribuição destes plasmídeos para a plasticidade genética de *Enterococcus* spp., quer associada à dispersão de genes de resistência a antibióticos, quer também à tolerância a biocidas.

4.2.4. O tipo de genes de resistência a antibióticos co-disseminados com genes de tolerância a biocidas reflete a sua abundância nas diferentes comunidades ecológicas.

A abundância de um determinado gene/elemento genético pode contribuir para o seu maior envolvimento em contextos de recombinação com diferentes elementos genéticos e,

consequentemente, originar um incremento na sua seleção e manutenção por diferentes compostos com impacto ecológico local:

- *Genes tet e/ou erm(B) (co-localizados com genes de tolerância a mercúrio, cobre, ou cloreto de benzalcônio)*: verificou-se que se encontram muito dispersos em diferentes nichos ecológicos, devido ao elevado uso de tetraciclinas e macrólidos na produção animal e na terapêutica humana. A sua inserção [ex: *tet(M)*, *tet(S)*, *erm(B)*] em transposões conjugativos muito recombináveis, favorece a sua inclusão em diferentes plasmídeos, assim como em outras partes do genoma.
- *Novo mecanismo de tolerância ao cobre transferido com resistência à ampicilina*: ocorreu em *E. faecium* das linhagens clonais ST18 e ST78, frequentemente associadas a infecção humana. A resistência à ampicilina é considerada um dos primeiros marcadores do sucesso destas linhagens, sendo a quase totalidade dos seus membros resistentes a este antibiótico. A descrição rara da transferência da resistência à ampicilina foi associada a transposões conjugativos envolvidos, frequentemente, em eventos de recombinação (Tn5382, Tn916, Tn5386) (Carias *et al.*, 1998; Rice *et al.*, 2007). Mais estudos são necessários para identificar este novo mecanismo de tolerância ao cobre e determinar o seu papel na patogenicidade de *E. faecium* (v.g., escape ao sistema imunitário).
- *Genes vanA (co-localizados com genes de tolerância cobre)*: foram observados apenas em estirpes do ambiente clínico (doentes hospitalizados e esgoto hospitalar), possivelmente por ser este o nicho que apresenta maior frequência de resistência aos glicopéptidos.

4.2.5. Módulos genéticos contendo genes de resistência a antibióticos e/ou tolerância a biocidas, flanqueados por sequências de inserção, contribuem para a plasticidade e evolução adaptativa de *Enterococcus* spp.

- A necessidade urgente de adaptação a diferentes condições de stress (presença de antibióticos ou biocidas) pode acelerar fenómenos de adaptação evolutiva, mediados por sequências de inserção (IS). Diferentes ISs estão envolvidas na plasticidade genética (incorporação e excisão de genes) de *Enterococcus* spp., destacando-se a IS1216, quer em plataformas de resistência a antibióticos (CTn6000), quer de tolerância a biocidas (plasmídeos do tipo pLG1 e Inc18).

- Genes de tolerância a biocidas estão incorporados em zonas altamente recombinadas, mediadas por ISs e recombinases de fagos, que podem favorecer uma continua evolução local por incorporação e excisão de módulos.
- A expressão de funções adaptativas (resistência/tolerância a diversos compostos por incorporação de novos genes) pode favorecer a replicação nas estruturas modulares superiores (por exemplo, plasmídeos) e, conseqüentemente, a sobrevivência dos hospedeiros bacterianos que os possuem, aumentando a tendência para manter essas mesmas estruturas modulares relevantes na adaptação a desafios ambientais em comunidades ecológicas particulares.

4.2.6. Diferentes membros do filo Firmicutes contribuem para a adaptação evolutiva de *Enterococcus* spp. a antibióticos e biocidas.

- Genes, ou módulos de genes associados à tolerância a biocidas (*tcrB*, *cueO*, *merA*) e resistência à tetraciclina (CTn6000) são partilhados entre *Enterococcus* spp. e diferentes gêneros bacterianos pertencentes às mesmas comunidades ecológicas, que, conseqüentemente, estão sujeitos aos mesmos desafios ambientais. A sobrevivência a estes desafios e a partilha de genes entre vários gêneros capazes de migrar entre diferentes ecossistemas (por exemplo, humanos, animais e ambiente aquático) deve ser tida em consideração, uma vez que todos podem contribuir para uma globalização ambiental da resistência a antibióticos e da tolerância a biocidas.

4.3. CONSIDERAÇÕES FINAIS

Diferentes lacunas na área de conhecimento dos biocidas têm vindo a ser apontadas por diferentes entidades, como a Comunidade Europeia (SCENIHR, 2010). A falta de informação neste tópico tem impedido a compreensão do impacto do uso de biocidas na saúde pública. É, entre outros aspectos, referida a inexistência de programas de vigilância, o que limita a obtenção de dados epidemiológicos robustos e o conhecimento sobre a seleção e disseminação de genes de resistência a antibióticos associados ao uso de biocidas. É também referida a ausência de métodos standardizados para avaliação da susceptibilidade dos microrganismos a estes compostos (SCENIHR, 2010).

Dentro do universo “*Enterococcus*”, um patogénico emergente nas últimas décadas e um género bacteriano com capacidade de penetrar em diversas comunidades biológicas e interagir, geneticamente, com diferentes microrganismos, os resultados deste estudo contribuíram para:

- a) Conhecer a epidemiologia da tolerância ao cobre, mercúrio e cloreto de benzalcónio em *Enterococcus* spp. usando, pela primeira vez, uma coleção com um vasto número de isolados representativos de diferentes comunidades ecológicas (n=920). Assim, ficou patente a distribuição de diferentes genes de tolerância a biocidas por nichos sujeitos a diversas pressões seletivas, por espécies e linhagens clonais.
- b) Compreender como o uso de biocidas pode contribuir para a seleção e manutenção de bactérias resistentes a antibióticos, através do conhecimento dos elementos genéticos envolvidos e na sua possível evolução adaptativa.

Uma melhor compreensão destes aspectos contribuirá para o planeamento robusto de estudos mais específicos do impacto de biocidas na evolução, emergência e patogenicidade de *Enterococcus* spp.

I

Permissões de inclusão de publicações e imagens



RightsLink®

[Home](#)
[Account Info](#)
[Help](#)


Title: Spread of multidrug-resistant Enterococcus to animals and humans: an underestimated role for the pig farm environment:

Author: Carla Novais, Ana R. Freitas, Eduarda Silveira, Patrícia Antunes, Ricardo Silva, Teresa M. Coque, Luísa Peixe

Publication: Journal of Antimicrobial Chemotherapy

Publisher: Oxford University Press

Date: 12/01/2013

Copyright © 2013, Oxford University Press

Logged in as:
Eduarda Silveira
Account #:
3000729325

[LOGOUT](#)

Order Completed

Thank you very much for your order.

This is a License Agreement between Eduarda Silveira ("You") and Oxford University Press ("Oxford University Press"). The license consists of your order details, the terms and conditions provided by Oxford University Press, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3294810436911
License date	Dec 23, 2013
Licensed content publisher	Oxford University Press
Licensed content publication	Journal of Antimicrobial Chemotherapy
Licensed content title	Spread of multidrug-resistant Enterococcus to animals and humans: an underestimated role for the pig farm environment:
Licensed content author	Carla Novais, Ana R. Freitas, Eduarda Silveira, Patrícia Antunes, Ricardo Silva, Teresa M. Coque, Luísa Peixe
Licensed content date	12/01/2013
Volume number	68
Issue number	12
Type of Use	Thesis/Dissertation
Requestor type	Academic/Educational institute
Format	Print
Portion	Full article
Will you be translating?	No
Author of this OUP article	Yes
Order reference number	
Title of your thesis / dissertation	TOLERÂNCIA A BIOCIDAS E DISPERSÃO DA RESISTÊNCIA A ANTIBIÓTICOS: UMA ABORDAGEM FENOTÍPICA E MOLECULAR EM Enterococcus spp DE DIFERENTES ORIGENS
Expected completion date	Mar 2014
Estimated size(pages)	200
Publisher VAT ID	GB 125 5067 30
Total	0.00 EUR

[ORDER MORE...](#)
[CLOSE WINDOW](#)



RightsLink®

[Home](#)
[Account Info](#)
[Help](#)


OXFORD JOURNALS
OXFORD UNIVERSITY PRESS

Author: Eduarda Silveira, Ana R. Freitas, Patrícia Antunes, Mariana Barros, Joana Campos, Teresa M. Coque, Luísa Peixe, Carla Novais

Publication: Journal of Antimicrobial Chemotherapy

Publisher: Oxford University Press

Date: first published online December 15, 2013

Copyright © 1969, Oxford University Press

Logged in as:
Eduarda Silveira
Account #:
3000729325

[LOGOUT](#)

Order Completed

Thank you very much for your order.

This is a License Agreement between Eduarda Silveira ("You") and Oxford University Press ("Oxford University Press"). The license consists of your order details, the terms and conditions provided by Oxford University Press, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3294800861734
License date	Dec 23, 2013
Licensed content publisher	Oxford University Press
Licensed content publication	Journal of Antimicrobial Chemotherapy
Licensed content title	
Licensed content author	Eduarda Silveira, Ana R. Freitas, Patrícia Antunes, Mariana Barros, Joana Campos, Teresa M. Coque, Luísa Peixe, Carla Novais
Licensed content date	first published online December 15, 2013
Type of Use	Thesis/Dissertation
Requestor type	Academic/Educational institute
Format	Print
Portion	Full article
Will you be translating?	No
Author of this OUP article	Yes
Order reference number	
Title of your thesis / dissertation	TOLERÂNCIA A BIOCIDAS E DISPERSÃO DA RESISTÊNCIA A ANTIBIÓTICOS: UMA ABORDAGEM FENOTÍPICA E MOLECULAR EM Enterococcus spp DE DIFERENTES ORIGENS
Expected completion date	Mar 2014
Estimated size(pages)	200
Publisher VAT ID	GB 125 5067 30
Total	0.00 EUR

[ORDER MORE...](#)
[CLOSE WINDOW](#)

Copyright © 2013 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#).
Comments? We would like to hear from you. E-mail us at customercare@copyright.com



RightsLink®

[Home](#)
[Create Account](#)
[Help](#)


**AMERICAN
SOCIETY FOR
MICROBIOLOGY**

Title: Different Genetic Supports for the tet(S) Gene in Enterococci

Author: Carla Novais, Ana R. Freitas, Eduarda Silveira et al.

Publication: Antimicrobial Agents and Chemotherapy

Publisher: American Society for Microbiology

Date: Nov 1, 2012

Copyright © 2012, American Society for Microbiology

User ID

Password

☐ Enable Auto Login

LOGIN

[Forgot Password/User ID?](#)

If you're a copyright.com user, you can login to RightsLink using your copyright.com credentials. Already **a RightsLink user** or want to [learn more?](#)

Permissions Request

Authors in ASM journals retain the right to republish discrete portions of his/her article in any other publication (including print, CD-ROM, and other electronic formats) of which he or she is author or editor, provided that proper credit is given to the original ASM publication. ASM authors also retain the right to reuse the full article in his/her dissertation or thesis. For a full list of author rights, please see: http://journals.asm.org/site/misc/ASM_Author_Statement.xhtml

[BACK](#)
[CLOSE WINDOW](#)

Copyright © 2013 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement.](#)
Comments? We would like to hear from you. E-mail us at customercare@copyright.com



RightsLink®

[Home](#)
[Account Info](#)
[Help](#)


Title: Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract

Author: Günter Klein

Publication: International Journal of Food Microbiology

Publisher: Elsevier

Date: 1 December 2003

Copyright © 2003, Elsevier

Logged in as:
Eduarda Silveira

[LOGOUT](#)

Order Completed

Thank you very much for your order.

This is a License Agreement between Eduarda Silveira ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3285850745423
License date	Dec 11, 2013
Licensed content publisher	Elsevier
Licensed content publication	International Journal of Food Microbiology
Licensed content title	Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract
Licensed content author	Günter Klein
Licensed content date	1 December 2003
Licensed content volume number	88
Licensed content issue number	2–3
Number of pages	9
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	Yes
Number of languages	1
Languages	Portuguese
Title of your thesis/dissertation	TOLERÂNCIA A BIOCIDAS E DISPERSÃO DA RESISTÊNCIA A ANTIBIÓTICOS: UMA ABORDAGEM FENOTÍPICA E MOLECULAR EM Enterococcus spp DE DIFERENTES ORIGENS
Expected completion date	Mar 2014
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 EUR
VAT/Local Sales Tax	0.00 EUR / 0.00 GBP
Total	0.00 EUR



RightsLink®

[Home](#)
[Account Info](#)
[Help](#)


Title: Antimicrobial activity of metals: mechanisms, molecular targets and applications

Author: Joseph A. Lemire, Joe J. Harrison, Raymond J. Turner

Publication: Nature Reviews Microbiology

Publisher: Nature Publishing Group

Date: May 13, 2013

Copyright © 2013, Rights Managed by Nature Publishing Group

Logged in as:
Eduarda Silveira
Account #:
3000729325

[LOGOUT](#)

Order Completed

Thank you very much for your order.

This is a License Agreement between Eduarda Silveira ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3294751381358
License date	Dec 23, 2013
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Microbiology
Licensed content title	Antimicrobial activity of metals: mechanisms, molecular targets and applications
Licensed content author	Joseph A. Lemire, Joe J. Harrison, Raymond J. Turner
Licensed content date	May 13, 2013
Type of Use	reuse in a dissertation / thesis
Volume number	11
Issue number	6
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	2
High-res required	no
Figures	Figure 2
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	TOLERÂNCIA A BIOCIDAS E DISPERSÃO DA RESISTÊNCIA A ANTIBIÓTICOS: UMA ABORDAGEM FENOTÍPICA E MOLECULAR EM Enterococcus spp DE DIFERENTES ORIGENS
Expected completion date	Mar 2014
Estimated size (number of pages)	200
Total	0.00 EUR

[ORDER MORE...](#)
[CLOSE WINDOW](#)

Copyright © 2013 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#).
Comments? We would like to hear from you. E-mail us at customercare@copyright.com



RightsLink®

[Home](#)
[Account Info](#)
[Help](#)


Title: Response of Gram-positive bacteria to copper stress
Author: Marc Solioz
Publication: Journal of Biological Inorganic Chemistry
Publisher: Springer
Date: Jan 1, 2009
 Copyright © 2009, SBIC

Logged in as:
 Eduarda Silveira
 Account #:
 3000729325

[LOGOUT](#)

Order Completed

Thank you very much for your order.

This is a License Agreement between Eduarda Silveira ("You") and Springer ("Springer"). The license consists of your order details, the terms and conditions provided by Springer, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3302601114887
License date	Jan 05, 2014
Licensed content publisher	Springer
Licensed content publication	Journal of Biological Inorganic Chemistry
Licensed content title	Response of Gram-positive bacteria to copper stress
Licensed content author	Marc Solioz
Licensed content date	Jan 1, 2009
Volume number	15
Issue number	1
Type of Use	Thesis/Dissertation
Portion	Figures
Author of this Springer article	No
Title of your thesis / dissertation	TOLERÂNCIA A BIOCIDAS E DISPERSÃO DA RESISTÊNCIA A ANTIBIÓTICOS: UMA ABORDAGEM FENOTÍPICA E MOLECULAR EM Enterococcus spp DE DIFERENTES ORIGENS
Expected completion date	Mar 2014
Estimated size(pages)	200
Total	0.00 EUR

[CLOSE WINDOW](#)

Copyright © 2014 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#).
 Comments? We would like to hear from you. E-mail us at customercare@copyright.com



Sequências nucleotídicas submetidas ao GenBank

Nucleotide

Display Settings: ☒ GenBank

Enterococcus faecalis strain C386 transposon Tn6000, complete sequence

GenBank: JN208881.1

[FASTA](#) [Graphics](#)Go to: 

LOCUS JN208881 30621 bp DNA linear BCT 30-OCT-2012

DEFINITION Enterococcus faecalis strain C386 transposon Tn6000, complete sequence.

ACCESSION JN208881

VERSION JN208881.1 GI:347952137

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM [Enterococcus faecalis](#)
 Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 30621)
 AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.
 TITLE Different Genetic Supports for the tet(S) Gene in Enterococci
 JOURNAL Antimicrob. Agents Chemother. 56 (11), 6014-6018 (2012)
 PUBMED [22908170](#)

REFERENCE 2 (bases 1 to 30621)
 AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.
 TITLE Spread of tet(S) among enterococci is associated with CTn6000
 JOURNAL Unpublished

REFERENCE 3 (bases 1 to 30621)
 AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.
 TITLE Direct Submission
 JOURNAL Submitted (05-JUL-2011) Microbiology, REQUIMTE Pharmacy Faculty, Porto University, Rua Anibal Cunha, 164, Porto 4050-047, Portugal

FEATURES

source Location/Qualifiers
 1..30621
 /organism="Enterococcus faecalis"
 /mol_type="genomic DNA"
 /strain="C386"
 /isolation_source="feces from a healthy human"
 /host="Homo sapiens"
 /db_xref="taxon:[1351](#)"
 /country="Portugal"
 /collection_date="2001"

[mobile element](#) 1..30621
 /note="conjugative transposon"
 /mobile_element_type="transposon:Tn6000"

[repeat region](#) 67..180
 /note="imperfect repeat"
 /rpt_type=direct

[CDS](#) 593..2005
 /note="ORF29; putative methyltransferase"
 /codon_start=1
 /transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33195.1](#)"
 /db_xref="GI:347952138"
 /translation="MVRYNWVVQNILNKEEGTLIIDKEIVLSAMKQKNIKTQTELAER LQMSKSQLSQMLSEGFSPLKTNVVKLTNELGLSYAEILKEELSDTMTTIVKKHATNN KQLELFNDDSLDRFKYKLDEFIDIQNTTPKRDTLVETFAGAGGLSLGLEKAGFNSVA DIEIDSTACSTLKLNRPNWNVIEGDINTIAQNGIFNHDQFTFRGELDLVSGGYPCQSF SYAGLRQGLNDTRGTLFHPYSQLLVQLQPKMFAENVKGLLNHDNGRTLETMIEIFED SGYQVFWNINLAWNYGVAQKRERIVIIIGIRNDLFEQQQYSFKFPKEYTYQPVLRDVLK DVPKSEGASYPASKIEVLNLVPPGGCWNLPENVAKSVMGASYHSGGRRGMARRLSW NEPSLTLTTSAPQKQTERCHPDETRPFVTREYARIQSFPDDWEFAGSTNNQYKQIGNA VPVNLAEAGKSVVNYLNQF"

[CDS](#) complement(2049..2768)
 /note="ORF28; putative methylase"
 /codon_start=1
 /transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33196.1](#)"
 /db_xref="GI:347952139"

CDS

```

/translation="MTEKAQNNDEIEKAKDFFREVIKHNHKNTEKLNINNFVNP
FLNKYLANYLGNDDPESVSKALIYPRVLGTSINTSFGSNLQKFINKTLEGYSTTTG
MDIEFIDKIDGRKKYCQIKAGPQTINYDDVTTIQNHFKSAINLARTNNIPIANMDCVV
GVFYGEDSELSAFYRTLAEDYTVLAGKDFWYHLTGDEDFYQKLTGDIGDIANEFDSTE
LLEDTIKALAEQLKNIEGIEE"

```

```

3031..4827

```

```

/note="ORF27"

```

```

/codon_start=1

```

```

/transl_table=11

```

```

/product="hypothetical protein"

```

```

/protein_id="AEP33197.1"

```

```

/db_xref="GI:347952140"

```

```

/translation="MTKFIDKINELERIEITKYYTEFRDGFKEIHDWAKSEGFLNEAK
LAKYEIDICSLIEKKPILSNNKSDTRFVPEYSITGIGNVPEITAFDEQFLYERRMI
ETDNIFLKVRYSDFLFEYGSEKVTTRKKYQISQFLLASLVESCRKYQTVHEDSNFILSI
SRLVEVSLSMNNKYLQQAIIQLIHSQLIESRDIIDNSIWACNLSQLVRIILDSKFKGYI
SEELYTCTLTILTNLKNKCFENHEHYHFLRFIEESIALGKFEAYNTEQIQNLRDIDIGK
SYLEAEYQGGRRQKSSLTAKFNLEQALEYKKNMGETEKINDLVLIKQTYETYQQSD
EMATIKIPFTIPTDYIDNCISPYIISNIHESLDKLAKSDEFIPNVSIKQQQSLQEAD
NNFFNLVSKSIIHGGNKIVNAETKEESKKFIFITSYVQELTNLEIFLKAIFEKLEIE
HSLSDAHLMEKFDWSKMLDERNYPLVKAGIENFFKEDYVSSIHLVLPQFESTFRFLA
KLGFATTSLKKDMVQYIITFNEFLVKDYIKTFLGEDIHITLIQIIMVEPMGLNLRNEIA
HGLIKQSDITKSKCILVIYFLILTRYDDNKE"

```

CDS

```

4918..5997

```

```

/note="ORF26; putative methlytransferase"

```

```

/codon_start=1

```

```

/transl_table=11

```

```

/product="hypothetical protein"

```

```

/protein_id="AEP33198.1"

```

```

/db_xref="GI:347952141"

