Pedro Miguel Santos

DEGRADATION OF AROMATIC COMPOUNDS IN *PSEUDOMONAS*

PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION

{DOCTORAL THESIS}

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DEGRADATION OF AROMATIC COMPOUNDS IN *PSEUDOMONAS*

Physiological And Molecular Characterization

by Pedro Miguel Santos

SUPERVISOR *Prof.* Elisabetta Zennaro,
Università di Roma Tre

CO-SUPERVISOR *Prof.* P. Moradas Ferreira,
Universidade do Porto

JURY Director of Instituto de Ciências Biomédicas de Abel Salazar (ICBAS)
Doctor Elisabetta Zennaro
Doctor Isabel Maria Sá Correia Leite de Almeida
Doctor Manuel José Magalhães Gomes Mota
Doctor Paolo De Marco
Doctor Pedro Gaspar Moradas Ferreira
Doctor Claudina Amélia Marques Rodrigues Pousada

DEGREE Doctor in Biomedical Sciences, Biochemestry
University of Porto, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS)

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Santos, P. M., J. M. Blatny, I. Di Bartolo, S. Valla, and E. Zennaro. 2000. Physiological analysis of the expression of the styrene degradation gene cluster in *Pseudomonas fluorescens* ST. Appl. Environ. Microbiol. **66:**1305-1310.

Santos, P. M., I. Di Bartolo, J. M. Blatny, E. Zennaro, and S. Valla. 2001. New broad-host-range promoter probe vectors based on the plasmid RK2 replicon. FEMS Microbiol. Lett. **195**:91-96.

Santos, P. M., G. Mignogna, H. J. Heipieper, and E. Zennaro. 2002. Occurrence and properties of glutathione S-transferases in phenol degrading *Pseudomonas* strains. Res. Microbiol. **153:**89-98.

Santos, P. M., L. Leoni, I. Di Bartolo, and E. Zennaro. 2002. Integration host factor is essential for optimal expression of the *styABCD* operon in *Pseudomonas fluorescens* ST (submitted).

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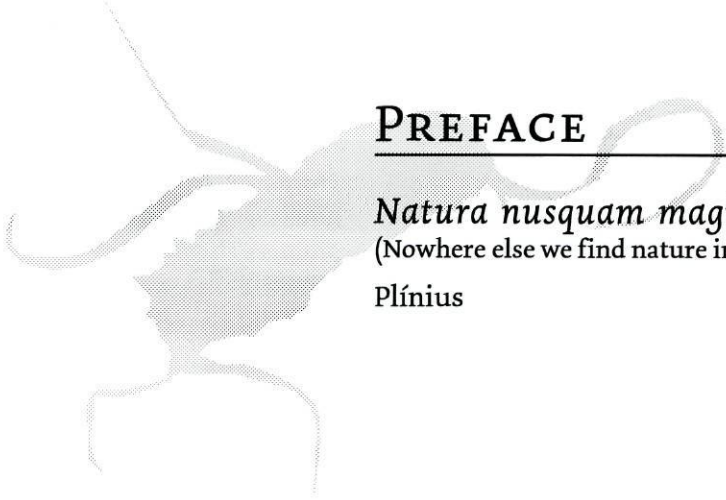

PREFACE

Natura nusquam magis est tota quam in minimis
(Nowhere else we find nature in its total like in the smallest creatures)
Plínius

For reasons related to the chemistry of life's origin and the physics of self-organization, the first living thing arose at the lower limit of life's conceivable, preservable complexity. Call this lower limit the "left wall" for architecture of complexity. Since so little space exists between the left wall and life's initial bacterial mode in the fossil record, only one direction for future increment exists - toward greater complexity at the right. Thus, every once in a while, a more complex creature evolves and extends the range of life's diversity in the only available direction. In technical terms, the distribution of complexity becomes more strongly right skewed through these occasional additions. But the additions are rare and episodic. They do not even constitute an evolutionary series but form a motley sequence of distantly related taxa. This sequence cannot be construed as the major thrust or trend of life's history. Think rather of an occasional creature tumbling into the empty right region of complexity's space. Throughout this entire time, the bacterial mode has grown in height and remained constant in position.