```

```

/translation="MIRITNLTGSLFDGIGVFPLAAQKQGITLSWAGEIEKAPIRIT
KKQFPHMIHLGDLTKLHGGKIPVDIVTFGSPCQNLSTIGNREGLAGKSNLFYEAIR
IIEEMRDA'NGKYPTLAIWENVMGAFSSNDRMDFKAVLEAFVQAEVPIPPSRQWANAG
MVRGRNVDCWRVLDQYWGVPQRRRIFLVADFRDFRAREILFDTQGMQSHLETCDQ
NRLSSPKDNRSLLTKAGRQLFLYPFQERRMRTYAKEKNTKGFVSSFGQSTDPFPTLA
GTVDTFAYWQEGFETEGFVRKLIPLCECERIMGLPENWTQYGFQDNQLISDSARYKALGN
SIVVPCAEFILSNVAKVYKGESYG"

```

CDS

```

6219..6626

```

```

/note="ORF25; putative anti-restriction protein; similar
to interrupted orf18"

```

```

/codon_start=1

```

```

/transl_table=11

```

```

/product="hypothetical protein"

```

```

/protein_id="AEP33199.1"

```

```

/db_xref="GI:347952142"

```

```

/translation="MDNTIQVYVTNLSRFEYDGLIIAAWFTLPVSLDELREKLQLETD
ESYEIEDWEAPFRLTDDGDILTNRLLALLIEENCHYDLFPYLDELIDLDFVESHEDGL
KRLNHFEHFEEKPTDPDILEDTFELSDGTFTYFTI"

```

CDS

```

6774..7088

```

```

/note="putative conjugation related protein; ORF23;
Tn916-like protein"

```

```

/codon_start=1

```

```

/transl_table=11

```

```

/product="hypothetical protein"

```

```

/protein_id="AEP33200.1"

```

```

/db_xref="GI:347952143"

```

```

/translation="MELKHVVPIMKDTFGHLEFAGEGNEQRRINGRLATVSRSYNLY
SDIQRADDIEVILPHTAGEKFFEVEESVKLINPRIIAEGYKIGERGFTNYILHADDMV
KA"

```

CDS

```

7104..7469

```

```

/note="ORF22; Tn916-like protein; putative conjugation
related protein"

```

```

/codon_start=1

```

```

/transl_table=11

```

```

/product="hypothetical protein"

```

```

/protein_id="AEP33201.1"

```

```

/db_xref="GI:347952144"

```

```

/translation="MRLAQGIIIDKEKTFGRLLKFSALRRVFLNEDGSVSTEVKERT
YDLKSREQGRMIQVSIPASIPREFDYNAEVELINPIADTVANATYRGADVVDWYIKAD
DLVLKNKTTPKIEPKNKQG"

```

CDS

```

7473..8849

```

```

/note="ORF21; Tn916-like protein; FtsK/SpoIIIE family
protein"

```

```

/codon_start=1

```

```

/transl_table=11

```

```

/product="hypothetical protein"

```

```

/protein_id="AEP33202.1"

```

```

/db_xref="GI:347952145"

```

```

/translation="MKLLQRKGKIRPSDKHLVFQFFFGAILSCWLLAFFPFLAFFS
RTTWQLLEEFKQHFSPYGSLSLVFSLLTVGFLLYLLYRYGFDYKSLTHRQKLARMIL"

```

ENGWLETKQVEEFQLFEDSPAKKKEKISYFAKFYRMHNLIVITVEITLGKYQDQLL
 RLEKKLESGLYCELVDEKELKDSFVEYTLTYDTIANRVSIIEVSVKDGAMQLMKNLAWA
 FDSLPHMLIAGGTGGGKTYFILTIESLLQTNAKLFILDPKNADLADLGTVMNDNVYQ
 KEEISACIDDFYARMMARSLDMKKMPHYKTGENYAYLNLEPNFLIFDEYVAYMEMLSA
 KENMAVMNKLKQIVMLGRQAGFFIILACQRPDAKYLDGIRDQFNFRVALGRMSELGY
 SMMFGDVVDKDFFLKQIKGRGYVDVGTNVISEFYTPLVPKGHDFLETIGALCHSKPLVK
 KEESEEDGV"

CDS
 8839..9123
 /note="ORF30"
 /codon_start=1
 /transl_table=11
 /product="hypothetical protein"
 /protein_id="AEP33203.1"
 /db_xref="GI:347952146"
 /translation="MEFDDCIYRLYELSRTENEELQQRFHSLASDVSKNGITGLVPIE
 EGGITDGVPLTVVLSILQSGLELATSPFDRTKIEALYNDLLSEGIDGYTK"

CDS
 9629..9823
 /note="ORF31"
 /codon_start=1
 /transl_table=11
 /product="hypothetical protein"
 /protein_id="AEP33204.1"
 /db_xref="GI:347952147"
 /translation="MSVVECIKSRISTISLPISGKNNVINGTQTQKVMATAHKSFL
 IWWVLATPTLINPPILTGG"

CDS
 10162..11145
 /note="ORF20; Tn916-like protein; putative transcriptional
 regulator"
 /codon_start=1
 /transl_table=11
 /product="hypothetical protein"
 /protein_id="AEP33205.1"
 /db_xref="GI:347952148"
 /translation="MLFDYVRIRFPTTNPEGVIENILKLMKMYLHEEYAFYSYEEQY
 VFGDIAMVSHDIEKGILVEMKGGCRQFEHFLAQRRTWVDFLDVFAVNGVYKRLD
 LAINDKVGLLNIPELTRKQCNEECISVFRSFKNYRSGELVRQHEKADMGDTLYIGSLK
 SEVYPCIQKDYQYVVLGIPLEENPVKNRFEIRLKNERALHAVIDLNNGNVGETTF
 SIINRYIRFVVDKSDNKRRSQWKINADWAFFLGENQRKLKLTSEPEPYSFDRTLNWL
 SRQVPTLKLAMKIDKLNQTSVIQNMIDYAELESDRQKKILQQQTLPEVEVIN"

CDS
 11159..11380
 /note="ORF19; Tn916-like protein; putative conjugation
 related protein"
 /codon_start=1
 /transl_table=11
 /product="hypothetical protein"
 /protein_id="AEP33206.1"
 /db_xref="GI:347952149"
 /translation="MNFQQLYNWFLSNAESLVLMAIVVIGVYLGFKREFSKLIGFLI
 IALIAVGLVFNAAGVKDVLNLNLFNRRIIGA"

CDS
 11397..11897
 /note="ORF18; putative anti-restriction protein ArdA"
 /codon_start=1
 /transl_table=11
 /product="hypothetical protein"
 /protein_id="AEP33207.1"
 /db_xref="GI:347952150"
 /translation="MDMQVYISNLGKYNEGEIVGAWFQPPIDMEEVKEKIGLNDQYEE
 YAIHDYELPFEIGEYTPISEINRLCALIEEIEDTEILKELKEIQSMWFSLEDLIEHK
 DDIIICYSDCDSMEDVATYYVEETGQLGEVPSNLQNYIDYQSLGRDMELEGNFLVTSNG
 VFEYLG"

gene
 12031..12840
 /gene="vap"

CDS
 12031..12840
 /gene="vap"
 /codon_start=1
 /transl_table=11
 /product="virulence associated protein"
 /protein_id="AEP33208.1"
 /db_xref="GI:347952151"
 /translation="MDIPKFIIDYFIHEEIVITDKGKDISVIKIEINDDLTVFEQWARH
 IREHYCSLEELDELREGTGLSRAEFLRDMKFPNPNFGLGSATMSGDFSEILIADYIEY
 ILNYIVPRVRYRQKFNRTSTQGSDLIGYKVESIETNSINDEMLIVEVKARNSESNE
 ATLQNAVDHSAKDLVRIAESLNASYRMLKNYNEEGMRLVRRFQNPTRPHKRTFAAA
 AVSSDKAFSRELLKEIDTSEHPSPDVQLIAVHSPKFLEFIKQMYVRAANVN"

gene
 12830..15967
 /gene="hel"

CDS
 12830..15967
 /gene="hel"
 /note="DEAD-DEAH box helicase"
 /codon_start=1

/transl_table=[11](#)
 /product="virulence associated protein"
 /protein_id="[AEP33209.1](#)"
 /db_xref="GI:347952152"
 /translation="MLIENNSKYLLKKVQAKSKMFEYNVPLEEHIQVEYMAQELIVLA
 IATIGDASNFIWDNRYTEFSSKLNELFENLNFSTIYFDSLLTSEIGEASYNDFGLL
 GAISFYLSDALGSTKVMINKIDWAMBLEINEIDTFLLYLLADRLDELKGYEFSSQYGN
 LFYYLIYDLSQFVLDGTPIDFEKLNLDKQEIYLSQNPRIILFGDSSLAVFIKKYKNSA
 LNLPLNYSDDTTIQDWEDLLNNHTIKELWPSQILLGEKNIFKGKSGVIQMPSTSSGKTT
 SIGLIIRSAFITHTRTKLAVVAPFKALCKEITLNLIEFFSNDEVIINELSDIAEKDDI
 AFFDNDIATNTLIILTPEKLLFLIRNNSEILEELNLIIFDEAHLFPDDSSRGNTYELLV
 STIRYYLNESSQKILISAVIANSEVINELNGTDGEVISNNIISSQRSIAIGDWMGQ
 TGQLYFLNQDNPIDDFFVPRIVDIREINRLDRERAKRYFPDVFRRKRLNKPYPDMQI
 YYALKMVHNGATAIFSGTKKTADKTIERLLEERGLDISAFSNEVSQNTKIASLI
 NMNLGSDTEYYISALKGFIHHGNIPLGIRLSVEYAMSQNLINFFVCTSTLAQGVNLP
 IKYLIISLYQKGSQMKVRDFHNLGRVGRAGKHIEGTVILSEPFHSNKSNNKWNRY
 KNMLDIDNSEDSESNLLKLVRIIDKFPPINLWNLVPQKYTNQEQFNDVINKIKTVI
 SNSNFPNKISDFNFELKIIDILKDVENYVMYFVKYFKDSNPNTNMVTQQTGLGYHLA
 SDSEKEKLVLEFVIKTYSLSTEAELSLYSISSLGFDNSKVLQWISEKIENTQAR
 SEFELIDIFPQILIFSENNIINRIDDTISFTQICELWTQGSYSDILNYCQSNQFQI
 SKRNQVLPLIQDIIIEICDNLAYSTILPLNGVATFMENSENDSTLHLIKSLQKKIRY
 GLPSNLSINIFELGFSRDRVIAQILARIENSSSFTITSISKRITKELLRQNKREIEEA
 LKVFPQYFLDILRKI"
 16114..16407
 /note="ORF17; Tn916-like protein; putative conjugation
 related protein"
 /codon_start=1
 /transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33210.1](#)"
 /db_xref="GI:347952153"
 /translation="MAWFVCSLFTVILLADIPPLSLIEGALLKYVGIPVGLTFWMSQK
 TFDGKKPYRFIQTVVTYAFRPKRTYAGKKVTFEKEKMDTATIRSEYIELSD"
 16694..18838
 /note="ORF16; Tn916-like protein; putative conjugation
 related protein"
 /codon_start=1
 /transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33211.1](#)"
 /db_xref="GI:347952154"
 /translation="MIGDNQVDYRFFIGFKLLVNEEVSLKNLKS AKNTLTTFREV
 NQKWMGDFVMPQDEVRRYTKMADLLQAKIERRFHIRPLDKNDFGYLIEHLYGKETSS
 FEEYEFPLPLKKLKHQTLVKRYDLLKPTRSLIEENQRYLKIQNEETTSYVAYFTIDS
 VGELDFPSNEIFYQQQQQDFPIDTNMNIIVANKKALSTVRNKKELKDLDHAWNS
 DNETGNNVLEALEDVNELEAELDQTKAMYKLSYVIRVAASSYEELKRRCKNEIKDFD
 DWSIKLVRPFGDMLGLHYEFLPSSKRYINDYVQYVTSDFLAGLGFASQMLGETEGY
 IGYNIETGKNVYLPKSLASQGVKGSVTNALASAFGLSGGGKSFNNLLVYYSVLFGA
 QALIVDPKAERGNNKTELPDMAEDINIVNLTSEEKNQGLDPYVLMKRKKDAESLAID
 ILTFLTGISSRDGERFPLLRKAIRSVTQKEIRGLWLVIDELREDDNPLSHSIADHIES
 FTDYDFAHLLFSDGTENSISLEKQLNIIQVADLVLPDAQSNFSDYTTLELLSVAMLI
 VISTFALDFIHSRDTFKKIVDLDEAWSFLQVAQGKTLNRLVRAGRAMNAGVYFVTQN
 ADDLIDEKLNIGLKFAPRSTDSQEIKKTLTFFGVDPNEDNQKRLRELENGQCCLIQ
 DLYGRVGTIQVHPVFEDLFHAFDTRPPVHQEE"
 18798..21086
 /note="ORF15; Tn916-like protein; putative conjugation
 related protein"
 /codon_start=1
 /transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33212.1](#)"
 /db_xref="GI:347952155"
 /translation="MPLILVPPSIKKSEVYTLKPKLKMGLVILLIGMTFLFLLLF
 TVAQAIGLVDDTDVQNLYSQYPLSHYQLDFYVDTDWDLWPNWKDGIGNQVQYGLYA
 MSDFIWTISLYLSNATGYLIKQAYKLDIFSDTANQIGENIQLAGISENGFSTSGFYV
 GFLLLFILIVGAYVAYVGLLKRETSKAIQAVTNFLVVFVLSAGFIAPFAPSYIQRINEF
 SADISQASLDLGGQIIFPHSSSQGTDSVDSIRDTLFSVQVEQPWLLQLFDNSNKEEIG
 ESRVNDLLAINPDNTNGKDREEAVKNEIEEHDNQQLTLTKTVNRLGTVSFLVLFNIGI
 SIFVFLLTGIMIFSQILFILYAMFLPIIFLLAMLPTPSGMGKAALMKLFNTIMLRAGI
 TLIIITVAFSLSTMLYSLTTSYPFFLVAFQIVTFAGIYLMGDMISLNLQANDSQOI
 SRGVMRRPKQYMRHARGARRLQRTISRTMIGGSLGYLAGKTKGRKNAKQLPSIAGTTPSN
 ARASVPSNQKNDRTPPTRSYQTGQQIGKVLDSKNRASNFVAQKKQVQKELPKTVT
 GNLHQITISDFKQGMDSERTESVTSKNRPVVQSRKTKQEKRGTTNPTTDKNHQRPAVD
 SVQAVNKIQQASTSNKRIDSVSRKNRPVQSRKTNRMKPIRSTVTRPPVQHRPSNKI
 QTVSQEVPQGTASKVQERITKQNRNRSIKQQPSIKQQAIPSRKQPTRYMTNQKQTST
 VRYSQNNRQKKNVRFTRPMKRK"
 21097..22116
 /note="ORF14; Tn916-like protein; putative conjugation
 related protein"
 /codon_start=1

/transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33213.1](#)"
 /db_xref="GI:347952156"
 /translation="MKWKLKIMVALTLFLSFFGLLSFVAIFVADEPQGNSSDSIVIQGSGLSVSPEVLKYRSLVEKYAKQENIEDYIHILLAI IQVESGGTLKDVMSSES KGLPPNTITDPEESIKQGVSYFAGLVRSAESLGVDDQSVIQAYNYGGSFLNYVAKNGKTYSFELAESFSNEQAKGQRVTYKNALSIPKNGGWRYNYGNMFYVLLVIQYLETASQKFDNQTVQIIMDEALKYQGYYPVFGGSNPNTSFDSCGITQWTFQKAGIILPRTAQAYEVMDHLPLSQAEPPGDLIFFHSTYATADYVTHVGIYVGDMRMYHAGDPIGYTDLNENYWQQHLIGA GRIKQ"
 22133..23050
 /note="ORF13; Tn916-like protein; putative conjugation related protein"
 /codon_start=1
 /transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33214.1](#)"
 /db_xref="GI:347952157"
 /translation="MKIHIEHKPKKEKANRQKKVKT IHLGTHKKS VLFWLLLLIGSFSFGLYKNFTAITDTHT IHTTVIEPVLVDTHAIESFTLNF IQEYYSWENNKEAIEQRPERINRYLTSELQELNVDTIRTDIPTSSSVSDCKIWQIQSIDEQNFVVVYTVKQIQKEGEEKTNKVASTYRITIHQDKNNNLVITSNPTVWAAPNPSEFPQDPTLVNDNNIDEQTKLEILD FLETFFKLYPNATKNELDYMMEDVLP IISQNFYSELVNPI IQKKEKQYLVKIAVRYLDNETKIENISQYQLTLEKKDNWKIIMNE"
 23894..25819
 /gene="tets"
 23894..25819
 /gene="tets"
 /note="tetracycline resistance protein"
 /codon_start=1
 /transl_table=[11](#)
 /product="TetS"
 /protein_id="[AEP33215.1](#)"
 /db_xref="GI:347952158"
 /translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTKTD TMFLERQRGITITITATS FQRENVKVINVDTPGHMDFLADVRSLSVLDGAILLISAKDGVQSQTRILFHALRKMNIPI IFFINKIDQNGINLPDVYQDIKDKLSDDII IKQTVNLNLKPYVIDYTEPEQWETVIVGNDYILLEKYTIGKTLNIAELEKEENERIQSCSLYPVYHGSAKNNIGIKQLIEVITSKLFSPQTQLNSDKLGNVFKVEYSDDGQRLVYVRLYSGTLH LRDSVNISEKEKIKVTEMYTSINGELRQIDKAEPGEI IILKNELKLNNVLGDKKRLPHREILENPLPMLQTTIEPCKSVQREKLLDALFEISDSDPLLOQYVVDVTHEIVLSFLG EVQMEVTC TLIQEKYHIEIETRKPTVIYMERPLKKSEFTIDIEVPPNPFWASIGLSVTP LPLGSGIQYESLVSLGYLNQSFQNAVMEGIRYGCEQGLYGWKLTDCKICFKYGLYSPVSTPADFRMLAPIVLEQAFRKSGTELLEPYLSFEIYVPQEYLSRAYNDASKYCANIL NTKLKGNEVILIGEIPARCIQEYRNSLTFFTNGRSVCLTELKGYQVTNIKSAFQPPRRP NNRIDKVRHMFNKNINLH"
 25943..26074
 /note="ORF6; conjugative transposon protein"
 /codon_start=1
 /transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33216.1](#)"
 /db_xref="GI:347952159"
 /translation="MREDTELKNFPLFCPKCRQEILIEITKFRITVITEPDAKTQSR"
 complement(26588..26947)
 /note="ORF9; transcriptional regulation"
 /codon_start=1
 /transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33217.1](#)"
 /db_xref="GI:347952160"
 /translation="MMDRKKHRTFDFKPLGLAIKEARLSKGLTREQVGSMIQIAPRYL TNIENTKGQPSFHVVDLVTTLLNISLDGFI LGEENSHKSSKRLQVEAQLDCLDEDELVTIGKVIQAIYGEKCKFN"
 27427..27927
 /note="ORF7; Tn916-like protein"
 /codon_start=1
 /transl_table=[11](#)
 /product="hypothetical protein"
 /protein_id="[AEP33218.1](#)"
 /db_xref="GI:347952161"
 /translation="MFIGIIFFLNGKGGVNDMSSSLFQAAIEMQFDYICKRSIDDERK DLLKSLSRISKEVAFSELDDYVVEQFASIDQYPSDFSYFELEGAEIAVKSMSLGEAL ERLPKKKRNII LFYFIEMNDAEIANLMGLSRSTVNEHRHKALHLIRKFRRRMRNEDN LFQSPL"
 27998..28123
 /note="ORF8; Tn916-like protein; transcriptional regulation"
 /codon_start=1

```

/transl_table=11
/product="hypothetical protein"
/protein_id="AEP33219.1"
/db_xref="GI:347952162"
/translation="MMQRLSLRFLKDEVGQKHLVVDEVLSARLETRLLTKILAFKI"
gene
28906..29181
/gene="xis6000"
CDS
28906..29181
/gene="xis6000"
/note="excisionase"
/codon_start=1
/transl_table=11
/product="Xis6000 protein"
/protein_id="AEP33220.1"
/db_xref="GI:347952163"
/translation="MKIEISLEDNSEIIEVLTEQILQRIEEQSTKFSSVNDLPPYPNR
KQVKRLRIGDDRNLNQWIASGLKVIPFGKEARFDRDDIKDFLHHLKV"
gene
29281..30513
/gene="int6000"
CDS
29281..30513
/gene="int6000"
/note="integrase"
/codon_start=1
/transl_table=11
/product="Int6000 protein"
/protein_id="AEP33221.1"
/db_xref="GI:347952164"
/translation="MIKKYTKKNGSTAYLVKVVLGVDP LTGKKKTTTKRGFKTQKAAK
QEVARLQLLAQEYDLVIESNRLFSIDIALEWFEQYKNTVKESTYVQVRVALNKHILPLF
GERRISKISIPYQKQVNHWSYKKYNSLIGMTNSIFQYAKSLRLIRNPMGIVIRP
KRQKQIDEEKYTAPFYTKAEVLYFLQIAKKYPDPLYPMFHILFTTGLRK GELLALRWK
DIDFKRGTL SVKQTLTTVEDWKLAFTPKTEKSLRTISIDNQTLSIFRQCILKQKAFK
LKTGRKPVENG AQLLFTTEENKPHYLDPLNHNLT KILKENKLKHM TVHGFRHTHCSLL
FESGASIK EYVRLGHTDVRTMDIYAHVSEQKKEETADRFADYMNK LHEEEDGMVKR
MVKTKKEQT PPIIMKSAKS"
repeat region
30443..30463
/note="imperfect repeat"
/rpt_type=direct
repeat region
30548..30564
/note="terminal repeat"
/rpt_type=direct
misc feature
30565..>30621
/note="similar to ribosomal protein L31"

```

ORIGIN

```

1 taaaattatc ggatttttga gatttacaag aacttctgat gtatttttgt actacttaaa
61 agagagggtc aaaaggatgg tcaaggacaa aaaaaagaaa acatccctatg ctcaggatac
121 tctctaaaaa ctgataaatc agcgcttttc ttattctgca gcgttttgtgt ctttgagacc
181 atactcaata ttgatatgat ttctggtagt ctttaacga actttatttc tttgttcgtt
241 cactaacaca ctctgatgag tcccctttca tacgactaag gaagggtcaa aggatgggtca
301 tttttatgac taaaaaactt cgccagtata ttaaggaata ttgttttgc agtatgttta
361 ataagtgaat ggtactttca aaaatcaaaa ggttttagac acttccaaag ttgaggtgtc
421 ttttttgaat aaacatagga attctgttaa atacgaaaaa gaactaacc tcatataaga
481 aaaattcaaa aaataagtta attagaatta catcttttga tactacaata aaaatcatc
541 ttctaaaaaa tattatttaa ttattgctga tatttgtatt gactagaaaa acatgggttcg
601 ctataattgg gttgttcaaa atattttaaa caaggaggag ggtactttga ttattgataa
661 agaaatagtt ctatcagcca tgaaacaaaa gaataataaa acccaaacag aactagcgga
721 acgactacaa atgagtaaaa gtcaattgtc tcaaatgtta tccgaagggt tttctccttt
781 aaaaactaat gttgtcaaac tcaactaatga gttaggacta tcctatgcag aaatacttaa
841 agaagaattg agtgatacaa tgactacaac tatagtaaaa aaacatgcaa ctaataataa
901 acagctagaa ttatttaacg atgattcatt agacagattt aaatacaagc tagatgagtt
961 tattgatatt caaaatacta ctccaaaacg cgattacacg cttgtagaaa catttgctgg
1021 tgcaggaggt ctatccttag gtttgaaaaa agctggattc aattctgttg cagatattga
1081 aatagactct acagcatgta gtactttaaa attaaatgcg cctaattgga atgtgattga
1141 aggagatatt aatactattg ctcaaaatgg tatctttaat catgatcagt ttacttttag
1201 aggagaacta gacgtattgt ctggcgggta tccttgtcaa agttttagtt atgctggctt
1261 acgtcaagga ctgaatgata ctagaggtag tctatttcat ccttattcac aattattagt
1321 acaactgcaa cctaaaatgt ttatagcaga aaatgtaaaa ggtcttttga atcatgataa
1381 tggccgtaca ttgaaacta tgatagaaat tttcgaggat tcagggtacc aagtatttg
1441 gaataatactg aatgcttgga attatggagt tgcacaaaaa agagagcgga ttgtcattat
1501 tggcattaga aacgaccttt ttgaacaaca acaatattct tttaaatttc ctaaagaata
1561 tacctatcaa cctgttctaa gggatgtatt aaaagatggt ccgaaaagtg aaggtgcttc
1621 ttatccagcg aatctcagtg aagtccttaa cttagtcctt cctggaggat gttgggttaa
1681 cttgcctgaa aatgttgcta aaagttacat ggggtcatcc tatcattctg gtgggtgctg
1741 aagaggaatg gctagacgct tatcttgtaa tgagccttct ctaacgttaa ctacatcacc
1801 cgcgacagaaa caaacagaa gttgccatcc tgatgaaact cgtccattta ctgttcgaga
1861 atatgcaaga atacaaaagt tcccagatga ttgggagttt gctggaagca caaacaatca
1921 atataaacaa attggaaatg ctgtcccggg aaatcttgcg gaagctattg gaaaatctgt
1981 agtaaatat ctgaatcaat tttaaaatat caaaaactag tacactaaaa aggtactagt
2041 ttttttaact actcttcaat tccttcaata ttttttagtt gttcagccaa agctttgatt
2101 gtttcgtcaa gaagctcagt actatcaaat tcattagcta tatctccaat accatcagtt

```

```

2161 agcttttgat aaaaatcttc atctcctggt aatgatgacc aaaaatcttt tccagctaag
2221 actgtatagt cctctgctaa cgttctataa aacgcactta attcgctgtc ttcgccataa
2281 aaaacaccta cgacacaatc catatttgca ataggaatat tattagtacg agctaaatta
2341 atcgctgatt taaaatgatt ttgaattgta gtcacatcat cataaattaat tgtttgaggg
2401 ccagctttaa ttgacagta tttttttctt ccacttattt tatcaataaa ttctatgtcc
2461 attccagtcg ttgtcagagc atatcccttc agagttttat tgataaaatt ttgaaggttg
2521 gaaccgaatg aagtattaat tgaagtacct aaaactcttg gatataatta tgccttcgaa
2581 acactttcag gatcatcggt ccctgttaaa taatttgcta aatacttggt cagaaatgga
2641 ttcacgttaa agttgtttat gttttttaac ttttctgtat ttttaatatg attcttagca
2701 attacttctc taaaaaagtc tttcgctttt tcaatgattt catcattggt ttgtgctttt
2761 tcagtcattt tcttcacctc gttattttct tataatttga gtataccctt ttggttattt
2821 catgtatagg tttccatggt cataaatata gtaaaacact ctttcattct tgcagttat
2881 acatttctaataaatgattaa ctatcttttt gaatctcttt tattcacttg cttcatccag
2941 ttaaaaaatta ttttttatat acttatattc cttoatagca tttgagaggg tataaataaa
3001 tttaaaaatta ggtatatcgg agataactag atgacgaaat ttatagataa aattaatgaa
3061 ctagaagaag tagaaattac caagtattat acagaattta gagatggttt taaagaatc
3121 catgattggg caaaatcgga aggattttta aatgaagcaa aattagcaaa atatgaaatt
3181 gatatttgggt cacttatcga aaaaaacca attctttcga ataataaaag cgacactaga
3241 tttgtccccc aatacagtat tacaggcatt ggaaatgttc ctgaaattac agctttcact
3301 gatgaacaat ttttatatta tgaaagaaga atgattgaaa ctgataatat ttttttaaaa
3361 gtaagatatt cagattttct ttttgagtat gggagtgaaa aagtaacgag aaaaaatat
3421 caaatttcac aatttttact tgcacacttt gtagaaggtt gtagaaataa tcagactgta
3481 catgaggata gtaattttat tttatctata tcaagattag ttgaggtttc gctttctatg
3541 aataataaaa aatacctgca acaagctatt caactaatac attcacaatt aattgaatcg
3601 agggatatag ataattcgat atgggctgtt aatctttctc aattagtaag aatcatttta
3661 gattctaaat taaaggttta ctttcagaa gaactttaca cctgtacatt aacaatattg
3721 acaaatctta aaaaacaaatg ttttgaaat catgagatc atttttatag attattttat
3781 gaggaatcta tagctcttgg aaagtttgag gcatacaata cagaacaaat acaaacctc
3841 cgtctggata ttgaaaaaag ctatgaatta gaggctgagt atcaaggcgg gagacaacaa
3901 aaaagctcat taacaaaagc ttttaactta gaacaagcac ttgaatacta taagaatatg
3961 ggagaaacag aaaaatttaa tgacttaaaa gttttaataa agcaaaacta tgaacatat
4021 caacaaatg atgaaatggc aacaataaaa attcctttta caattcctac agactatatc
4081 gataattgta tttcacctta tattatttct aacattcatg aatctcttga taagcttgca
4141 aaaagtgcg aattttattc aaatgtcagt ataattaaag agcaacaatc acttcaagaa
4201 cgagataata atttttttaa tcttgtctca aaaagtataa ttcagtgtgg aacaaaaatc
4261 gtaaacgcag aaactaaaga agagtcaaaa aagttcattt tctacttag ttatgtacaa
4321 gaattgcac taaatttaga gattttttta aaggctattt ttgaaaaatt gatagaagaa
4381 catagccttt ctgccgatca tcttatggaa aaatttgatt catggaaaaa gcttgatgag
4441 agaactacc cttctgtaaa agcaggaatt gaaaattttt ttaagaaga ctatgttagc
4501 tctattcata ttttagttcc tcaatttgaa tctacattta gacggtttct tgctaaattg
4561 ggattcgcga ctacatcact caaaaaagat atggttcagt atgaaataac atttaacgaa
4621 tttttagtta aagattacat taaaactttt ctaggagagg atattcatac ttttaattcaa
4681 ataattatgg ttgaaccaat ggggtctaat ctacgaaatg aaattgcaca tggcttaatt
4741 aaacaatctg acattacgaa atcaaaaatgt atcttagtta tttatctatt ttttaattta
4801 acaagatacg atgataataa agagtaattct ttttatattt cacccttata atcaaataga
4861 aatcacaa ca tttttttgga cacttcatat tgggtgtctt tttttattta caaggagatg
4921 ataagaataa caaatcttac tttaggaagc ctctttgacg gtatcggtgt cttccctctt
4981 gccgctcaaa aacagggcat tacactatca tgggcaggtg aaatcgaaaa agcaccgatt
5041 cgtattacaa agaaacagtt tccccatag attcatttag gagatttaac caaactacat
5101 ggtggaaaaa ttccaccggt tgatattgtc acgtttggat ccccttgta gaacctctca
5161 accatttgaa atcgtgtaag attagctggc aaaaaatcca atttatttta tgaagctatc
5221 agaattattg aagaaatgag ggatgccacc aatggaaagt atccaactct cgctatttgg
5281 gaaacgta tgggagcttt ttcacgaat gaccgtagg attttaaggc cgtcttgaa
5341 gccttcgttc aagccgaagt tccaattcct ccctctcgct aatgggcaaa tgcgggtatg
5401 gtgcgagga gaaatgttga tgtgtgttg cgagtccttg atagtcaata ttgggtgtc
5461 cccaacggc gccgaagaat ctttctcgct gcagatttta gagacttcg tgcagagaa
5521 atattatttg acaccaag aatgcagtcg catcttgaaa ctgogacca gaacaggttg
5581 tctgtcccca aagacaatcg aagccttact actaaagcag ggcggcaact atctcttat
5641 cccttccaag agcgccgaat gogtacgtat gcaaaagaaa aaaataacca aggattcgtc
5701 tccagtttcg gacaatcaac tgaccctttt ccactctct tagcaggtac tgttgatacc
5761 tttgcttatt ggcaagaagg atttgaaact gaagggtttg ttcggaagct cattccactt
5821 gaatgtgagc gaatcatggg tttgcctgaa aattggacac aatatggatt tgataatcaa
5881 ttaatcagtg actctgccg atacaaagca ctaggaaact ctatcgttgt tccctgtgcy
5941 gaattttatt tctggaatgt cgcaagggtc tatggtaaag gagaatcgta tgggttaaca
6001 cgcgacgct taaacgctgc ccaacaatta ttatgtacca attcaatcaa tcagttgtct
6061 gcggcgatgc ataacttcca atttttacia catattgacg gccgagaagt gcgtggacgt
6121 caagcaatcg cagaacaatt gatggaagtc gaagaatacc tactgattgt taaaaatcaa
6181 atacaaacta ttcggaatatt atacaaggag aaataaaaat ggataacaca attcaagtat
6241 acgtcaccaa ccttagtcga tttgagtatg acggactaat tattgccgct tggttcacc
6301 tacctgtttc tttgatgaa ctaagagaaa agctacaatt ggaacagat gaaagttagt
6361 aaattgaaga ttgggaagcc cttttccgat taacagacga tggggatatt ctcacactca
6421 atcgttttagc gcttttaact gaggaaaact gtcactatga cttatttctt tatttagatg
6481 aattgattga cttagatgtc tttgaatctc atgaggatgg cttaaagcga ttaaatcatt
6541 tcgagcactt tgaagaaaag ccaactgatc cagatatattt ggaagatacc tttgagttat
6601 ccgatggcac ctatttttacc atttaaaagca gtttgactaa tccaatagtt gagctgcttt
6661 tttgttttga aaggaagtgt atcaatgag gttcccgcc ggtacacaaca
6721 acaattttta agcttatttc aatacaaaat tttgaatgaa aagaggaaaa acaatggaat
6781 taaaacacgt agtaccgatt atgaaagaca cattttgaca cttagaattt gcggcggaag
6841 gaaatgttga acaacgacgc atcaatgggtc gtttagctac tgtttctcgt agctataacc
6901 tatattcaga tatacaacgt gctgatgata ttgaggtgat tttacctcat acagccggag

```

```

6961 aaaaattctt tgaagtggaa gaatcggta aattgattaa cccacgcatt atcgcagaag
7021 gttataagat tggcgaacga ggattcacga attacatttt acatgcagac gatattggtca
7081 aagcctaaag gaggaataa cacatgagat tagcacaagg aattattatc gacaagaaa
7141 aaacggttgg acggttgaaa ttttctgcac tacgtcggga agtttttcta aacaatgaag
7201 atggcagtgat atcaactgaa gtcaaagaac gaacctatga tttaaaatca cgtgaacaag
7261 gagcaatgat aagcgataaa attcccgctt caattccatt acgagaattt gattacaatg
7321 ctgaagtggga attaatcaac ccaatcgcag acaccgtagc caatgcgact tatcgtgggg
7381 cggatgttga ttggtacatt aaggccgatg atttggttct aaagaataaa accacaccga
7441 aaattgagcc aaagaataaa caggggatag ccatgaaact cttacaaaaga aaaggaaaaa
7501 gaatcagacc aagcgataaa cacttggctt ttcagttttt ctttgggtgt atattaagtt
7561 gttggttact tgcatttttt ccctttcatt tggctttttt cagtcgaact acttggcaac
7621 tagaggaatt taaacaacac tttttcagtc cctatggtag tctatcactc gtcttctcac
7681 ttcttactgt tggttttctc ctctattttt tgtatcggta tgggttcgat tattacaaga
7741 gtttaaccga tcgttaacaa ctgcctcgaa tgattctaga aaacggatgg ttagaacaaa
7801 aacaagtggga agaatttcaa ctctttgaag attccctgcg taaaaagaaa gaaaaaatca
7861 gctactttgc taagtcttac tatcgaatgc acaacggact gattgttatt actgttgaag
7921 tcacattagg taaataccaa gatcaattgt tacgcttaga gaagaaattg gaatcaggct
7981 tgtactgtga gctagtgtga aaagaactca aagactcttt tgtggaatag accctactct
8041 atgacacgat tgccaatcga gtttcaattg aagaggtttc tgtgaaagat ggggctatgc
8101 aattgatgaa aaatttggcg tgggctttcg attctttgcc tcatatgttg attgcaggtg
8161 gaaccggtgg tgggaaaacg tttttcattc ttacgattat tgagtcctt cttcaaacca
8221 acgccaaagt gtttatcctt gatcccaaaa atgccgattt agcggactta ggaacagtga
8281 tggataatgt ctattaccaa aaagaagaaa tatctgcatg tatcgatgat ttttatgctc
8341 gcatgatggc gcgtagtctc gatatgaaga aaatgcctca ttataaaaac ggcgaaaatt
8401 acgcttatct aaacctagaa ccaaacttcc ttatttttga tgagtattgt gcgtatatgg
8461 aaatgttgag tgctaaagaa aatatggcag tcatgaataa attaaaaaaa atcgctatgt
8521 taggtcggca agcgggattc tttattatct tggcttgcga gcgaccggat gcaaaatact
8581 tacaagatgg gattcgtgat cagtttaatt ttctgtggc acttgggcgt atgagttagc
8641 ttgggtattc catgatgttt ggagatgtcg ataaagattt ctttttaaaa caaattaaag
8701 gccaggagata tgtagatgtt ggaaccaatg tcattagcga attctacaca ccattgttct
8761 ctaaaaggaca tgattttttt gaaacgattg gtgccttggt tcaactgaaa ccattagtaa
8821 aaaaggagga atctgaagat ggagtttgat gattgcattt atcgtttgta tgaactttca
8881 agaactgaga atgaagaatt acaacaacgt tttcactctc tagcttctga tgtcagtaaa
8941 aatgggatta ctggtttagt ccctatcgaa gaaggcgga ttacagatgg cgttccactc
9001 actgtgtgtt tatctatctt acaatcaggg ttagaactag ccactctctc ttttgaccgg
9061 acaaaaattg aagccttgta taatgactta ctttcagaag gtatcgatgg ctatagcaaa
9121 tagcacggat agaagcggaa ttttttaaca aactgcggta tagttaatgt aacaagatgg
9181 taggaattgc catgtctcac tttaccgcta ctgtttttac cagtttgcat ccagcctatt
9241 tgatccgcta tatatctttt agtggatttg tcaactgcgt tttctatttt tcaagtctcg
9301 atactgcccc tacttctatt tatgtctttt taggattaaa ctttcttctc tatccatttg
9361 ctatgtttgt ttatgattcg attgtctctc ttttaattgg gaataatgtg tggattacca
9421 gtggcatttt tgccttcatt tggggcttca tcaaaatttt attaatttac ttcttttagt
9481 ttttgattgc gccaatggc atocctattc tgtattttac aaatogttag agaattagat
9541 tataaaggtc ttccattcgt cccgtagaaa agatgtgaag gatcgaagga gcctgtgact
9601 gatactttat atcttttaga ggggaacat gagcgtagtc gaatgtataa aatcaagaat
9661 atcaacaatt tcactgcccc ttagtggaaa gaacaacgta ataaacggaa cacagacaga
9721 aaaagtcatg acggcagcgc ataagtcatg gctttttctt atctgggtgg tgtggccttg
9781 cacgcccacc ttgattaacc cccccattct aacagggggg tagaaataca aaaaataata
9841 gcaataaagg cttaggaact ttgatacaac aaactttcta tcgctttatg aggtgtcaac
9901 tttttttgaa tagttgacat cttttttggt cactcaaaaa agaaaggaga agttgtgttg
9961 ataaacctca aacaacaagt attagaacta aaagaacgga gaaaaaacta tgggtgatcg
10021 caaaaataac tagctactgc agttggtatc tcaagacaat accttagtga aattgaaaca
10081 gaaaaagtgc ttcttccag tgagttactt tcagaattag atgttctgct cgaacgtttc
10141 aatccagagt taccttggga aatgctattc gactatgtac ggattcgatt tccaaccact
10201 aatccagaag gagtgatcga gaatatctta aaactaaaaa tggaagtata gttacatgag
10261 gaatacgtct tttactcgtg tgaggaacaa tatgtttttg gtgatatcgc ggtcatggta
10321 tctcatgata ttgaaaaagg tattctcgta gaaatgaaag gcaaaggctg tcggcaattt
10381 gaacattttt tgttggcaca acgaagaacg tgggtagact tcttcttaga tgtctttgca
10441 gtaaatggtg tttataaacg ttggatttta gcgattaacg ataaagtcgg cttattaaat
10501 attccagaac tcacggttaa atgtcagaac gaagaatgta tttctgtctt tcgtagcttt
10561 aaaaattatc gttcaggtga attggttcgc caacatgaaa aagctgatag gggtgatagc
10621 ttgtatattg gttcgtctaa atcagaagtc tattttttga tctatcaaaa agattatgag
10681 caatatgtaa aacttgggat tccattagaa gagaatccag tcaaaaatcg ttttgagatt
10741 cgtttgaaaa atgaacgtgc cttacacgca gtgattgatt tattaaacaa tggaaacgtt
10801 ggcgaacga ccttctccat tatcaaccga tatattcggg ttgtggataa agattccaac
10861 aaacgtcgtg gtcaatggaa aattaatgcy gattgggcat ttttctagag agaaatcag
10921 cgaaaactaa aactaacttc agaacctgaa ccctattcgt ttgaccgaac cttgaattgg
10981 ttgtcacgac aagtcacttc taccttaaaa ttgcaaatga aaatcgacaa actcaatcaa
11041 acgagtgatc tccaaaaatat gattgattat gcggaattat cggatcgtca gaaaaagatt
11101 ttacaacaac aaaccttcc ttagagggaa gttattatta attaaaggag aatgtgtgat
11161 gaactttgga caaaatttat ataattggtt tctttccaat gcggaatcat tagtattaat
11221 ggcaattggt gtgattggtg tgtatttagg ttttaaaaga gaattttcta aattgatttg
11281 cttcttgatc attgccttaa ttgcggttgg cttggtattt aatgctgctg gtgtaaaaga
11341 tgtcttattg aatcttttca atcgtattat cgggtgcttag aagggaatga aaccaaatgg
11401 atatgcaagt ttatatctcc aacttaggaa aatacaacga aggcgaaata gtcggtgctt
11461 ggttccaacc acctattgat atgggaagag tcaaaagaaa aattggattg aatgatcaat
11521 acgaagaata tgcaattcat gattatgagt tgccttttga aattggtgaa tacacacca
11581 tttcagaaat taatcgatta tgtgccttaa ttgaagagat tgaggacaca gaaattttga
11641 aagaattaaa agaaattcag agcatgtggt tttctagttt agaagacttg attgaacaca
11701 aagacgatat tatctgttat tcagattgtg attcaatgga agatgtagcg acttattatg