Bacteria represent the great success story of life's pathway. They occupy a wider domain of environments and span a broader range of biochemistries than any other group. They are adaptable, indestructible and astoundingly diverse. We cannot even imagine how anthropogenic intervention might threaten their extinction, although we worry about our impact on nearly every other form of life (adapted from Gould, 1994).

From a human's perspective, the selective forces that shaped the metabolic diversity of bacteria as they are exerted in nature are often subtle. Some clues may come from considering that bacteria perceive agents of environmental selection differently according to their different phenotypical attributes. These attributes might include the overall catabolic versatility of an organism, whether or not a particular organism is normally found in association with plants or with lignin-degrading fungi, and whether an organism has a sedentary (nonmotile) or pioneering (motile) lifestyle. Above all, it is clear that bacteria know who they are: they have an identity. If we can better understand how these identities are shaped, then we will have moved closer to describing fundamental principles of metabolic capacities (Harwood and Parales, 1996).

DEGRADAÇÃO DE HIDROCARBONETOS AROMÁTICOS EM *PSEUDOMONAS*

caracterização fisiológica e molecular

No ambiente, os compostos tóxicos aromáticos são lentamente biodegradados pelos microrganismos devido a diversos factores, nomeadamente a limitações na disponibilidade de nutrientes e na acessibilidade dos substratos, e a características físico-químicas desfavoráveis do meio em que se se encontram. Acresce que a expressão dos genes catabólicos é regulada em função das condições ambientais tendo os mecanismos de regulação evoluido de modo a melhorar o estado energético ("fitness") dos microrganismos e não sua a capacidade biodegradtiva. Os mecanismos regulatórios mais relevantes e que podem fortemente condicionar a eficiência de biodegradação são: indução pelo substrato, repressão catabolica e a resposta ao stress causado pelo substrato tóxico.

O objectivo desta tese é contribuir para um maior conhecimento e compreensão de alguns dos mecanismos principais que controlam a relação célula-ambiente durante a degradação de compostos aromáticos. O modelo experimental usado é a degradação do estireno em *Pseudomonas fluorescens* ST. Um dos aspectos interessantes deste modelo consiste no facto do controlo da expressão dos genes catabólicos envolver um sistema regulatório de dois componentes, um tipo de regulação pouco comum entre genes envolvidos na degradação de hydrocarbonetos aromáticos. Antes deste trabalho também não existia qualquer informação sobre o mecanismo de regulação ou de modulação da expressão dos genes envolvidos no catabolismo do estireno em diferentes condições de crescimento.

Os resultados obtidos indicam que a expressão dos genes do catabolismo do estireno (*styABCD*) está sujeita a repressão catabólica enquanto que os genes reguladores são expressos mesmo na ausência do indutor (estireno) (1). Foi ainda demonstrado que a expressão dos genes catabólicos é regulada a nível transcricional por StyR (o regulador específico) e por outros factores de transcrição. Em particular, foi demonstrado que é necessária uma específica estrutura na região do promotor de *styABCD* para se verificar a expressão óptima deste sistema catabólico, e uma proteina semelhante a histonas, IHF, contribui fortemente para o posicionamento correcto dos elementos regulatórios em *cis* e em *trans* (2).

Por fim os estudos realizados sobre a toxicidade e compostos aromáticos sugeriram que, em alguns casos, a expressão de agrupamentos catabólicos ou a activação de deteminadas proteinas protectoras, tais como a glutationa S-transferase, podem desempenhar um papel importante entre os mecanismos de defesa celular que permitem contrariar os efeitos nefastos destes compostos (3).

1. **Santos, P. M., J. M. Blatny, I. Di Bartolo, S. Valla, and E. Zennaro**. 2000. Physiological analysis of the expression of the styrene degradation gene cluster in *Pseudomonas fluorescens* ST. Appl. Environ. Microbiol. **66**:1305-1310.