```

```

11761 tggaggaaac aggccaaactt ggtgaagttc cttctaacct tcaaaattat attgactatc
11821 aatcacttgg acgagatatg gagttggaag ggaacttttt agttacgagt aatggggtgt
11881 ttgaatatat aggatagcat agaaaaatct agcatacatc ctaatattta ctaatctttt
11941 atataaacaa tttctttctg ttataaaaca attaatcccc ttttaaaatt aatgatataa
12001 tgttattaat tttaaaaggg gttgagtttt atggatatct cgaattcatc tgattacttt
12061 attcatgaag aaatcgttat cactgacaaa ggaaaagata tctctgttat aaaaattgaa
12121 ataaatgatg atctgaccgt tttcgaacaa tgggcaagac atcttagaga gcattattgt
12181 tcacttgaag aattggatga actaaagaaa ggtactggtc tttctagagc tgaattccct
12241 agagatatga agtttcccaa tccaaatttt ggattgggtt cagcaacaat gtccggtgat
12301 ttttcagaaa tattaaattgc agattacatt gaatacatct tgaattatat agttccaaga
12361 gtacgctaca gacaaaaatt caatcgaaat acttccacac aaggaagtga ctttaattggg
12421 tacaaagtag agtctattga aactaatagt atcaatgatg aaatgttaat tggttgaagta
12481 aagcgtagaa atagtgaatc aaatccagaa gcaactctac aaaatgctgt agatcattct
12541 gcaaaagacc ttgttagaat agctgaatct ttaaatgcct cttacagaat gttaaagaac
12601 tataatgaag aggaagggtat gcgttttagta agaagatttc aaaatcctac agacagacct
12661 cataagagaa cttttgctgc agcagctggt agttcggata aagctttttc tagagagtta
12721 ttaaaagaaa tcgatacttc agaacatcct agtccagatg ttcagttaat agctgtacac
12781 tctcctaagt ttttaagaat tattaacaaa atgttatgtg gggctggcaa tggtaattga
12841 aaataattca aaatattact taaaaaaagt tcaagcaaaa tcaaaaatgt ttgaatacaa
12901 tgttcctctt gaagagcata tccaagtaga atatatggca caagaattaa tagtccttgc
12961 aatagcgaca attggtgatg catctaattt tatttgggat aatagatata ctgaattttc
13021 tttcaaaatta aataatgaat tatttgaaaa tttaaatttc acatcaattt actttgattc
13081 actacttact tcagagattg gtgaagcaag ttataatgat tattttggat tattaggggc
13141 aatttctttc tatctttcgg atgcacttgg aagtacaaaa gtaatgataa acaagataga
13201 ttgggcaatg gatttagaaa ttaatgaaat tgatactttt cttttatatac tattagcaga
13261 cagattggac gaattaaagg ggtacgagtt ttccagtcga tatggtaatc tcttctatta
13321 tctaataatat gacttatctc aattttactt agatggtaca cccattgatt ttgaaaaatt
13381 aatgacttta aaacaagaaa tatatctatc tcaaaatcca cgagagattc tttttggaga
13441 ttcatatttg gcagttttta ttaaaaagta taaaaattct gctttgaatc ttttgcccaa
13501 tttattctgat acgacaatac aagattggga agatttattg ttaacaatc atacaataaa
13561 agaatttggt cctagtcaaa ttttgctagg agaaaagaat atttttaaag tataactctg
13621 agtcattcaa atgcctacga gttctggcaa aacaacttca attggtttaa taattagatc
13681 agcttttatt acccatcgaa caaaacttgc cgttgtgtgt gctccattta aagctctctg
13741 caaagaaaata actctaaatt taatagaatt cttttcaaat gatgaggtaa tcataaacga
13801 actgtcggat atgacgaaa aagatgatat tgcgttcttt gataatgata tcgctacaaa
13861 cacattgatt atactcactc ctgaaaaatt actgttctta attagaaata atagtgaat
13921 tttggaagaa cttaacttga ttatttttga tgaagctcac ttattcgatg atagctcgcg
13981 aggtactaat tatgaattgc ttgtttcaac aatcagatat tacttaaatg aatcttcaca
14041 aaaaatactg atctcagcgg ttattgcgaa tagtgaagta atcaatgaat gggttaattg
14101 tactgatgga gaagttattt ctaacaacaa tattatttct tcccaacgat caatagcaat
14161 aggtgattgg atgggacaaa ctggacagtt atattttcta aatcaagata atcctgacat
14221 agatgatttt tatgtcccaa gaattgttga tatacgtgaa ataaatagac ttgatagaga
14281 aagagcaaaa aggtattttc ctgacgttga ttttcgtaaa agaaaactca ataaacctta
14341 cgatatgcaa atttattatg cattaaaaat gggtcacaaat ggagcaacag ctatatcttc
14401 tggaacaaaa aaaactgctg ataagaccat cgaagacta cttgaacttg aagaagagg
14461 actagatata tcagcctttt caaatgaagt tagtcaaaat gagaatacaa aaatagcaag
14521 tcttattaat atgaaactag gaagtatac tgaattatat atttcagcct tgaaggcat
14581 atttattcat catggttaata ttcctctagg aattcgatta tcagttgaat atgcaatgtc
14641 gcagaattta atcaattttg tagtttgtac ttccacctta gctcaaggag tgaatttacc
14701 aattaaatc cttattattt caagtctcta tcaaggaaaa agtcaaatga aggttcgtga
14761 ttttcacaaat ttattagtta gagtgggtag agcaggttaag catattgaag gaacggttat
14821 attatcagaa ccttttattc attctaacaa atcaaatggg aaatggaaca gatacaaaaa
14881 tatgctagat attgataatt cagaagacag tgagagtaat ttattaaagc ttgttaggcc
14941 tattataatt gataaattcc cccctattaa tctttggaat gtactgccac aaaagtatac
15001 taatcaagaa cagtttaatt atgtaaatca taagataaaa accgtaatta gcaactcaaa
15061 ttttccgaat aaaatatcag acttcaactt cgaattaaag ataattttag atatttttaa
15121 agatgtcgaa aactatgtaa tgtactttgt gaaatacttt aaagattcaa atccaaattt
15181 tactaatatg gtaactcagc aaactttagg ttatcatttg gctagcgata gcgaaaaaga
15241 aaaattagtt gaactgtttg aggttaattaa gacttattct ctttcttcca ctgaagctga
15301 actatctctt tattcaatat cctccttagg atttgataac tctaaagtct tgtctcaatg
15361 gatttcagag aaaattgaaa acattactca agcaaggtca gaatttgaat tgattgacat
15421 aatctttcct cagttaatat tattctcaga aaataatatt ataaatagga ttgatgatac
15481 tttctatttt actcagatat gtgaattatg gactcaagga caaagctatt ctgatatttt
15541 aaactattgt caaagataac aattccaaat tagtaagcgt aatcagctag ttccgctcat
15601 attacaagat atcattgaaa tatgtgataa catactagct tacagcacta tattacctct
15661 aaatggagtt gcaacattta tggaaaattc cgaaaatgat tcaacacttc atttgattaa
15721 aagtctacaa aagaaaatac gttatggact tcctagcaat ctatcaataa atattttcga
15781 gcttgggttt tctgctcag taattgctca aattttggct cgaattattg aaaaattctc
15841 tttcttttaca attacttcaa ttagtaaaag gataactaaa gaattactta gacaaaataa
15901 acgagagatt gaagaagcac taaaagtttt tcctcaatat tttctggata tattgagaaa
15961 aaataaaact attgagagca cttttatagg tgttcttttt tggaggtgtt taaactgaaa
16021 aaactaaaaa gctataccag tatttggctc gttgaaaaag tgattttatgc catcaacgac
16081 tttaaacttc cctttccaat tagtttttagt cagatggcctt ggtttgtttg tagtctgttt
16141 acggtgattc tactggctga tatccctcct ctttcattga ttgagggggc gttattgaaa
16201 tatgtcggca ttcctgtttg cttgacttgg ttcattgagtc aaaagacgtt cgatgggaag
16261 aaaccctatc gttttatttc aaccgttgc acttatgcct tccgtcccaa acgaacctat
16321 gctggaaaaa aagtaacctt tgaaaaagag aaaatggatg aaactgccac aattgtaagg
16381 agtgaataca ttgaattatc cgattaaata cattgaaaac aacctcgtat tcaatcacga
16441 aggcgaatgt tttgcctatt ttgaattgtt gccctataac tattcctttt tgacccctga
16501 acagaaactt caagtccaca cgaattttcg acaactcatt gcacaaaatc gtgaaggtaa

```

```

16561 gattcatgcc ttacaataa gtaccgaacg atcgattcgt tcgattcaag aaaaaagtaa
16621 agaacggtc actggtcggg tgaagacat tgcttttcag cggattgatg aacaaaccga
16681 agcgtagta aacatgattg gtgacaatca agtggactat cgctttttta ttggctttta
16741 attactggtc aatgaagagg aagtttcctt aaagaatttg aaaaaatctg ccaaaaaatc
16801 actcactaca ttttttagag aagtgaatca aaaatggatg ggtgattttt tcgtgatgcc
16861 acaagatgag ttccgctcgt acacaaaaat ggctgacctc ttacaagcaa agattgaacg
16921 acgtttccat atcagaccct tagataaaaa tgattttggc tatttgattg aacatctgta
16981 tggcaagaaa acaagttcct ttgaggaata cgaattccct ttacctctca aaaaattgaa
17041 acacaaaaca ctcgtaaaac gatatgattt actgaaaccc actcgttcct tgattgaaga
17101 aaaccaacgc tatttgaaaa ttcaaaatga agaaacgacg tcttatgttg ctcactttac
17161 gattgattcg attgtggggc aattggattt tccttccaat gagattttct attaccagca
17221 acaacaattc gacttcccca ttgataccaa tatgaacatt gaaattgttg ccaataaaaa
17281 agccttgtct actgttcgga acaagaaaaa agaactcaaa gacttggacg atcatgcgtg
17341 gaatagtgaac aatgaaacag gaaacaacgt ttgggaagcc ttagaagatg tcaatgagtt
17401 agaagctgaa ttagaccaga ccaaagaagc catgtacaaa ctaagctatg ttattcgagt
17461 ggagcttctc tcctatgaag aattaaaacg tcgttgcaat gagatcaaaag atttctatga
17521 cgattggagt attaaattgg ttcgtccctt tggcgatatg ttgggcttac attatgaatt
17581 tcttccttca agcaaacgat acatcaacga ctatgtgcaa tatgtgactt ctgactttct
17641 agctggctta ggttttgggt cttcccaaat gttaggagaa acagaaggaa tttacattgg
17701 ctacaacatc gagactgga aaaaatgtgt tcttaaacct agtttagcta gtcaagggtg
17761 caaagggtcg gtgacgaatt ctctggtctc ggcttttctt ggttctttag gtggtgaaa
17821 atccttctct aataacttgt tgggtatta ctcggtttta tttggggcac agcgcttaat
17881 tgttgatcca aaggccgaac gtggcaattg gaaagaaacc ttgccagaca tggccgatga
17941 aattaatatt gtcaatttaa ccagtgaaga gaaaaatcaa ggcttgcttg atccctatgt
18001 tctcatgaaa cggaagaaag acgctgaaag tttagcgatt gatattctca cttttctaac
18061 tggaaattct agtcgagat gggaacgttt cctcttttta ctcgatccgt
18121 cactcaaaaa gagattcgtg gtttgggtt agtcattgat gaattacgag aagatgacaa
18181 cccactatcg cattcgattg cggatcacat tgaaagtttt accgattatg actttgccca
18241 tttactattt agtgatggga caacgaaaaa ctcgattagt tttagaagaa agttaaatat
18301 tattcaagtg gcagatttgg ttttaccaga cgcccaatct aatttttcag attacaccac
18361 tcttgaatta ttgagtgtgg ctatgttaat tgcatttcg acttttgctt tggatttcat
18421 tcattctgat cgaaccaa ttaaaattgt ggatttggac gaagcgtggt cgtttttaca
18481 agtagctcaa ggaaaaacct tgtcgaatcg cttagttcgt gcaggacgtg caatgaatgc
18541 ggggtgttat ttgtcacgc aaaatgcgga cgacttaatc gatgaaaaat tgaaaaacaa
18601 tattgggtta aagtttgctt ttcgttcaac ggatagtcag gaaatcaaaa aacactgac
18661 tttctttggt gtggatccca acgacgagga caatcaaaaa cggttgcgag aattagaaaa
18721 tggccaatgc ttgattcaag atttatatgg gcgtgttggg acaattcaag tccatccagt
18781 ctttgaagat ttattccatg cctttgatac tcgtccccc gcctatcaag aagagtgagg
18841 tgtatacatt gaaaccaaaa ttgaaaaaaa tgctaggtgt cattcttcta attggaggaa
18901 tgactttttt atttctctta ctttttggtta ctgtcgctca ggcgattgga ctagttagtg
18961 ataccgtgga cgtacaaaat ttgtattcac aatatccttt gtctcattat caattggatt
19021 tctacgttga taccgattgg gactggttgc cttggaattg gaaagatggc attggcaatc
19081 aagtgcataa tggcttatag gctatgtctg attttatttg gaccattagt ttgtacctct
19141 ccaatgcgac ggggtacttg atcaaaacag cttacaaact ggatttcacg tcagacacag
19201 cgaatcaaat tgggtgaaat attcaaatat tagcaggcat atcagaaaat ggcttttcga
19261 cttctggtct ttatgttggc tttctgttgt tgtttattct gattgtaggg gcctatgttg
19321 cttatgtcgg atgtctcaaa cgagagacat ccaaagcaat tcaagcagtc accgaatttc
19381 ttgtggtgtt tgttttgcg gccggcttta ttgcttttgc cccttcgtac attcaacgaa
19441 ttaatgaatt ttctgccgat atttcgcaag ccagtttgga tttagggggg caaattatatt
19501 ttctctatct ctcttcacaa ggaacagata gtgtagacag tattcgagat accttgtttt
19561 cggtaacaag cgaacaacct tggcttttac tacaatttga taactctaat aaagaagaaa
19621 ttggcgaaag tcgggtcaat gacctgttgg caatcaatcc tgataccaat aatgggaaag
19681 atcgtgagga agcagtaaaa aatgaaattg aagaacacga caaccaacaa ctaaccttaa
19741 caaaaacggt caatcgatta gggacggtta gttttttagt cttgtttaac attgggattt
19801 ccattttcgt tttcttattg actggcatta tgattttcag tcaaatcttg tttattctct
19861 atgcaatggt cttgcctatt attttcttgt tggctatggt gccaaccttt agtggcatgg
19921 gaaaagcggc attgatgaaa ctctttaata ccattatggt gcgagctgga atcacgttga
19981 ttattacggt ggcgtttagt ttatccacca tgttgtatag cctaaccacc agttatccct
20041 ttttctagt tgccttttta cagattgtga cttttgcggg tatctactta aagatgggcg
20101 atattatgag ttgttttaat cttcaagcca acgattccca acaaaatagt cgagggtta
20161 tgagacgacc aaaacaatat atgcaccgag gagcacgacg acttcaacga accattagtc
20221 gaaccatgat cgggtgttct cttgggtatt tggcaggcaa aaccaaaagg agaaaaaatg
20281 ccaagcaact tccttcaatt gcaggtacaa cgcttccaa tgctcgtgcg tctgtccctt
20341 ccaatcaaca aaaaatgat cgcacacaac gaccaaccac tcgaagttat cagactggcg
20401 acaaaattgg gaaagtcttg gatagtaaaa atcgagcgag caattttgtg gctcaaaaga
20461 aacaacaagt caaagagtta cctaaaacag ttacagggaa tcttcatcaa accatttctg
20521 attttaaaca agggatgtca gatgaacgaa cggaatcagt aacaagtaaa aacagaccgg
20581 ttgtacaatc tcgaaaaaca aaacaagaaa aaaagcgagg aacaacaaat cctacgactg
20641 acaaaaacca tcaacgacca gctgttgatt cggttcaagc agtgaataag atacaacaag
20701 caagtacgtc aaacaacaga atagattcag tatcgagaaa aaatagaccg gttattcaat
20761 ctcgaaaaac aaatcgagaa atgaaaccaa ttcgttctac tgttactcgt ccacctgtac
20821 aacacagacc atcgaaataag atocaaacag tatcgcaaga agtaccacaa ggacagacag
20881 cttccaaagt acaagagcga ataaccacaa aaaggaaccg ctcgataaaa caacaacctt
20941 ctatcaagca acaagctcct atttctcgca agcaaccaac aaggtagatg acaaatcaga
21001 acaaaacacg gtcaactgtc cgttatttctc aaataaaccg tcaaaagaaa aatgtccgtt
21061 tcacacgtcc aatgaaaaga aagtaggtca atcgctatga atgggaaact aaaaataatg
21121 gtggctttta cgctctttct gtccttcttt ggcttattat cttttgtggc aatttttgtg
21181 gcagatgaac ctcaaggtaa ttcttctgat tcattgttta ttcaaggaa tggtttgtct
21241 gtttcaccag aggtcctaaa gtatcgctcc ttagtagaaa aatacgcaaa acaagagaa
21301 attgaggact atatccatat tctcttagcg attattcaag ttgaaagtgg cggcactcta

```

```

21361 aaagatgtca tgcaatcgct agaatacaaaa ggcttacctc caaatacgcg tactgatccc
21421 gaagaatcaa taaaaacaag tgtttcttat tttgcaggac tggtagcttc tgcggaatcg
21481 ttaggtgttg accaacaaga cgtgatccaa gcgtacaatt acggtgggag ctttttaaat
21541 tatgtcgcga agaattggga aacttattcc tttgagttag cggaaagcct ttctaatagaa
21601 caagccaaag gtcaacgagt gacttataaa aatgcacttt ctattcctaa aaatggcggc
21661 tggcggtata tctatggcaa tatgttttat gtgctattag ttatccaata tttagagaca
21721 gcttcgcaaa agtttgataa tcaaacggtt cagataatta tggatgaagc gttgaaatat
21781 caaggctatc cctatgtttt tggcggttcc aatccaataa cctcttttga ctgtagcggc
21841 attaccaaat ggacctttc gaaagctggc atcattttgc ctgtagctgc ccaagcaca
21901 ttagaggtga tggatcattt gctttgtgca caagctgaac caggagattt aattttcttt
21961 cactcgacct atgcaactgc cgattatgtg acacatgtcg gcatttatgt aggagatatg
22021 agaattgtacc acgctgggtg tccgattggt tacaccgact taaatgaaaa ctattggcag
22081 caacatttaa ttggtgctgg acgaataaaa caataagaaa tgaggacaat tcatgaaaa
22141 tcatattgaa cacaacctc aaaaagaaaa agcaaacaga caaaagaaag tcaaaacct
22201 ccaactgggc actcacaaaa agagtgtcct tttcttggg ctgttattga ttggtagctt
22261 tagttttggc ctgtataaaa actttaccgc tattgataca cacacgattc atgagacaa
22321 agtcattgaa cctgtgctt tggataccca tgcgattgag agttttactc ttaatttcat
22381 tcaagaatat tatcttggg aaaataacaa agaagccatt gagcaacgac ctgaacgaat
22441 caatcgctac ctgacttcgg aattacaaga attgaatgta gatacaattc ggaccgatat
22501 tccacacgag cttctgtgca gtgattgtaa aatttggcag atacaatcca ttgatgaaca
22561 gaattttgta gtagtttata cggcgaagca acaaatataa gaaggcgaag aaacgaaaaa
22621 tgtggtctct acctatcgaa tcacgattca tcaagataag aataacaatc tagttattac
22681 aagtaatcca accgtctggg cagcaccgaa tccctctgaa tttcaaccag atacacttgt
22741 aaacgataat aatattgatg aacaaacgaa gctagaatc ctcgattttt tagaaacttt
22801 ctttaagtgt tatcccaatg ctactaagaa tgaattggat tattatatga ttgaagatgt
22861 tttaccaata atcagtcaca actttattta ctctgagttg gtgaatccaa ttattcagaa
22921 gaaagagaaa cagtatctag taaaaatagc cgttcgatat ttagacaatg aaacaaagat
22981 tgaaaatatt tcccaatacc aattaacttt agagaagaag gataactgga aaataataat
23041 gaatgaataa agaattatga gatgaaacaa atacttgaat atttagtata agcaaaaaaa
23101 attgtatgtc actatagcaa acttataatg atttagcata aataatccaa aatatagcgt
23161 gaaatcaacc ttctatgaga agtataagca aattcacctt tattctaact tataactatt
23221 ttcattacat tacttaaaaa ggtctctcca attttttcca aataaacagt ataactttta
23281 atgatatttt ccaagtcaaa cttaatatga acaattcaaa actattggaa ccactgcaaa
23341 atattttgtt aagcagataa tatttcaaaa attatacgtg atatatattg aaaaaggttg
23401 tggacagttg tctatttgac ctgaaaagta atataatagc tggatgaata aagaaacctc
23461 tatgaacttg taaatcgtaa ttaaatcgct gaattttgat agccattctg aagagttatc
23521 ttaactact acactaactt ccattaatat ctcattaat tccctttcgt tcaaaaaata
23581 tggttggctt tacaagcatt ctatcatatg atagaattaa gataacattc aaaaggagat
23641 aaataaaaat cggcaaggtg ttcttaataa aactgtcaat tagatagcgg gaataattaa
23701 ttaagcatcc ttatttaaaa taaggactgg tttttgtgca ccagttaaag aatactttta
23761 tcatttttatc ataaagcatc tgtggctatc agtggttagt atacagctat tttgtatta
23821 aaatcccagt gataagagta tttatcactg ggattttttc tgctttttga gcttttgaat
23881 ggaggaaata aaattgaaaa ttattaatat cgttatctta gcacatgttg atgcagaaa
23941 aactactttg acagaagagt tactatacag tagcggagca attaaagagt taggaagtgt
24001 agatagcggg acaacgaaaa cggatactat gtttttgtaa cgccagagag gtattactat
24061 tcagaccgca ataacatctt ttcaacggga aaatgttaaa gtaaatattg tagatactcc
24121 tggacacatg gatttttttg cagatgtata ccgttcatta tctgttttgg atggagctat
24181 tttgctaata cctgcaaaag atggagtaca gtcacaaact cgtatactat tccatgcact
24241 tagaaagatg aacataccta taatattttt tattaacaaa attgatcaaa atggaataaa
24301 tttgccagat gtttatcaag atattaagga caaactttct gagcacatca taattaagca
24361 gactgtgaat ctaaaattga aaccttatgt aatgatttat actgaaccag aacaattgga
24421 gacagtaatt gtgggaaatg attattttat agaaaaatat accattggga aaacattgaa
24481 tattgcagaa cttgaaaagg aggaaaacga aagaattcaa agttgctcct tatatcctgt
24541 ttatcacgga agtgcaaaag ataattattg aattaaacaa cttagagagg taattactag
24601 caaattattt taccaccac aactcaattc agataaactt tgtggaaatg ttttaaaagt
24661 agaattattc gatgatggtc aacggcttgt ctatgtactg ctttatagtg gaacgctaca
24721 tttgcgagac tcagtcaata tatcagaaaa ggaaaaaata aaagttacag aaatgtatc
24781 ttcaataaat ggagaattac gccagataga taaggcagag cctgggtgaga ttattatttt
24841 aaaaaatgag cttttaaaaa taaataacgt acttggagat aaaaaagat taccacatag
24901 agaaattctt gagaattcct ttcctatggt acaacaaca attgaaccat gtaaatcagt
24961 acaaaagaaa aagttactag atgcactttt tgaatatcc gatagtgat cccttctaca
25021 atattatgta gatacagtaa ctacagaaat tgtgctatct tttttagggt aggtccaaat
25081 ggaggtaact tgtactctga ttcaagaaaa atatcatatt gagatagaaa caagaaaacc
25141 aactgtcatt tatattgaaa gaccttaaaa aaaatctgaa tttaccattg atatcgaaat
25201 acctccaaat ctttctctgg cttctatttg tttatctgta acaccacttc ctttgggtag
25261 tggcattcag tatgagagcc tggtttctct aggttattta aatcaatcat ttcaaaatgc
25321 agttatggaa ggtatagct atgggtgtga acaaggattg tacggttgga aattaacaga
25381 ctgtaagatc tgttttaagt atgggtctata ttacagccct gtcagtacgc cagcagattt
25441 ccgaatgctt gcgcctattg tactagagca ggcttttaga aagagtggta cagagtattt
25501 agagccatat cttagcttcg aaatttatgt accacaagaa tatctttcga gagcatataa
25561 tgatgcttcc aaatattgtg caaatatttt aaatactaag ttaaaaggtg acgaggtcat
25621 tctcattggg gaaattccag cccgttgat tcaagagtat cgaacagttt taactttctt
25681 tacaatgga cgcagtgtct gtttaacaga gttaaaaggt tatcaggtta ctaacattaa
25741 gtctgcttcc caaccacgct gtccaataa tagaataagc aaagtaaggc atatgtttta
25801 taaaaatcaac ttacattgat agtgaactaa agagaaattt ttaaaatgaa tattgagttg
25861 ttaattagaa caggaggata tagatattgt aaatactagg tggatactgt gccctatatg
25921 tggcaataag acccgtttaa aaatgagga agatactgaa ttaaaaaatt tccctctatt
25981 ttgtcctaaa tgcagacaag aaattttaat tgaataacg aagtttagaa taactgtaat
26041 tacagagcca gacgcaaaag cgcagagccg ataattcgag atatcaaaat ctcattttat
26101 cggctttttc ttttctatgc ataccattga gcttagttct tgatatcctt tataagaaaa

```

```

26161 taagtgtact gatttttttt gcagtagctg tattacagta gacatccctc aagcataaaa
26221 agtatacaat taaaattcaa tacttgatta ttgatgaagt acagagttac tcgtcatttc
26281 aaaactagta tatcagtc aa gtttttccta aggaacaaaa acgaacttcg ctatgggaat
26341 caaaaatttt tagtcggatg aagtgtatg caataaatcc agaaaatttt cccaacaaca
26401 aaaaatgaac tgtactttgc agcgataaga gtaaccaaag aactatttat cattcgtctc
26461 agttcctaag tcaatcaaa gtaaaattcat tttctattta tgattaacaa aaatcagaaa
26521 aaattcatta catctttgta atcaatcttt cctgatagta ttaattaatc catttgtgat
26581 tattgaacta gttaaattta cttttttctc cgtaaattgc ttgaatcact ttcccaatcg
26641 tcactaatc atcttcatcc agacaatcca actgtgcttc tactgaaga cgtttggagc
26701 ttttatgact gttttcttcc cctaaaataa atccatccaa agaaaatttt agcaagtca
26761 ctaaatcata tacaacatga aaacttggtc gttgtccttt attttcaatg ttctgcaaat
26821 agcgtggcgc aatctggatc attgatoccta cctgctcacg agtcaatcct ttactaagac
26881 gggcttcttt aatcgcaaga cccaatggtt tgaatcaaaa agttctatgt ttttttctgt
26941 ccatcatata tcacctatcg gcattatact gttctatctc aaatgtaaac aggccttata
27001 taaagcagta tatagcttaa ataaatccta tttttttcaa attgctaaaa gaaaaaaaaa
27061 atcagtcctt tagccgattt ctttctctatg cacaaaaata tatctggagt acactaaacg
27121 acgattttctg aatcgtcatt tttttatgtg taaagctaaa gacgatgttt ttgggagaat
27181 aaaccatgac tcaatgacag catccattgg atttatttac taaatttatc aaaaaaattt
27241 tttctatttc aaagaatcct ttccgattaa aatgtagttt cctggttcta ttaaaaggga
27301 actattttgtg tgaattactt tgtccatatt gtcggatatg gacttttttg tgtttttcta
27361 aaagtttttt tcaaaaaattc cgtcattttc tctctttacg tcgggttata ttacgaaagg
27421 agataaatgt tcatttgacg catcttcttt ctcaacggga aaggaggtgt gaatgacatg
27481 agttcttccc tgttccaagc tgccattgaa atgcaatttg actatatttg taaacgatcc
27541 attgatgacg aacgtaagga ctttttaaa gctctcagtc ggatatcaaa aaaagaagtc
27601 gctttttcag aactagatga ttatgtagtg gaacagtttg ctagtattga ccagtatcca
27661 tccgatttca gttactttga attggagggt gcagaaaattg ctgtttaaag tatgtctcta
27721 ggagaagcat tagagcgttt gccaaaagaa aaaagaaaca ttatcttgtt ttattacttt
27781 attgaaatga atgacgcaga aattgcaaat ttgatgggat tgagtcgctc aacggtaaat
27841 gagcatcgac acaaaagcgtt acatttgatt cgaaaattca gaaggagat gcgcaatgaa
27901 gacaacctat cccagagtc cctttgacgt gattgtacag gcaattgatg gtgatgtggt
27961 ggcaattaat tcccaataag aacatttttc tccgtatatg atgcaacgct ctctccggtt
28021 tctgaaagat gaagtagggc aaaaacatct ggtggtagat gaagtattat ctgctcgtct
28081 tgaaacacgt ttgcttacaa aaatcttagc atttaagatt tgatagcttc atcctctgtg
28141 gaggtgggtc aacctctcga acttctctga ttgtttatta ttccatgaaa gtttactggt
28201 ttaagcaaat tttcatgggc tagtaagcca cttgctcctt gacaaactaa tagcataaatt
28261 agatacgttc agtttttaga gaagcgtgtt aagaaagtgc gcatgatatt tcatttgaaa
28321 ggagcgatgc acacttttgt tacagctttc attttgggaa aatggaaagt cgaggatctc
28381 taagctggat aatgatacac ctaccttgaa cgcctcaccc cattaggtag ctctccgcgt
28441 atgagaagag atgaaacttc tgtgggtctt ccagacgctc ctaattatga gtactaccga
28501 ttgttacgag gatttataca cctgttacta agaccagata ctgatttctg cctttgaatt
28561 gttagatgca aaatctgta gtttaatgac atcgccaga ttcatTTTTT caatggatcg
28621 ctccgctgct ttccattttt tgatggaact taaactgata cctgtgtggt cagaaagttc
28681 ttttgctggt tttcttttca aaattttacg aattaaatga attgtotgca ttacctgttt
28741 cataagggat tagcctcctt ttcatagaaa attttaccat gccactgtag tgggtttcaa
28801 gcttatttca aggttactat agtggctctg cggaattttt gataggagag agccaaaaat
28861 acaaaaatag ataggttttt aaactttctt aaacgaggtg atgaaatgaa aattgaaatt
28921 tcattagaag acaatagatg aataatcgaa gttttaaccg aacagatact gcagcgaatt
28981 gaagaacaat caacaaaatt ttcttctgtc aatgacttac caccctatcc gaatcgtaag
29041 caagtcacaaa aaagattacg tattggagat gaccgattaa atcagtggtat tgccctcagga
29101 ttaaaagtca tccctttttg aaaagaagct cgttttgatc gcatgatata caaagatttc
29161 ttacaccact taaaagttaa agtttgacga ttacgcctaa tccgcctatg ataccagtat
29221 ggaaggagga aaaacgattt ccctttgata tactggcgaa tcattcaaa gagaactccc
29281 atgattaaaa aatatacaaa aaagaatggg tctactgctt atctggttaa agtctattta
29341 ggcgttgatc cgcttactgg aaagaagaaa acaacaacca agcaggggtt caaaacccaa
29401 aaagcagcca aacaagaagt agcacgttta cagttactcg cacaagaata tgatcttgtc
29461 atcagagtcta accgatttgt ttccgatatc gcttttagat gggttgaaat atataaaaaat
29521 acggtcaaa gaaagtaccta tgttgttcaa agagtgcgat taaacaaaca tattcttcca
29581 ctatttggtg aacggagaat ttctaaaaat tccattccgt attgtcagaa acaagtcaat
29641 cattggtatt cctattacaa aaagtattct aatctgattg gtatgacaaa ctccattttt
29701 caatacgcca aaagctttag actgattcgt agaaatccaa tggaaaggct tattcgacca
29761 aagagacaaa aacaaattga tgaagaaaaa tatarcgctc cttctatata gaaagcagaa
29821 gttctatact tcttcgaaat tgctaaaaaa tctcctgac cattgtaccc aatgtttcat
29881 attttaactt ttacaggctt acgtaaagggt gaattactag ctttacgatg gaaagacatt
29941 gatttcaaac gaggaacctt ttctgttaag caaacattaa cgactgttga agattggaaa
30001 tttagctttc aaaccccgaa aacagaaaaa agtttgcgaa ctatttcgat tgataatcaa
30061 actttatcaa tctttcgaca atgtattctt aaacaaaaag cctttttttt aaaaacaggt
30121 agaaaacctg tggaaaatgg cgcacaactt cttttcacga cagaagaaaa taaaccgcgt
30181 tactgaattt ctttaataca taatttaact aaaatactga aagaaaaata gttaaagcat
30241 atgactgttc atgggttttc tcatacgcgt tgtagtttat tgtttgaatc aggagcttct
30301 atcaaagaag tacaagttcg tttgggtcat acggatgttc gaactaccat ggatatttac
30361 gccatgtga gtgaacagaa aaaagaagaa acggcagatc gttttgctga ttacatgaat
30421 aagttgcacg aggaagaaga tgggatgggt caaaacgaaa aaaagagcag
30481 actccaccta taatgaagtc tgctaaaagc tgataaatca gcgtttttct tattccgcag
30541 cgtttgctgc tttgagaccg ttttttttgt tgaacggtc cacacgtccg tctgcttgtg
30601 tgaatttttg acgaccagtg t

```

//

Nucleotide

Display Settings: ☒ GenBank

Enterococcus faecium strain A119 transposon Tn6000, partial sequence

GenBank: JN208880.1

[FASTA](#) [Graphics](#)Go to: ☐

LOCUS JN208880 6862 bp DNA linear BCT 30-OCT-2012

DEFINITION Enterococcus faecium strain A119 transposon Tn6000, partial sequence.

ACCESSION JN208880

VERSION JN208880.1 GI:347952132

KEYWORDS .

SOURCE Enterococcus faecium

ORGANISM [Enterococcus faecium](#)
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 6862)
AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.
TITLE Different Genetic Supports for the tet(S) Gene in Enterococci
JOURNAL Antimicrob. Agents Chemother. 56 (11), 6014-6018 (2012)
PUBMED [22908170](#)

REFERENCE 2 (bases 1 to 6862)
AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.
TITLE Spread of tet(S) among enterococci is associated with CTn6000
JOURNAL Unpublished

REFERENCE 3 (bases 1 to 6862)
AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.
TITLE Direct Submission
JOURNAL Submitted (05-JUL-2011) Microbiology, REQUIMTE Pharmacy Faculty, Porto University, Rua Anibal Cunha, 164, Porto 4050-047, Portugal

FEATURES

source Location/Qualifiers
1..6862
/organism="Enterococcus faecium"
/mol_type="genomic DNA"
/strain="A119"
/isolation_source="nontreated drinking water from fountain"
/db_xref="taxon:[1352](#)"
/country="Portugal"
/collection_date="2008"

[mobile element](#) <1..>6862
/note="conjugative transposon"
/mobile_element_type="transposon:Tn6000"

[misc feature](#) 1..>293
/note="similar to hypothetical protein; ORF14; disrupted by group II intron; putative conjugation related protein"

[gene](#) 1007..2836
/gene="rvt"

[CDS](#) 1007..2836
/gene="rvt"
/note="group II intron reverse transcriptase"
/codon_start=1
/transl_table=[11](#)
/product="reverse transcriptase"
/protein_id="[AEP33191.1](#)"
/db_xref="GI:347952133"
/translation="MTVKVSNKKALRFNEYDQTQKIQDELYQLSAEGSHVFRDLMSYI
TQENILLAYRNKISNKGSKTAGTNKRTIIDVGEENPYQLVQYVQNRFNFPQHSIRR
VEIPKPNKTRPLGIPITIEDRLVQQCIQILEPILEAKFHKHSYGFRPERSHHAIAI
FQQWTFKGFHYVVDIDIKGFFDNVNHGKLLKQLWTMIRDKTFISILSRMLKAEVKG
GKSTKGTPOGGILSPLLANVVLNELDWWIDSQWDGFPTRKRYSSLLSKTQSIRKYNL
KEIKIVRYADDFKIMCKDYHTAQKIFLATQWLKVRLLDISPEKSKVTNLRKNYSDF
LGFKLKVKKGKANGYTNRSRMCDAKINAVDKLNKNIKTIAANPTVDNVNKNYSVVLG
LHNYKYIATLVNLDVFDIAFTVNSSLDCRTKNIRNKHGTLTSTYQKFYKQYNWKKRFI
ANMILFPIAGIKFTMPRIFFPKDWNRYTPEGRLFHQRLKMDMHTVYYLLENSNPNSV
EFNDNRISLFIAQQGKCHVSGENLSKDNMEVHHIKPLSLGGNDNYKNLVIVTKETHKL
IHATKRETIHVHLPVLVKKERSLEKLNKLALAGNSEIEMNYC"

[misc feature](#) <2932..2997
/note="similar to hypothetical protein; ORF14; disrupted

by group II intron; putative conjugation related protein"
 3014..3931
 /note="ORF13; putative conjugation related protein"
 /codon_start=1
 /transl_table=11
 /product="hypothetical protein"
 /protein_id="AEP33192.1"
 /db_xref="GI:347952134"
 /translation="MKIHIEHKPKKEKANRQKKVKTIIHLGTHKKSVLFLWLLIGSFS
 FGLYKNFTAIDTHTIHETTVIEPVLVDTHAIESFTLNFIEYYSWENNKEAIEQRP
 INRYLTSELQELNVDTIRTDIPTSSSVSDCKIWQIQSIDEQNFVVVYTVKQIQKEGEE
 TKNVASTYRITIHQDKNNLVITSNPTVWAAPNPSEFQPDTLVNDNNIDEQTKLEILD
 FLETFFKLYPNATKNELDYMMEDVLPISQNFYSELVNPIIQKKEKQYLKIAVRY
 LDNETKIENISQYQLTLEKKDNWKIIMNE"

mobile element 4208..5532
 /mobile_element_type="insertion sequence:ISEfm2"

repeat region 4221..4233
 /rpt_type=inverted

gene 4302..5480
 /gene="tnpA"

CDS 4302..5480
 /gene="tnpA"
 /note="ISEfm2 transposase"
 /codon_start=1
 /transl_table=11
 /product="transposase"
 /protein_id="AEP33193.1"
 /db_xref="GI:347952135"
 /translation="MNDFTTEILKTLANKGDLNELFRVHLEKAVNTLLKTELTAFLDY
 EKYDRIGFNTGNSRNGSYDRTVKTEYGEHLHQIPDRNGEFKQQTVPAYRRNTDLE
 TVIHLFRKGITMSEIADLIEKMYGHHYTPQTMNSMTKVLTEEVNAFKVRTLNDKYVAI
 FMDATYIPLKRQTVSKEAIYIAIGIREDTGKEVLSYAIAPTSTYVWNELLQDIYSRG
 VHDVLLFITDGLKGMKDTIHQIYPKAKYQHCCVHVSRNIAHKVRVKDRKEICDDFKAV
 YQASSKEANTFLGSMIEKWQKTYPKVTQSLIKNQDLLTFYEFPPGIRRSIYSTNLIE
 SFNKQIKKYSRRKQDFQNEESLERFLVSIFDTYNQKFLNRSHKGFQQTDTTLASMFTE
 "

repeat region 5506..5518
 /rpt_type=inverted

gene 6108..>6862
 /gene="tetS"

CDS 6108..>6862
 /gene="tetS"
 /note="tetracycline resistance protein"
 /codon_start=1
 /transl_table=11
 /product="TetS"
 /protein_id="AEP33194.1"
 /db_xref="GI:347952136"
 /translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTKTD
 TMFLERQRGITITQTAITSFQRENVKVNIVDTPGHMDFLADVRSLSVLDGAILLISAK
 DGVQSQTRILFHALRKNPIIIFFFINKIDQNGINLPDVYQDIKDKLSDDIIIKQTVNL
 NLKPYVIDYTEPEQWETVIVGNDYLLKEYTIGKTLNIAELEKEENERIQSCSLYPVYH
 GSAKNNIGIKQLIEVITSKLFSPQLNSDKLCG"

ORIGIN

```

1 aagcgttgaa atatcaagc tatccctatg tttttggcgg ttccaatcca aatacctctt
61 ttgactgtag cggcattacc caatggacct ttcagaaagc tggcatcatt ttgcctcgta
121 ctgccaagc acaatacagc gtgatggatc atttgccttt gtcacaagct gaaccaggag
181 atttaatttt ctttactcgc acctatgcaa ctgccgatta tgtgacacat gtcggcattt
241 atgtaggaga tatgagaatg taccacgctg gtgatcgtgc gagacgttcc taactgaaaa
301 ggtaggcaga ttacaacctc gcaaaaacaa aagttgtaac gagaactgat aattcgaaaa
361 gtcgaatgac ggagtaacgc cctgaaaggc tttagctaaa actgcgacag gcgataaatt
421 attaaggatt tcatggctcg gagctcgagc aagtcgactg agaagacgcc ctaatcgat
481 tttgagaata cgaaaggaaa tgcgaaactag aggtagtcga gtgtccgata ggtcgggctt
541 ccataaaaata ctcatggtaa gaatgtgcga aaagcactga cgaaattctg aatgtacgtg
601 tctacaccgc gaagttgtct aacggcagct actacaaatc gtagggtttg tggggtggag
661 taaaactggt cactatgaaa ctccctatat cgttacaggc gatatcaagc aaacaggcac
721 agagggatta cctaaaagta tgatgtacgg atagaattat cggaacgtcg taagctgaaa
781 acacggatag ctctattcgt aaatgaagtg tttgtaagga aaagccttga ctataactta
841 cattaatttc agtgaaagtg gtggcacagt acctacgaaa cagtataaac aagctgtgga
901 gggatagcca ctagtcaaca gtaattcaat ctatttgact gttcatgaat taggttcggg
961 taagactaag atagggaaac ttcattatta cggaaggaa cactaatga cagtcaaagt
1021 tcaataacaaa aaggcactga ggttcaatga gtattacgat actcagaaaa ttcaagacga
1081 actctatcaa ttatctgcgc aaggcagtc tgtgtttaga gatttaatga gctatatcac
1141 acaagaagaa aatattttat tggcttatcg aaatatcaaa agcaataaag gcagtataaac
1201 tgctggaaca aataaaagaa cgattattga tgttggtgaa gaaaacccat atcaattagt
1261 tcaatacgtt caaaatcgat ttaacaactt tcaacctcac tccatcagaa gactcgagat
1321 tccaaagcca aatggtaaaa ccagaccgct aggaatccca acaattgagg atagattagt
1381 acagcaatgc ataaagcaga tccttgaacc aattttgaa gcaaaattcc ataagcatc
1441 ttatggattt cgaccagaaa gaagttcgca ccatgccata gcgatatttc agcaatggac
1501 atttaaaaga tttcactatg tagtggacat tgatattaag ggcttccttg acaatgtaaa