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{Abstract}

DEGRADATION OF AROMATIC HYDROCARBONS IN *PSEUDOMONAS*

physiological and molecular characterization

Many harmful aromatic compounds are known to be slowly biodegradable by microorganisms in the natural environment. This is due to a number of different reasons, such as unfavorable physicochemical conditions, availability of nutrients, and accessibility of the substrates. Moreover, expression of the catabolic genes is environmentally regulated. Such regulatory mechanisms have evolved to improve ecological fitness rather that biodegradation efficiency. Among the regulatory devices that might affect biodegradation efficiency, the most relevant are substrate-dependent induction, catabolite repression, and stress response to substrate toxicity. The aim of this thesis is to contribute to a better understanding of some of the main mechanisms which control the relationship cell-environment during aromatic compounds degradation. The experimental model used in this thesis is styrene degradation in *Pseudomonas fluorescens* ST. One interesting feature of this system concerns the fact that a two-component regulatory system controls the expression of the catabolic genes. This kind of regulation, for genes involved in aromatic hydrocarbon degradation, has been reported only in a few cases. However, no data were available on the regulation mechanism nor on the modulation of styrene gene expression in different growth conditions.

The results showed that the styrene catabolic genes (*styABCD*) expression was controlled by catabolite repression whereas the respective regulatory genes were also expressed in the presence of substrates other than styrene (1). It was also demonstrated that the regulation of *styABCD* is not only dependent on the StyR protein (the specific regulator) but also on the presence of several other transcriptional factors. In particular, a specific DNA structure of the *styABCD* promoter is required for the optimal expression of this catabolic system and it was demonstrated that a histone-like protein, IHF, greatly contributes for the right positioning of the *cis* and *trans* regulatory elements (2).

Finally, studies on the toxicity effects of the aromatic compounds suggested that in some cases the expression of catabolic operons or activation of general protective agents, such as glutathione S-transferase, may play an important role as cellular defense mechanisms against aromatic compounds toxicity (3).

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{Résumé}

DÉGRADATION DES HYDROCARBURES AROMATIQUES CHEZ *PSEUDOMONAS*

caractérisation physiologique et moléculaire

Dans l'environnement, plusieurs composés aromatiques sont biodégradés lentement por les microorganismes, en raison de facteurs divers tels que la disponibilité des nutriments, l'accessibilité des substrats et les conditions physico-chimiques défavorables de l'environnement où ils se trouvent l'expression des gènes cataboliques est régulée por les conditions environnementales. Ces mécanismes de régulation ont évolué afin d'améliorer l'état énergétique ("fitness") plutôt que la capacité de biodégradation. Les mécanismes de régulation les plus importants et qui peuvent affecter fortment la capacité de biodégradation incluent l'induction par le substat, la répression par catabolites et la réponse au stress causé par le substrat toxique.

Ce travail de thèse a pour objectif de contribuer à une meilleure compréhension de certains mécanismes qul contrôlent la relation cellule-environnement pendant la dégradation de composés toxiques. Le modèle expérimental utilisé dans ce travail est la dégradation du styrene chez *Pseudomonas fluorescens* ST. Ce modèle est intéressanr du fait que le contrôle de l'expression des gènes cataboliques se fait par un système régulateur à deux composants. Ce type de régulation dans le cas de gènes impliqués dans la dégradation de composés aromatiques n'est pas trop commun. D'autre part, il n'existe pas beaucoup d'informations sur le mécanisme de régulation ou sur la modulation de l'expression des gènes cataboliques en différentes conditions de culture.

Les résultats obtenus montrent que l'expression des gènes cataboliques du styrene, *styABCD*, est contrôlée selon la répression par le catabolite tandis que les gènes régulateurs sont exprimés en présence de substrats autres que le styrene (1). Il a été aussi démontré que l'expression des gènes cataboliques est régulée au niveau transcriptionnel, dépendant non seulement de StyR (régulateur spécifique) mais aussi de la présence d'autres facteurs de transcription. Il a été observé, en particuller, qu'une structure spécifique de l'ADN du promoteur *styABCD* est nécessaire pour une expression optimale de ce système catabolique. La formation de cette structure dépend d'une protéine "histone-like", IHF, qui, entre autres, positionne correctement les éléments régulateurs en *cis* et en *trans* (2). D'autre part, des études sur les effets toxiques des composés aromatiques ont indiqué que l'expression des certains systèmes cataboliques auxillaires ou l'activation de certaines protéines protectrices, comme la glutathion S-transferase peuvent avoir un rôle important dans la défense des cellules contre la toxicité des composés aromatiques (3)

1. **Santos, P. M., J. M. Blatny, I. Di Bartolo, S. Valla, and E. Zennaro**. 2000. Physiological analysis of the expression of the styrene degradation gene cluster in *Pseudomonas fluorescens* ST. Appl. Environ. Microbiol. **66**:1305-1310.