```

```

1561 tcatggaaaa ttgcttaagc aactctggac aatgaaaatt cgagacaaga cattttatttc
1621 aatattgtcg agaagtctta aggcagaggt aaaaggcatt ggaaaatcaa ccaaaggaac
1681 cccacaaggt ggtattcttt ctctctcttt agctaattgt gtgctaaacg aattagattg
1741 gtggatagat agtcagtggt acggattttcc aacccaaaaga aaatacagta gtctgtctgag
1801 taaaactcaa tcaataagaa agtattcaaa cttgaaagaa atcaaaattg tccgttacgc
1861 agatgatttc tcatagcgga gcaaagatta tcatactgcc cagaaaaattt tctctgcaac
1921 aaaacaatgg ttaaaagtac gacttgattt agacataagt cctgaaaagt caaaagtaac
1981 taatctgaga aaaaattatt ctgactttct aggattcaag ttgaaaagtga aaaaaggaaa
2041 agcaaacgga tataccaact gaagccgaat gtgtgataag gcaaagatta atgcggtaga
2101 taaactaaag aacaatatca aaacaatcgc cgctaatacca actgtagata atgtcaataa
2161 gtacaactct gttgttctag gtttgcataa ctattataaa attgcgactc tgggtcaactt
2221 agatttttgt gatattgctt tcacagtaaa taaaagtctt gattgtcgaa ccaaaaaatat
2281 tcgcaataag cacggaacgc taaccagtac ctatcaaaaa ttctataaac aatataactg
2341 gaagaaacga ttcattagcg acatgatttt atttccaata gccgggataa aattttacaat
2401 gccagaatc tttccgaagg actggaatcg atatacgctt gaaggtaggc tgtttattca
2461 tcaaaagatta aagatggata tgcataccgt ttattacctg ctggaaaatt ccaatccgaa
2521 taagtctgta gaatttaacg ataatcgaat ctctctgttc atagcccaac aaggaaaatg
2581 tcatgttaag gtgaaaattt tatcaaaaga taatatggaa gttcatcaca ttaagccgtt
2641 atcacttggt gggaaatgata actataagaa tcttgtgatt gttacaaaag aaacacacaa
2701 attgattcat gcgactaaaa gagaaacgat agttcattat ttacctttgg tattaaaaaa
2761 ggaaagatca ttggaaaagc tgaataaatt acgtgcttta gctgggaata gtgaaataga
2821 aatgaattac tttgtatgga acgcccgtgt aggggaaact ctcatgcacg gtgtgaagcg
2881 ggggaaaaga tggagataac ctcaaagtct tactatcgct tatcgattgg ttacaccgac
2941 ttaaatgaaa actattggca gcaacattta attgggtctg gacgaataaa acaataagaa
3001 ataggacaaa ttcattgaaa ttcatattga acacaaacct aaaaaagaaa aagcaaacag
3061 acaaaagaaa gtcaaaaacc tccacttggg cactcacaaa aagagtgtcc ttttcttgtg
3121 gctgttattg attggtagct ttagttttgg cctgtataaa aactttaccg ctattgatac
3181 acacacgatt catgagacaa cagtcattga acctgtgctt gtggataacc atgcgattga
3241 gagttttact cttaatttca ttcaagaata ttattcttgg gaaaataaca aagaagccat
3301 tgagcaacga cctgaacgaa tcaatcgtaa cctgacttcg gaattacaag aattgaatgt
3361 agatacaatt cgggacgata ttcccacgag ctcttctgtc agtgattgta aaatttggca
3421 gatacaatcc attgatgaac agaattttgt agtagtttat acggtcaagc aacaaattaa
3481 agaaggcgaa gaaacgaaaa atgtggcttc tactatcgca atcacgattc atcaagataa
3541 gaataacaat ctagtattta caagtaatcc aacgctctgg gcagcaccga atccctctga
3601 atttcaacca gatacacttg taaacgataa taatattgat gaacaaacga agctagaaat
3661 tctcgatttt ttagaaactt tctttaagtt gtatcccaat gctactaaga atgaattgga
3721 ttattatatg atggaagatg ttttaccaat aatcagtcga aactttattt actctgagtt
3781 ggtgaatcca attattcaga agaaagagaa acagtatcta gtaaaaatag ccgttcgata
3841 tttagacaat gaaacaaaga ttgaaaatat ttcccaatac caattaaact tagagaagaa
3901 ggataactgg aaaataataa tgaatgaata aagaatatta agatgaaaac aatacttgaa
3961 tatttagtat aagcaaaaaa aattgtatgt cactatagca aacttataat gatttagcat
4021 aaataatcca aaatatagcg tgaatcaac ctctatgag aagtataagc aaattcacct
4081 ttatttcaac ttataactat ttctattaca ttacttaaaa aggtctctoc aattttttcc
4141 aaataaacag tataactttt aatgatattt tccaagtcaa acttaatat aaacaattcaa
4201 aactattgag agtgtaaaat attttgtgta aatagaaaaa aggaagtcoc ttctgtagaa
4261 tagagttacc acaacacatt cacagaaaag aggacttcca tatgaacgat ttactacag
4321 aaattctaaa gactctagcg aacaaaggcg atttgaatga attattccgt gtccatttgg
4381 aaaaagctgt caatacgcct ctcaaaacgg agttaacggc tttcctcgat tacgaaaagt
4441 acgatcgat tggttttaac acgggtaatt ctcgtaacgg ctctatgac cgtacggtca
4501 agaccgagta cggggaactt catctccaga ttccgcgcga ccgcaacggc gaggttcaagc
4561 aacagactgt tctgactgtt agacggacga atgatacctt agaggagacc gtcattcacc
4621 tcttccgaaa aggtattacc atgtcggaaa tcgcagactt gattgagaaa atgtatgggc
4681 atcactacac gccccaaacc atgtccaaca tgactaaagt gctgactgaa gaagtaaatg
4741 catttaaaag tagaactcta aatgataagt atgtcgctat tttcatggat gctacttata
4801 ttcctttaaa acggcaaaag gtctccaaag aagcaatcta tattgccatt ggtataacga
4861 aagacggcac taaagaagta ctaagttatg cgattgtctc gactgaatcg acctacgttt
4921 ggaatgagtt gttacaggac atttactcca gaggggttca tgatgtctta ctgtttatta
4981 ccgatggctt aaaaggtcatg aaagatacga ttaccagat ttatcctaaa gctaaatacc
5041 aacattgctg tgttcacgtt tcccgtata ttgctcataa agtacgtgtc aaagatcgaa
5101 agaaaatctg tgatgttttt aaggctgttt atcaagctag ctcaaaaggaa gaggcataa
5161 ctttttttag gagtatgatt gagaagtggc agaaaactta tcctaaagtg acgcagtcac
5221 tgataaaaaa tcaagattta ttgacttttt atgagtttcc gcctggatgc cgccgaagca
5281 ttactcaac taatttaact gaatctttca ataaacaaat caagaaatgc agccgcagaa
5341 aagatcagtt tcaaaatgaa gaatcactag agcgtttctt agtatccatc tttgatacat
5401 acaatcaaaa attcttaaat agaagccata aaggcttcca acaagtgcac gatacattag
5461 cttcaatggt tactgagtaa cccattattt tgcagaagga cgagttattt acacaaaatt
5521 attgacgctc ccaaactatt ggaaccactg caaaatattt tgtaagcag tatatatctc
5581 aaaaattata cgtaaatata ttgtagaagg gttgtggaca gttgtcctat tgacctgaaa
5641 agtaataata tagctggatg aataaagaat cccttatgaa cttgtaaatc gttattaaat
5701 cgctgaattt tgatagccat tctgaagagt tatcttcaac tactacacta acttccatta
5761 atttctcatt aatttccctt tctgtcaaaa aatatggttg gctttacaag cattctatca
5821 tatgatagaa ttaagataac attcaaaagg agatgataaa aatcggcaca ggtattctta
5881 aataaactgt caattagata gcgggaataa ttaattaagc atccttattt aaaataagga
5941 ctggtttttt gtgccaggtt taagaatact tttatcattt tatcataaag catctgtggc
6001 tatcagtggt tagtatacag ctatttttgt attaaaaatc cagtgtaga agtatttatc
6061 actgggattt tttctgcttt ttgagctttt gaatggagga aataaaaattg aaaattatta
6121 atatcggtat cttagcacat gttgatgcag gaaaaactac tttagacaga agcttactat
6181 acagtagcgg agcaattaaa gaggtaggaa gtgtagatag cgttacaacg aaaacggata
6241 ctatgttttt ggaacgccag agaggattta ctattcagac cgcaataaca tcttttcaac
6301 gggaaaatgt taaagtaaat attgtagata ctctggaca catggatttt ttggcagatg

```

```
6361 tataccgttc attatctggt ttggatggag ctatcttgct aatctctgca aaagatggag
6421 tacagtcaca aactcgtata ctattccatg cacttagaaa gatgaacata cctataatat
6481 tttttattaa caaaattgat caaaatggaa taaatttgcc agatgtttat caagatatta
6541 aggacaaact ttctgacgac atcataatta agcagactgt gaatctaaat ttgaaacctt
6601 atgtaataga ttatactgaa ccagaacaat gggagacagt aattgtggga aatgattatt
6661 tattagaaaa atataccatt gggaaaacat tgaatattgc agaacttgaa aaggaggaaa
6721 acgaaagaat tcaaagttgc tccttatatc ctgtttatca cggaagtgca aagaataata
6781 ttggaattaa acaacttata gaggtaatta ctagcaaatt attttcacc acacaactca
6841 attcagataa actttgtgga aa
```

//

Nucleotide

Display Settings: ☒ GenBank

Enterococcus faecalis strain WBH16 plasmid insertion sequence IS1216 transposase gene, partial cds; and TetS (tetS) gene, partial cds

GenBank: JN980097.1

[FASTA](#) [Graphics](#)[Go to:](#) ☒

LOCUS JN980097 1552 bp DNA linear BCT 19-MAR-2012

DEFINITION Enterococcus faecalis strain WBH16 plasmid insertion sequence IS1216 transposase gene, partial cds; and TetS (tetS) gene, partial cds.

ACCESSION JN980097

VERSION JN980097.1 GI:380040898

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM [Enterococcus faecalis](#)
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 1552)
AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.
TITLE Spread of tet(S) among enterococci is mediated by CTn6000 and plasmids harbouring different composite sequence
JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1552)
AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.
TITLE Direct Submission
JOURNAL Submitted (01-NOV-2011) Microbiology, REQUIMTE, Pharmacy Faculty, Rua Jorge Viterbo Ferreira #228, Porto 4050-313, Portugal

FEATURES
source Location/Qualifiers
1..1552
/organism="Enterococcus faecalis"
/mol_type="genomic DNA"
/strain="WBH16"
/isolation_source="clinical sample"
/db_xref="taxon:[1351](#)"
/plasmid="unnamed"
/country="Serbia"
/collection_date="2002"
/note="CC87/ST28"

[mobile element](#) <1..129
/mobile_element_type="insertion sequence:IS1216"

[CDS](#) complement(<1..61)
/codon_start=1
/transl_table=[11](#)
/product="transposase"
/protein_id="[AFD32986.1](#)"
/db_xref="GI:380040900"
/translation="MTMNHFKGKQFQQDVIIIVAV"

[repeat region](#) 118..129
/note="IR left"
/rpt_type=inverted

[gene](#) 749..>1552
/gene="tetS"

[CDS](#) 749..>1552
/gene="tetS"
/note="tetracycline resistance protein"
/codon_start=1
/transl_table=[11](#)
/product="TetS"
/protein_id="[AFD32985.1](#)"
/db_xref="GI:380040899"
/translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTKTD
TMFLERQRGITIQTAITSFQRENVKVNIVDTPGHMDFLADVRSLSVLDGAILLISAK
DGVQSQTRILFHALRKMNPIIIFFINKIDQNGINLPDVYQDIKDKLSDDIIIKQTVNL
NLKPYVIDYTEPEQWETVIVGNDYLLKTYTIGKTLNIAELEKEENERIQSCSLYPVYH
GSAKNNIGIKQLIEVITSKLFSPQTQLNSDKLCGNVFKVEYSDDGQRLVYV"

ORIGIN
1 ccacggctac aataatcaca tcctgctgaa attgctttcc tttaaaatga ttcacgtc
61 ttctctctgc tatctttttc tattattcta ccttatttga tagtagattt aaaactttgc

```
121 aacagaaccc ttttaatgat attttccaag tcaaacttaa tattaacaat tcaaaactat
181 tggaaacct gcaaaatatt ttgttaagca gtatatatct caaaaattat acgtaatata
241 tttgtagaaa ggttggtggac agttgtccta ttgacctgaa aagtaatata atagctggat
301 gaataaaagaa tcccttatga acttgtaaat cgttattaaa tcgctgaatt ttgatagcca
361 ttctgaagag ttatcttcaa ctactacact aacttcatt aatttctcat taatttcctt
421 ttcgttcaaa aaatatgggt ggcttttaca gcattctatc atatgataga attaagataa
481 cattcaaaag gagataataa aaatgcggca aggtattctt aaataaactg tcaattagat
541 agcgggaata attaattaag catccttatt taaaataagg actgggtttt tgtgccagt
601 ttaagaatac ttttatcatt ttatcataaa gcactctgtg ctatcagtgt gtagtatata
661 gctatttttg tattaaaatc ccagtataaa gagtatttat cactgggatt tttctgctt
721 tttgagcttt tgaatggagg aaataaaatt gaaaattatt aatatcggta tcttagcaca
781 tgttgatgca ggaaaaacta ctttgacaga aagcttacta tacagtagcg gagcaattaa
841 agagtttaga agttagata gcggtacaac gaaaacggat actatgtttt tggaacgcca
901 gagaggattt actattcaga ccgcaataac atcttttcaa cgggaaaatg ttaaagtaaa
961 tattgtagat actcctggac acatggattt ttggcagat gtataccgtt cattatctgt
1021 tttggatgga gctattttgc taatctctgc aaaagatgga gtacagtcac aaactcgtat
1081 actattccat gcacttagaa agatgaacat acctataata ttttttatta acaaaattga
1141 tcaaaatgga ataaatttgc cagatgttta tcaagatatt aaggacaac tttctgacga
1201 catcataatt aagcagactg tgaatctaaa ttgaaacct tatgtaatag attatactga
1261 accagaacaa tgggagacag taattgtggg aaatgattat ttattagaaa aatataccat
1321 tgggaaaaca ttgaatatg cagaacttga aaaggaggaa aacgaaagaa ttcaaagttg
1381 ctccttatat cctgtttatc acggaagtgc aaagaataat attggaatta aacaacttat
1441 agaggtaatt actagcaaat ttttttcacc cacacaactc aattcagata aactttgttg
1501 aaatgttttt aaagtagaat attcagatga tggtaacg cttgtctatg ta
```

//

Nucleotide

Display Settings: ☒ GenBank

Enterococcus faecium strain E241 plasmid TetS (tetS) gene, partial cds; and insertion sequence IS1216 transposase gene, partial cds

GenBank: JN980096.1

[FASTA](#) [Graphics](#)[Go to:](#) ☒

LOCUS JN980096 1544 bp DNA linear BCT 19-MAR-2012

DEFINITION Enterococcus faecium strain E241 plasmid TetS (tetS) gene, partial cds; and insertion sequence IS1216 transposase gene, partial cds.

ACCESSION JN980096

VERSION JN980096.1 GI:380040895

KEYWORDS .

SOURCE Enterococcus faecium

ORGANISM [Enterococcus faecium](#)
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 1544)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.

TITLE Spread of tet(S) among enterococci is mediated by CTn6000 and plasmids harbouring different composite sequence

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1544)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.

TITLE Direct Submission

JOURNAL Submitted (01-NOV-2011) Microbiology, REQUIMTE, Pharmacy Faculty, Rua Jorge Viterbo Ferreira #228, Porto 4050-313, Portugal

FEATURES

source Location/Qualifiers

1..1544

/organism="Enterococcus faecium"

/mol_type="genomic DNA"

/strain="E241"

/isolation_source="hospital residual water"

/db_xref="taxon:[1352](#)"

/plasmid="unnamed"

/country="Portugal"

/collection_date="2002"

/note="ST17/CC17"

[gene](#) complement(<1..792)

/gene="tetS"

[CDS](#) complement(<1..792)

/gene="tetS"

/note="tetracycline resistance protein"

/codon_start=1

/transl_table=[11](#)

/product="TetS"

/protein_id="[AFD32983.1](#)"

/db_xref="GI:380040896"

/translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTKTD
TMFLERQRGITITQAITSFQRENVKVINIVDTPGHMDFLADVYRSLSVLDGAILLISAK
DGVQSQTRILFHALRKMNPIPIFFINKIDQNGINLPDVYQDIKDKLSDDIIKQTVNL
NLKPYVIDYTEPEQWETVIVGNDYLLKTYIGKTLNIAELEKEENERIQSCSLYPVYH
GSAKNNIGIKQLIEVITSKLFSPQQLNSDKLCGNVFKVEYSDDGQR"

[mobile element](#) 1412..>1544

/mobile_element_type="insertion sequence:IS1216"

[repeat region](#) 1412..1433

/note="IR left"

/rpt_type=inverted

[CDS](#) 1480..>1544

/codon_start=1

/transl_table=[11](#)

/product="transposase"

/protein_id="[AFD32984.1](#)"

/db_xref="GI:380040897"

/translation="MTMNHFKGKQFQQDVIIIVAVG"

ORIGIN

1 ccgttgacca tcatctgaat attctacttt aaaaacattt ccacaaagtt tatctgaatt

61 gagttgtgtg ggtgaaaata atttgctagt aattacctct ataagttgtt taattccaat

121 attattcttt gcacttcctg gataaacagg atataaggag caactttgaa ttctttcgtt

```
181 ttcctccttt tcaagttctg caatattcaa tgttttccca atggtatatt tttctaataa
241 ataatcattt cccacaatta ctgtctccca ttgttctggt tcagtataat ctattacata
301 aggtttcaaa tttagattca cagtctgctt aattatgatg tcgtcagaaa gtttgtcctt
361 aatatcttga taaacatctg gcaaatttat tccattttga tcaattttgt taataaaaaa
421 tattataggt atgttcacat ttctaagtgc atggaatagt atacgagttt gtgactgtac
481 tccatctttt gcagagatta gcaaaatagc tccatccaaa acagataatg aacggtatag
541 atctgccaaa aaatccatgt gtccaggagt atctacaata tttactttaa ctttttccc
601 ttgaaaagat gttattgctg tctgaatagt aatacctctc tggcgttcca aaaacatagt
661 atccgttttc gttgtaccgc tatctacact tcctaactct ttaattgctc cgctactgta
721 tagtaagctt tctgtcaaaag tagtttttcc tgcatacaaca tgtgctaaga taccgatatt
781 aataattttc aattttattt cctccattca aaagctcaaa aagcagaaaa aatcccagtg
841 ataaatactc ttatcactgg gattttaata caaaaatagc tgtatactac acactgatag
901 ccacagatgc tttatgataa aatgataaaa gtattcttaa actgggcaca aaaaaccagt
961 ccttatttta aataaggatg cttaattaat tattcccgtc atctaattga cagtttattt
1021 aagaatacct tgccgcattt ttattatctc cttttgaatg ttatcttaat tctatcatat
1081 gatagaatgc ttgtaaagcc aaccatattt tttgaacgaa agggaaatta atgagaaatt
1141 aatggaagtt agttagtag ttgaagataa ctcttcagaa tggctatcaa aattcagcga
1201 ttttaataacg atttacaagt tcataaggga ttctttattc atccagctat tatattactt
1261 ttcagggtcaa taggacaact gtccacaacc tttctacaaa tatattacgt ataatttttg
1321 aaatatatac tgcttaacaa aatattttgc agtgggtcca atagttttga attgttaata
1381 ttaagtttga cttggaaaat atcattaaaa gggttctgtt gcaaagtttt aaatctacta
1441 tcaaataaag tagaataata gaaaaagata gcaggaggaa tgacgatgaa tcattttaaa
1501 ggaaagcaat ttcagcagga tgtgattatt gtagccgtgg gcta
```

//

Nucleotide

Display Settings: ☒ GenBank

Enterococcus sp. SN373 insertion sequence IS1216 transposase gene, partial cds; cadE gene, complete sequence; tetS gene, partial sequence; and TetS gene, partial cds

GenBank: JN980095.1

[FASTA](#) [Graphics](#)[Go to:](#) ☒

LOCUS JN980095 1676 bp DNA linear BCT 19-MAR-2012

DEFINITION Enterococcus sp. SN373 insertion sequence IS1216 transposase gene, partial cds; cadE gene, complete sequence; tetS gene, partial sequence; and TetS gene, partial cds.

ACCESSION JN980095

VERSION JN980095.1 GI:380040892

KEYWORDS .

SOURCE Enterococcus sp. SN373

ORGANISM [Enterococcus sp. SN373](#)
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 1676)
AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L. and Coque,T.M.
TITLE Spread and Diversification of tet(S) Conjugative Transposon CTn6000
JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1676)
AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L. and Coque,T.M.
TITLE Direct Submission
JOURNAL Submitted (01-NOV-2011) Microbiology, REQUIMTE, Pharmacy Faculty, Rua Jorge Viterbo Ferreira #228, Porto 4050-313, Portugal

FEATURES
source Location/Qualifiers
1..1676
/organism="Enterococcus sp. SN373"
/mol_type="genomic DNA"
/strain="SN373"
/isolation_source="piggery; food for animals"
/db_xref="taxon:[1156716](#)"
/plasmid="unnamed"
/country="Portugal"
/collection_date="2006"

[mobile element](#) complement(<1..134)
/mobile_element_type="insertion sequence:IS1216"

[CDS](#) complement(<1..66)
/codon_start=1
/transl_table=[11](#)
/product="transposase"
/protein_id="[AFD32981.1](#)"
/db_xref="GI:380040893"
/translation="MTMNHFKGKQFQQDVIIIVAVGY"

[repeat region](#) complement(113..134)
/note="IR left"
/rpt_type=inverted

[gene](#) 187..261
/gene="cadE"

[misc feature](#) 187..261
/gene="cadE"
/note="nonfunctional cadmium efflux system accessory protein due to mutation"

[gene](#) 873..>1676
/gene="tetS"

[CDS](#) 873..>1676
/gene="tetS"
/note="tetracycline resistance protein"
/codon_start=1
/transl_table=[11](#)
/product="TetS"
/protein_id="[AFD32982.1](#)"
/db_xref="GI:380040894"
/translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTKTD
TMFLERQRGITITQTAITSFQRENVKVNIVDTPGHMDFLADVRSLSVLDGAILLISAK
DGVQSQTRILFHALRKMNPIIFFINKIDQNGINLPDVYQDIKDKLSDDIIIKQTVNL
NLKPVIDYTEPEQWETVIVGNDYLLLEKYTIGKTLNIAELEKEENERIQSCSLYPVYH

GSAKNNIGIKQLIEVITSKLFSPPTQLNSDKLCGNVFKVEYSDDGQRLVYV"

ORIGIN

```

1  gtagccacag  gctacaataa  tcacatcctg  ctgaaattgc  tttcctttaa  aatgattcat
61  cgtcattcct  cctgctatct  ttttctatta  ttctacctta  tttgatagta  gatttaaaac
121  ttgcaacag  aaccatcaaa  tagatatact  tcattcaaac  aatcgtttga  atgtgaggtg
181  agatgaatga  aaaaagtttg  ccaagtgact  gtcgtacata  aagaaaaagt  aaatgggata
241  aaaaaataat  tggatcaaaa  agatatcttc  caagtcaaac  ttaataattaa  caattcaaaa
301  ctattggaac  cactgcaaaa  tattttgtta  agcagtatat  atttcaaaaa  ttatacgtaa
361  tatatttgta  gaaaggttgt  ggacagttgt  cctattgacc  tgaaaagtaa  tataatagct
421  ggatgaataa  agaatccctt  atgaacttgt  aaatcgttat  taaatcgctg  aattttgata
481  gccattctga  agagtattct  tcaactacta  cactaacttc  cattaatttc  tcattaattt
541  ccctttcgtt  caaaaaatat  ggttggcttt  acaagcattc  tatcatatga  tagaattaag
601  ataacattca  aaaggagata  ataaaaatgc  ggcaaggtat  tcttaaataa  actgtcaatt
661  agatagcggg  aataattaat  taagcatcct  tatttaaaat  aaggactggt  tttttgtgcc
721  cagtttaaga  atacttttat  cattttatca  taaagcatct  gtggctatca  gtgtgtagta
781  tacagctatt  tttgtattaa  aatcccagtg  ataagagtat  ttatcactgg  gattttttct
841  gctttttgag  cttttgaatg  gaggaataa  aattgaaaat  tattaatatc  ggtatcctag
901  cacatgttga  tgcaggaaaa  actactttga  cagaaaagctt  actatacagt  agcggagcaa
961  ttaaagagtt  aggaagtgtg  gatagcggta  caacgaaaac  ggatactatg  tttttggaac
1021  gccagagagg  tattactatt  cagaccgcaa  taacatcttt  tcaacgggaa  aatgttaaag
1081  taaatattgt  agatactcct  ggacacatgg  attttttggc  agatgtatac  cgttcattat
1141  ctgttttgga  tggagctatt  ttgctaactc  ctgcaaaaga  tggagtacag  tcacaaactc
1201  gtatactatt  ccatgcactt  agaaagatga  acatacctat  aatatTTTTT  attaacaaaa
1261  ttgatcaaaa  tggataaaat  ttgccagatg  tttatcaaga  tattaaggac  aaactttctg
1321  acgacatcat  aattaagcag  actgtgaatc  taaatttgaa  accttatgta  atagattata
1381  ctgaaccaga  acaatgggag  acagtaattg  tgggaaatga  ttatttatta  gaaaaatata
1441  ccattgggaa  aacattgaat  attgcagaac  ttgaaaagga  ggaaaacgaa  agaattcaaa
1501  gttgctcctt  atatcctgtt  tatcacggaa  gtgcaaagaa  taatattgga  attaaacaac
1561  ttatagaggt  aattactagc  aaattatTTT  caccacaca  actcaattca  gataaacTTT
1621  gtggaaatgt  ttttaaagta  gaatatTCAG  atgatggTCA  acggcttgTC  tatgta

```

//

Nucleotide

Display Settings: ☒ GenBank**Enterococcus faecalis strain SN369 plasmid insertion sequence IS1216 transposase gene, partial cds; cadE and tetS genes, complete sequence; and TetS gene, partial cds**

GenBank: JN980094.1

[FASTA](#) [Graphics](#)[Go to:](#) ☒

LOCUS JN980094 1671 bp DNA linear BCT 19-MAR-2012

DEFINITION Enterococcus faecalis strain SN369 plasmid insertion sequence
IS1216 transposase gene, partial cds; cadE and tetS genes, complete
sequence; and TetS gene, partial cds.

ACCESSION JN980094

VERSION JN980094.1 GI:380040889

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM [Enterococcus faecalis](#)
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;
Enterococcus.

REFERENCE 1 (bases 1 to 1671)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L.,
Roberts,A.P. and Coque,T.M.

TITLE Spread of tet(S) among enterococci is mediated by CTn6000 and
plasmids harbouring different composite sequence

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1671)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L.,
Roberts,A.P. and Coque,T.M.

TITLE Direct Submission

JOURNAL Submitted (01-NOV-2011) Microbiology, REQUIMTE, Pharmacy Faculty,
Rua Jorge Viterbo Ferreira #228, Porto 4050-313, Portugal

FEATURES Location/Qualifiers

source 1..1671
/organism="Enterococcus faecalis"
/mol_type="genomic DNA"
/strain="SN369"
/isolation_source="piggery manure"
/db_xref="taxon:1351"
/plasmid="unnamed"
/country="Portugal"
/collection_date="2006"

[mobile element](#) complement(<1..129)
/mobile_element_type="insertion sequence:IS1216"

[CDS](#) complement(<1..61)
/codon_start=1
/transl_table=11
/product="transposase"
/protein_id="AFD32979.1"
/db_xref="GI:380040890"
/translation="MTMNHFKGKQFQQDVIIIVAV"

[repeat region](#) complement(107..129)
/note="IR left"
/rpt_type=inverted

[gene](#) 182..256
/gene="cadE"

[misc feature](#) 182..256
/gene="cadE"
/note="nonfunctional cadmium efflux system accessory
protein due to mutation"

[gene](#) 868..>1671
/gene="tetS"

[CDS](#) 868..>1671
/gene="tetS"
/note="tetracycline resistance protein"
/codon_start=1
/transl_table=11
/product="TetS"
/protein_id="AFD32980.1"
/db_xref="GI:380040891"
/translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTTKTD
TMFLERQRGITITAITSFQRENVKVNIVDTPGHMDFLADVYRSLSLVDGAILLISAK
DGVQSQTRILFHALKMNIPIIFFINKIDQNGINLPDQYQDIKDKLSDDIIKQTVNL

NLKPYVIDYTEPEQWETVIVGNDYLLEKYTIGKTLNIAELEKEENERIQSCSLYPVYH
GSAKNNIGIKQLIEVITSKLFSPQTQLNSDKLCGNVFKVEYSDDGQRLVYV"

ORIGIN

```

1  ccacggctac  aataatcaca  tcctgctgaa  attgctttcc  tttaaaatga  ttcacgtcga
61  ttctctctgc  tatctttttc  tattattcta  ctttatttga  tagtagatgt  aaaactttgc
121 aacagaaacca  tcaaatagat  tacttcatt  caaacaatcg  tttgaatgtg  aggtgagatg
181 aatgaaaaaa  gtttgccaag  tgactgtcgt  acataaagaa  aaagtaaag  gaataaaaaa
241 taaattggat  caaaaagata  ttttccaagt  caaacttaat  attaacaatt  caaaactatt
301 ggaaccactg  caaaaatatt  tgtaaagcag  tatatatatt  aaaaattata  cgtaatatat
361 ttgtagaaaag  gttgtggaca  gttgtcctat  tgacctgaaa  agtaataata  tagctggatg
421 aataaagaat  cccttatgaa  cttgtaaatc  gttattaaat  cgctgaattt  tgatagccat
481 tctgaagagt  tatcttcaac  tactacacta  acttccatta  atttctcatt  aatttccctt
541 tcgttcaaaa  aatatgggtg  gctttacaag  cattctatca  tatgatagaa  ttaagataac
601 attcaaaaag  agataataaa  aatgcggcaa  ggtattctta  aataaactgt  caattagata
661 gcgggaataa  ttaattaagc  atccttattt  aaaataagga  ctggtttttt  gtgcccagtt
721 taagaatact  tttatcattt  tatcataaag  catctgtggc  tatcagtgtg  tagtatacag
781 ctatttttgt  attaaaatcc  cagtataag  agtatttatc  actgggattt  tttctgcttt
841 ttgagctttt  gaatggagga  aataaaattg  aaaattatta  atatcggtat  cttagcacat
901 gttgatgcag  gaaaaactac  ttgacagaa  agcttactat  acagtagcgg  agcaattaa
961 gagttaggaa  gtgtagatag  cggtacaacg  aaaacggata  ctatgttttt  ggaacgccag
1021 agaggatta  ctattcagac  cgcaataaca  tcttttcaac  gggaaaatgt  taaagtaaat
1081 attgtagata  ctctgggaca  catggatttt  ttggcagatg  tataccgttc  attatctgtt
1141 ttggatggag  ctatttttgt  aatctctgca  aaagatggag  tacagtcaca  aactcgtata
1201 ctattccatg  cacttagaaa  gatgaacata  cctataatat  tttttattaa  caaaattgat
1261 caaaatggaa  taaatttgcc  agatgtttat  caagatatta  aggacaaact  ttctgacgac
1321 atcataatta  agcagactgt  gaatctaaat  ttgaaacott  atgtaataga  ttatactgaa
1381 ccagaacaat  gggagacagt  aattgtggga  aatgattatt  tattagaaaa  atataccatt
1441 gggaaaacat  tgaatattgc  agaacttgaa  aaggaggaaa  acgaaagaat  tcaaagttgc
1501 tccttatatc  ctgtttatca  cggaagtgca  aagaataata  ttggaattaa  acaacttata
1561 gaggtaatta  ctagcaaat  attttcaccc  acacaactca  attcagataa  actttgtgga
1621 aatgttttta  aagtagaata  ttcagatgat  ggtcaacggc  tggctctatgt  a

```

//

Nucleotide

Display Settings: ☒ GenBank

Enterococcus sp. SN151 insertion sequence IS1216 transposase gene, partial cds; tetS gene, complete sequence; TetS and hypothetical protein genes, complete cds; and insertion sequence IS1216 transposase gene, partial cds

GenBank: JN980093.1

[FASTA](#) [Graphics](#)[Go to:](#)

LOCUS JN980093 3722 bp DNA linear BCT 19-MAR-2012

DEFINITION Enterococcus sp. SN151 insertion sequence IS1216 transposase gene, partial cds; tetS gene, complete sequence; TetS and hypothetical protein genes, complete cds; and insertion sequence IS1216 transposase gene, partial cds.

ACCESSION JN980093

VERSION JN980093.1 GI:380040884

KEYWORDS .

SOURCE Enterococcus sp. SN151

ORGANISM [Enterococcus sp. SN151](#)
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 3722)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.

TITLE Spread of tet(S) among enterococci is mediated by CTn6000 and plasmids harbouring different composite sequence

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 3722)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.

TITLE Direct Submission

JOURNAL Submitted (01-NOV-2011) Microbiology, REQUIMTE, Pharmacy Faculty, Rua Jorge Viterbo Ferreira #228, Porto 4050-313, Portugal

FEATURES

source Location/Qualifiers

1..3722

/organism="Enterococcus sp. SN151"

/mol_type="genomic DNA"

/strain="SN151"

/isolation_source="piggery waste lagoon"

/db_xref="taxon:[1156715](#)"

/plasmid="unnamed"

/country="Portugal"

/collection_date="2006"

[mobile element](#) complement(1..121)

/mobile_element_type="insertion sequence:IS1216"

[CDS](#) complement(<1..53)

/codon_start=1

/transl_table=[11](#)

/product="transposase"

/protein_id="[AFD32975.1](#)"

/db_xref="GI:380040885"

/translation="MTMNHFKGKQFQQDVIIIV"

[repeat region](#) complement(99..121)

/note="IR left"

/rpt_type=inverted

[misc feature](#) 122..334

/note="nonfunctional conjugation related protein due to insertion sequence; similar to CTn6000 orf13"

[gene](#) 1178..3103

/gene="tetS"

[CDS](#) 1178..3103

/gene="tetS"

/note="tetracycline resistance protein; similar to CTn6000 sequence"

/codon_start=1

/transl_table=[11](#)

/product="TetS"

/protein_id="[AFD32976.1](#)"

/db_xref="GI:380040886"

/translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTKTD
TMFLERQRGITITAITSFQRENVKVNIVDTPGHMDFLADVRSLSVLDGAILLISAK
DGVQSQTILFHALRKMNIPIIFFINKIDQNGINLPDVYQDIKDKLSDDIIIKQTVNL

NLKPYVIDYTEPEQWETVIVGNDYLLEKYTIGKTLNIAELEKEENERIQSCSLYPVYH
 GSAKNNIGIKQLIEVITSKLFSPQTQLNSDKLCGNVFKVEYSDDGQRLVYVRLYSGTLH
 LRDSVNISEKEKIKVTEMYTSINGELRQIDKAEPGEIIILKNELKLNVLGDKKRLP
 HREILENPLPMLQTTIEPCKSVQREKLLDALFEISDSDPLQYYVDVTVEIVLSFLG
 EVQMEVTCCTLIQEKYHIEITRKPTVIYMERPLKKSEFTIDIEVPPNPFWASIGLSVT
 PLPLGSGIQYESLVSLGYLNQSFQNAVMEGIRYGEQGLYGWKLTDCKAICFKYGLYYS
 PVSTPADFRMLAPIVLEQAFKSGTELEPYLSFEIYVPQEYLSRAYNDASKYCANIL
 NTKLKGNVEVILIGEIPARCIQEYRNSLTFFTTNGRSVCLTELKGYQVTNIKSAFQPRRP
 NNRIDKVRHMFNKLNLH"
 3223..3354
 /note="conjugative transposon protein; similar to CTn6000
 orf6"
 /codon_start=1
 /transl_table=11
 /product="hypothetical protein"
 /protein_id="AFD32977.1"
 /db_xref="GI:380040887"
 /translation="MREDTELKNFPLFCPKCRQEILIEITKFRITVITEPDAKTQSR"
 complement(3625..>3722)
 /mobile_element_type="insertion sequence:IS1216"
 complement(3625..3646)
 /note="IR right"
 /rpt_type=inverted
 complement(3679..>3722)
 /codon_start=3
 /transl_table=11
 /product="transposase"
 /protein_id="AFD32978.1"
 /db_xref="GI:380040888"
 /translation="VCTEIKVLLGIPA"

ORIGIN

```

1 acaataatca catctgctg aaattgctt cctttaaaat gattcatcgt cattcctcct
61 gctatctttt tctattattc taccttattt gatagtagat ttaaaacttt gcaacagaac
121 cgattattat atgatggaag atgttttacc aataatcagt caaaacttta ttactctga
181 gttggtgaat ccaattattc agaagaaaga gaaacagtat ctagtataaa tagccggtcg
241 atatttagac aatgaacaa agattgaaa tatttcccaa taccaattaa ctttagagaa
301 gaaggataac tggaaaataa taatgaatga ataaagaata ttaagatgaa aacaatactt
361 gaatatttag tataagcaaa aaaaattgta tgtcactata gcaaacttat aatgatttag
421 cataaataat ccaaaataat gcgtgaaatc aaccttctat gagaagtata agcaaattca
481 cctttattct aacttataac tattttcatt acattactta aaaagggtctc tccaattttt
541 tccaaataaa cagtataact ttaaatgata ttttccaagt caaaacttaat attaacaatt
601 caaaactatt ggaaccactg caaaatattt tgtaagcag tatatatattc aaaaattata
661 cgaatataat ttgtagaag gttgtggaca gttgtcctat tgacctgaaa agtaataata
721 tagctggatg aataaagaat cccttatgaa cttgtaaatc gttattaaat cgctgaattt
781 tgatagccat tctgaagagt tatcttcaac tactacacta acttccatta atttctcatt
841 aatttcctt tcgttcaaaa aatatggttg gctttacaag cattctatca tatgatagaa
901 ttaagataac attcaaaag agataataaa aatgcggcaa ggtattctta aataaactgt
961 caattagata gcgggaataa ttaattaagc atccttattt aaaataagga ctggtttttt
1021 gtgcccagtt taagaatact tttatcattt tatcataaag catctgtggc tatcagtggtg
1081 tagtatacag ctattttttt attaaaatcc cagtgataag agtatttatc actgggattt
1141 tttctgctt ttgagatttt gaatggagga aataaaattg aaaattatta atatcggtat
1201 cttagcacat gttgatgcag gaaaaactac tttgacagaa agcttactat acagtagcgg
1261 agcaattaaa gagttaggaa gtgtagatag cgttacaacg aaaacggata ctatgttttt
1321 ggaacgccag agaggatta ctattcagac cgcaataaca tcttttcaac gggaaaatgt
1381 taaagtaaat attgtagata ctctgggaca catggatttt ttggcagatg tataccgttc
1441 attatctgtt ttggatggag ctatttttgc aatctctgca aaagatggag tacagtcaca
1501 aactcgtata ctattccatg cacttagaaa gatgaacata cctataatat tttttattaa
1561 caaaattgat caaaatggaa taaatttgcc agatgtttat caagatatta aggacaaact
1621 ttctgacgac atcataatta agcagactgt gaatctaaat ttgaaacctt atgtaataga
1681 ttatactgaa ccagaacaat gggagacagt aattgtggga aatgattatt tattagaaaa
1741 atataccatt gggaaaacat tgaatattgc agaacttgaa aaggaggaaa acgaaagaat
1801 tcaaaagtgc tccttataat ctgtttatca cggaaagtga aagaataata ttggaattaa
1861 acaacttata gaggaatta ctagcaaat attttcacc acacaactca attcagataa
1921 actttgtgga aatgttttta aagtagaata ttcagatgat ggtcaacggc ttgtctatgt
1981 acgtctttat agtggacgc tacatttgcg agactcagtc aatatatcag aaaaggaaaa
2041 aataaaagt acagaaatgt atacttcaat aaatggagaa ttacgccaga tagataaggc
2101 agagcctggg gagattatta ttttaaaaaa tgagctttta aaactaaata acgtacttgg
2161 agataaaaaa agattaccac atagagaaat tcttgagaat cctcttccta tgttacaac
2221 aacaattgaa ccatgtaaat cagtacaaag agaaaagtta ctatagtcac tttttgaaat
2281 atccgatagt gatcccttc tacaatatta ttagatata gtaactcacg aaattgtgct
2341 atctttttta ggtgaggtcc aaatggaggt aacttgtact ctgattcaag aaaaatatca
2401 tattgagata gaaacaagaa aaccaactgt catttatatg gaaagaccat aaaaaaatc
2461 tgaatttacc attgatatcg aagtacctcc aaatcctttc tgggcttcta ttggtttatc
2521 tgtaacacca ctctctttg gtatggcat tcagtatgag agcctggttt ctctaggtta
2581 tttaaatcaa tcatttcaaa atgcagttat ggaaggtata cgctatgggt gtgaacaagg
2641 attgtacggg tggaaattaa cagactgtaa gatctgtttt aagtatggtc tatattacag
2701 ccctgtcagt acgccagcag atttccgaat gcttgcgcct attgtactag agcaggcttt
2761 tagaaagagt ggtacagagt tattagagcc atatcttagc ttcgaaattt atgtaccaca
2821 agaatatctt tcgagagcat ataatgatgc ttccaaatat tgtgcataa ttttaaatca
2881 taagttaaaa ggtaacgagg tcattctcat tggtgaaatt ccagccggtt gtattcaaga

```

```

2941 gtatcgaaac agtttaactt tctttacaaa tggacgcagt gtctgtttta cagagttaaa
3001 aggttatcag gttactaaca ttaagtctgc tttccaacca cgtcgtccaa ataatagaat
3061 agacaaagta aggcataatgt ttaataaaat caacttacat tgatagttaa ctaaagagaa
3121 atttttaaaa tgaatatgag ttgttaatta gaacaggagg atatagatat tgtaaatact
3181 agtgatactg tgcctatat gtggcaataa gaccggttta aaatgaggga agatactgaa
3241 ttaaaaaaatt tccctctatt ttgtcctaaa tgcagacaag aaattttaat tgaataacg
3301 aagtttagaa taactgtaat tacagagcca gacgcaaaga cgcagagccg ataattcgag
3361 atatcaaaat ctcattttat cggctttttc ttttctatgc ataccattga gcttagttct
3421 tgatatacctt tataagaaaa taagtgtact gatTTTTTTT gcagtagctg tattacagta
3481 gacatccctc aagcataaaa agtatacaat taaaattcaa tacttgatta ttgatgaagt
3541 acagagttac tcgtcatttc aaaactagta tatcagtcaa gtttttccta aggaacaaaa
3601 acgaacttcg ctatgggaat caaaaaattt tgaacggtt tcttggtaaa gaataaaatc
3661 ccttacggta tctatgattt aagctgggat tcccaataat accttgattt cagtacagac
3721 cg

```

//

Nucleotide

Display Settings: ☒ GenBank**Enterococcus gallinarum strain F329 plasmid insertion sequence IS1216 transposase gene, partial cds; tetS gene, partial sequence; and TetS gene, partial cds**

GenBank: JN980092.1

[FASTA](#) [Graphics](#)[Go to:](#) ☒

LOCUS JN980092 1991 bp DNA linear BCT 19-MAR-2012

DEFINITION Enterococcus gallinarum strain F329 plasmid insertion sequence
IS1216 transposase gene, partial cds; tetS gene, partial sequence;
and TetS gene, partial cds.

ACCESSION JN980092

VERSION JN980092.1 GI:380040881

KEYWORDS .

SOURCE Enterococcus gallinarum

ORGANISM [Enterococcus gallinarum](#)
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;
Enterococcus.

REFERENCE 1 (bases 1 to 1991)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L.,
Roberts,A.P. and Coque,T.M.

TITLE Spread of tet(S) among enterococci is mediated by CTn6000 and
plasmids harbouring different composite sequence

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1991)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L.,
Roberts,A.P. and Coque,T.M.

TITLE Direct Submission

JOURNAL Submitted (01-NOV-2011) Microbiology, REQUIMTE, Pharmacy Faculty,
Rua Jorge Viterbo Ferreira #228, Porto 4050-313, Portugal

FEATURES

source Location/Qualifiers

1..1991

/organism="Enterococcus gallinarum"

/mol_type="genomic DNA"

/strain="F329"

/isolation_source="poultry carcasse"

/db_xref="taxon:1353"

/plasmid="unnamed"

/country="Portugal"

/collection_date="1999"

[mobile element](#) complement(<1..131)

[CDS](#) /mobile_element_type="insertion sequence:IS1216"

complement(<1..63)

/codon_start=1

/transl_table=11

/product="transposase"

/protein_id="AFD32973.1"

/db_xref="GI:380040882"

/translation="MKMNHFKGKQFQQDVIIIVAVG"

[repeat region](#) 110..131

/note="IR left"

/rpt_type=inverted

[misc feature](#) 132..344

/note="nonfunctional conjugation related protein due to
insertion sequence; similar to CTn6000 orf13"

[gene](#) 1188..>1991

/gene="tetS"

[CDS](#) 1188..>1991

/gene="tetS"

/note="tetracycline resistance protein; similar to CTn6000
sequence"

/codon_start=1

/transl_table=11

/product="TetS"

/protein_id="AFD32974.1"

/db_xref="GI:380040883"

/translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTKTD
TMFLERQRGITITAITSFQRENVKVNIVDTPGHMDFLADVRSLSVLDGAILLISAK
DGVQSQTRILFHALRKMNIPIIFFINKIDQNGINLPDVYQDIKDKLSDDIIKQTVNL
NLKPYVIDYTEPEQWETVIVGNDYLLKTYTIGKTLNIAELEKEENERIQSCSLYPVYH
GSAKNNIGIKQLIEVITSKLFSPSTQLNSDKLGNVFKVEYSDDGQRLVYV"

Nucleotide

Display Settings: ☒ GenBank

Enterococcus gallinarum strain F38 plasmid insertion sequence IS1216 transposase gene, partial cds; tetS gene, partial sequence; and TetS gene, partial cds

GenBank: JN980091.1

[FASTA](#) [Graphics](#)[Go to:](#) ☒

LOCUS JN980091 1993 bp DNA linear BCT 19-MAR-2012

DEFINITION Enterococcus gallinarum strain F38 plasmid insertion sequence
IS1216 transposase gene, partial cds; tetS gene, partial sequence;
and TetS gene, partial cds.

ACCESSION JN980091

VERSION JN980091.1 GI:380040878

KEYWORDS .

SOURCE Enterococcus gallinarum

ORGANISM [Enterococcus gallinarum](#)
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;
Enterococcus.

REFERENCE 1 (bases 1 to 1993)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L.,
Roberts,A.P. and Coque,T.M.

TITLE Spread of tet(S) among enterococci is mediated by CTn6000 and
plasmids harbouring different composite sequence

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1993)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L. and
Roberts,A.P.

TITLE Direct Submission

JOURNAL Submitted (01-NOV-2011) Microbiology, REQUIMTE, Pharmacy Faculty,
Rua Jorge Viterbo Ferreira #228, Porto 4050-313, Portugal

FEATURES

source Location/Qualifiers

1..1993

/organism="Enterococcus gallinarum"

/mol_type="genomic DNA"

/strain="F38"

/isolation_source="poultry carcassee"

/db_xref="taxon:1353"

/plasmid="unnamed"

/country="Portugal"

/collection_date="1999"

[mobile element](#) complement(<1..133)

[CDS](#) /mobile_element_type="insertion sequence:IS1216"

complement(<1..66)

/codon_start=1

/transl_table=11

/product="transposase"

/protein_id="AFD32971.1"

/db_xref="GI:380040879"

/translation="MKMNHFKGKQFQQDVIIIVAVGY"

[repeat region](#) complement(113..133)

/note="IR left"

/rpt_type=inverted

[misc feature](#) 134..347

/note="nonfunctional conjugation related protein due to
insertion sequence; similar to CTn6000 orf13"

[gene](#) 1191..>1993

/gene="tetS"

[CDS](#) 1191..>1993

/gene="tetS"

/note="tetracycline resistance protein; similar to CTn6000
sequence"

/codon_start=1

/transl_table=11

/product="TetS"

/protein_id="AFD32972.1"

/db_xref="GI:380040880"

/translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTKTD
TMFLERQRGITITAITSFQRENVKVNIVDTPGHMDFLADVRSLSVLDGAILLISAK
DGVQSQTTRILFHARLKMNIPIIFFINKIDQNGINLPDVYQDIKDKLSDDIIKQTVNL
NLKPVIDYTEPEQWETVIVGNDYLLKYTIGKTLNIAELEKEENERIQSCSLYPVYH
GSAKNNIGIKQLIEVITSKLFSPQTQLNSDKLGNVFKVEYSDDGQRLVYV"

ORIGIN

```

1  gtagccacag gctacaataa tcacatcctg ctgaaattgc tttcctttaa aatgattcat
61  cttcattcct cctgtatctt ttttctatta ttctacctta ttgatagta gatttaaaac
121 tttgcaacag aaccgattat tatatgatgg aagatgtttt accaataatc agtcaaaact
181 ttattttact tgagttgggtg aatccaatta ttcagaagaa agagaaacag tatctagtaa
241 aaatagccgt tcgatattta gacaatgaaa caaagattga aaatatttcc caataccaat
301 taacttttaga gaagaaggat aactggaaaa taataatgaa tgaataaaga atattaagat
361 gaaaacaata cttgaatat ttagtataagc aaaaaaaatt gtatgtcact atagcaaaact
421 tataatgatt tagcataaat aatccaaaat atagcgtgaa atcaaccttc tatgagaagt
481 ataagcaaat tcacotttat tctaacttat aactattttc attacattac ttaaaaagggt
541 ctctccaatt ttttccaat aaacagtata acttttaatg atattttcca agtcaaactt
601 aatattaaca attcaaaact attggaacca ctgcaaaata ttttgtaaag cagtatatat
661 ttcaaaaatt atacgtaata tatttgtaga aaggttggtg acagttgtcc tattgacctg
721 aaaagtaata taatagctgg atgaataaag aatcccttat gaacttgtaa atcgttatta
781 aatcgctgaa ttttgatagc cattctgaag agttatcttc aactactaca ctaacttcca
841 ttaatttctc attaatttcc ctttcgttca aaaaatatgg ttggctttac aagcattcta
901 tcatatgata gaattaagat aacattcaaa aggagataat aaaaatgcgg caaggatttc
961 ttaataaacc tgtcaattag atagcgggaa taattaatta agcatcctta tttaaaataa
1021 ggactgggtt tttgtgcccc gtttaagaat acttttatca ttttatcata aagcatctgt
1081 ggctatcagt gtgtagtata cagctatttt tgtattaaaa tcccagtgat aagagtattt
1141 atcactggga ttttttctgc tttttgagct tttgaatgga ggaaataaaa ttgaaaatta
1201 ttaatatcgg tatcttagca catggtgatg caggaaaaac tactttgaca gaaagcttac
1261 tatacagtag cggagcaatt aaagagttag gaagtgtaga tagcggtaga acgaaaacgg
1321 atactatggt tttggaacgc cagagaggta ttactattca gaccgcaata acatcttttc
1381 aacgggaaaa tgttaaagta aatattgtag atactcctgg acacatggat tttttggcag
1441 atgtataccg ttcattatct gttttggatg gagctatttt gctaactctc gcaaaagatg
1501 gagtacagtc acaaaactcg atactattcc atgcacttag aaagatgaac atacctataa
1561 tattttttat taacaaaatt gatcaaaatg gaataaattt gccagatggt tatcaagata
1621 ttaaggacaa actttctgac gacatcataa ttaagcgagc tgtgaatcta aatttgaaac
1681 cttatgtaat agattatact gaaccagaac aatgggagac agtaattgtg ggaaatgatt
1741 atttattaga aaaatatacc attgggaaaa cattgaatat tgcagaactt gaaaaggagg
1801 aaaacgaaag aattcaaagt tgctccttat atcctgttta tcacggaagt gcaaagaata
1861 atattggaat taaacaactt atagaggtaa ttactagcaa attattttca cccacacaac
1921 tcaattcaga taaactttgt ggaaatgttt ttaaagtaga atattcagat gatggtcaac
1981 ggcttgtcta tgt

```

//

ORIGIN

```

1  gccacggct  acaataatca  catcctgctg  aaattgcttt  cctttaaaat  gattcatctt
61  cattcctcct  gctatctttt  tctattattc  taccttattt  gatagtagat  ttaaaacttt
121  gcaacagAAC  cgattattat  atgatggaag  atgttttacc  aataatcagt  caaaacttta
181  tttactctga  gttggtgaat  ccaattattc  agaagaaaga  gaaacagtat  ctagtaaaaa
241  tagccgttcg  atatttagac  aatgaaacaa  agattgaaaa  tatttcccaa  taccaattaa
301  ctttagagaa  gaaggataac  tggaaaaata  taatgaatga  ataaagaata  ttaagatgaa
361  aacaataact  gaatatttag  tataagcaaa  aaaaattgta  tgtcactata  gcaaacttat
421  aatgatttag  cataaataat  ccaaaatata  gcgtgaaatc  aaccttctat  gagaagtata
481  agcaaattca  cttttattct  aacttataac  tattttcatt  acattactta  aaaaggctct
541  tccaattttt  tccaaataaa  cagtataact  tttaatgata  ttttccaagt  caaacttaat
601  attaacaatt  caaaactatt  ggaaccactg  caaaatattt  tgtaagcag  tataattttc
661  aaaaattata  cgtaatatat  ttgtagaaag  gttgtggaca  gttgtcctat  tgacctgaaa
721  agtaatataa  tagtgggatg  aataaagaat  cccttatgaa  cttgtaaatc  gttattaaat
781  cgctgaattt  tgatagccat  tctgaagagt  tatcttcaac  tactacacta  acttccatta
841  atttctcatt  aatttccctt  tcgttcaaaa  aatatggttg  gctttacaag  cattctatca
901  tatgatagaa  ttaagataac  attcaaaagg  agataataaa  aatgcggcaa  ggtattctta
961  aataaactgt  caattagata  gcgggaataa  ttaattaagc  atccttattt  aaaataagga
1021  ctgggttttt  gtgccaggtt  taagaatact  tttatcattt  tatcataaag  catctgtggc
1081  tatcagtggt  tagtatacag  ctatttttgt  attaaaaatc  cagtataaag  agtatttato
1141  actgggattt  tttctgcttt  ttgagctttt  gaatggagga  aataaaattg  aaaattatta
1201  atatcggtat  cttagcacat  gttgatgcag  gaaaaactac  tttgacagaa  agcttactat
1261  acagtagcgg  agcaattaaa  gagttaggaa  gtgtagatag  cggtaacaag  aaaacggata
1321  ctatgttttt  ggaacgccag  agaggtatta  ctattcagac  cgcaataaca  tcttttcaac
1381  gggaaaatgt  taaagtaaAT  attgtagata  ctctgggaca  catggatttt  ttggcagatg
1441  tataccgttc  attatctggt  ttggatggag  ctattttgct  aatctctgca  aaagatggag
1501  tacagtcaca  aactcgtata  ctattccatg  cacttagaaa  gatgaacata  cctataatat
1561  tttttattaa  caaaattgat  caaaatggaa  taaatttgcc  agatgtttat  caagatatta
1621  aggacaaact  ttctgacgac  atcataatta  agcagactgt  gaatctaaat  ttgaaacctt
1681  atgtaataga  ttatactgaa  ccagaacaat  gggagacagt  aattgtggga  aatgattatt
1741  tattagaaaa  atataccatt  gggaaaacat  tgaatattgc  agaacttgaa  aaggaggaaa
1801  acgaaagaat  tcaaagttgc  tccttatatc  ctgtttatca  cggaagtgca  aagaataata
1861  ttggaattaa  acaacttata  gaggtaatta  ctagcaaatt  attttcccc  acacaactca
1921  attcagataa  actttgtgga  aatgttttta  aagtagaata  ttcagatgat  ggtcaacggc
1981  ttgtctatgt  a

```

//

Nucleotide

Display Settings: ☒ GenBank

Enterococcus faecalis plasmid insertion sequence ISEnta1 transposase gene, partial cds; and TetS (tetS) gene, partial cds

GenBank: JN998895.1

[FASTA](#) [Graphics](#)[Go to:](#) ☒

LOCUS JN998895 1577 bp DNA linear BCT 19-MAR-2012

DEFINITION Enterococcus faecalis plasmid insertion sequence ISEnta1 transposase gene, partial cds; and TetS (tetS) gene, partial cds.

ACCESSION JN998895

VERSION JN998895.1 GI:380005295

KEYWORDS .

SOURCE Enterococcus faecalis

ORGANISM [Enterococcus faecalis](#)
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus.

REFERENCE 1 (bases 1 to 1577)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.

TITLE Spread of tet(S) among enterococci is mediated by CTn6000 and plasmids harbouring different composite sequences

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1577)

AUTHORS Novais,C., Freitas,A.R., Silveira,E., Baquero,F., Peixe,L., Roberts,A.P. and Coque,T.M.

TITLE Direct Submission

JOURNAL Submitted (07-NOV-2011) Microbiology, REQUIMTE. Pharmacy Faculty, Rua Jorge Viterbo Ferreira 228, Porto 050-313, Portugal

FEATURES

source Location/Qualifiers

1..1577

/organism="Enterococcus faecalis"

/mol_type="genomic DNA"

/strain="SN245"

/isolation_source="pig manure"

/db_xref="taxon:[1351](#)"

/plasmid="unnamed"

/country="Portugal"

/collection_date="2006"

[mobile element](#) complement(<1..134)

[CDS](#) /mobile_element_type="insertion sequence:ISEnta1"

complement(<1..65)

/codon_start=1

/transl_table=[11](#)

/product="transposase"

/protein_id="[AFD29104.1](#)"

/db_xref="GI:380005296"

/translation="MKMTQFKGKQFQKDVIIIVAVG"

[repeat region](#) complement(118..134)

/note="IR left"

/rpt_type=inverted

[misc feature](#) 135..173

/note="similar to cadE"

[misc feature](#) 174..>1577

/note="similar to Tn6000"

[gene](#) 784..>1577

/gene="tetS"

[CDS](#) 784..>1577

/gene="tetS"

/note="tetracycline resistance protein"

/codon_start=1

/transl_table=[11](#)

/product="TetS"

/protein_id="[AFD29105.1](#)"

/db_xref="GI:380005297"

/translation="MKIINIGILAHVDAGKTTLTESLLYSSGAIKELGSVDSGTTTKTD
TMFLERQRGITITAITSFQRENVKVNIVDTPGHMDFLADVRSLSVLDGAILLISAK
DGVQSQTRILFHALRKMNIPIIFFINKIDQNGINLPDVYQDIKDKLSDDIIIKQTVNL
NLKPYVIDYTEPEQWETVIVGNDYLLEKYTIGKTLNIAELEKEENERIQSCSLYPVYH
GSAKNNIGIKQLIEVITSKLFSPQTQLNSDKLCGNVFKVEYSDDGQRL"

ORIGIN

```
1 tagcgcgacgg ctacgataat cacgtcctttt tgaaattggt ttcctttgaa ctgagtcato
61 ttcatctctc caagctatct ttttcaatta ttttacctca aaatgagctt atcggaaact
121 ttgcaacaga acccaaaaag taaatggaat aaaaaataaa ttggatcaaa aagatatttt
181 ccaagtcaaa cttaatatat acaattcaaa actattggaa ccaactgcaa atattttgtt
241 aagcagtata ttttcaaaa attatacgta atatatttgt agaaagggtg tggacagttg
301 tcctattgac ctgaaaagta atataatagc tggatgaata aagaatccct tatgaacttg
361 taaatcgta ttaaatcgct gaattttgat agccattctg aagagttatc ttcaactact
421 acactaactt ccattaattt ctcattaatt tccctttcgt tcaaaaaata tggttggctt
481 tacaagcatt ctatcatatg atagaattaa gataacattc aaaaggagat aataaaaaatg
541 cggcaaggta ttcttaataa aactgtcaat tagatagcgg gaataattaa ttaagcatcc
601 ttatttaaaa taaggactgg ttttttgtgc ccagtttaag aatactttta tcattttatc
661 ataaagcatc tgtggctatc agtgtgtagt atacagctat ttttgatta aaatcccagt
721 gataagagta tttatcactg ggattttttc tgcttttga gcttttgaat ggaggaaata
781 aaattgaaaa ttattaatat cggatatcta gcacatgttg atgcaggaaa aactactttg
841 acagaaagct tactatacag tagcggagca attaaagagt taggaagtgat agatagcggg
901 acaacgaaaa cggatactat gtttttggaa cgcagagag gtattactat tcagaccgca
961 ataacatctt ttcaacggga aaatgttaaa gtaaatattg tagatactcc tggacacatg
1021 gattttttgg cagatgtata ccgttcatta tctgttttgg atggagctat tttgctaato
1081 tctgcaaaag atggagtaca gtcacaaact cgtatactat tccatgcact tagaaagatg
1141 aacataccta taatattttt tattaacaaa attgatcaaa atggaataaa tttgccagat
1201 gtttatcaag atattaagga caaactttct gacgacatca taattaagca gactgtgaat
1261 ctaaatttga aaccttatgt aatagattat actgaaccag aacaatggga gacagtaatt
1321 gtgggaaatg attattttat agaaaaatat accattggga aaacattgaa tattgcagaa
1381 cttgaaaagg aggaaaacga aagaattcaa agttgctcct tatatcctgt ttatcacgga
1441 agtgcaaaaga ataattattg aattaacaa cttatagagg taattactag caaattattt
1501 tcacccacac aactcaattc agataaactt tgtggaatg tttttaagt agaattattca
1561 gatgatggtc aacggct
```

//