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GLOSSARY

A	adenine
ADP	adenosine diphosphate
Ala (A)	alanine
AMP	adenosine monophosphate
cAMP	cyclic AMP
Arg (R)	arginine
Asn (N)	asparagine
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumine
bp	base pairs
C	cytosine
CoA	coenzyme A
Cys (C)	cysteine
CRC	catabolite repression control
C1,2O	catechol 1,2-dioxygenase
C2,3O	catechol 2,3-dioxygenase
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
FAD	flavin adenine dinucleotide (oxidized)
FADH2	flavin adenine dinucleotide (reduced)
FMN	flavin mononucleotide (oxidized)
G	guanine
Gln (Q)	gluatamine
Glu (E)	glutamate
Gly (G)	glycine
GSH	reduced glutatione
GST	glutatione S-transferase
His (H)	histidine
Ile (I)	isoleucine
Kbp	kilo base pairs
Kda	kilo dalton
Leu (L)	leucine
$logP_{ow}$	logarithm of the partition coefficient of a compound in a mixture n-octanol and water
Lys (K)	lysine
Met (M)	methionine
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NP-40	nonidet P40, non-ionic detergent
PADH	phenylacetaldehyde dehydrogenase
Phe (F)	phenylalanine
Pro (P)	proline
PstyA	promoter of *styABCD* operon
PstySR	promoter of *stySR* operon
PTS	phospho-transfer system
RNA	ribonucleic acid
mRNA	messanger RNA
rRNA	ribosomal RNA
RNAase	ribonuclease
RNAP	RNA polymerase
Ser (S)	serine
SMO	styrene monoxygenase enzyme constituted by two subunits
SOI	styrene oxide isomerase
sp.	species
styA	gene encoding for StyA
StyA	subunit of SMO
styABCD	catabolic operon for upper pathway of styrene degradation
styB	gene encoding for StyB
StyB	subunit SMO
styC	gene encoding for StyC
StyC	SOI
styD	gene encoding StyD
StyD	PADH
styR	gene encoding for StyR
StyR	positive regulator from FixJ family
styS	gene encoding StyS
StyS	hybrid histidine kinase
stySR	regulatory operon for upper pathway of styrene degradation
T	thymine
Thr (T)	threonine
Trp (W)	tryptophan
Tyr (Y)	tyrosine
Val (V)	valine

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General Introduction

...Every living thing is a sort of imperialist, seeking to transform as much as possible of its environment into itself.....

Bertrand Russell

1. *Environment and Biodegradation*

1.1 Environment

The term "environment" is interpreted differently by different individuals. A definition that is disarmingly simple originates from Albert Einstein: "The environment is anything which isn't me". This definition explains succinctly why society has so many environmental problems. Indeed, the environment is the "tragedy of the commons"; the fact that it belongs to nobody and to everybody result in the fact that we all exploit the environment to the limit (Verstraete and Top, 1992). Modern society is facing an increasing number of environmental problems.

The philosophy behind environmental technological solutions must be holistic. Indeed, too often, one cleans water by stripping the pollutants into the air, or removes organics from water which are then dumped in the soil. Environmental strategies have to be conceived with respect to the 'whole' of the environment in a long-term perspective (Verstraete and Top, 1992).

1.2. Environmental Biotechnology

In environmental biotechnology, microorganisms are not used as producers (of a chemical compound of interest), but rather as agents for the breakdown of toxic compounds. Nevertheless, there is still a huge lack of knowledge with respect to microbial diversity and interactions. In this sense, we are not able to evaluate the short, medium and long term consequences of applying newly developed products without evaluating possible co-lateral effects of such an action. Several historical examples exist, like the DTT use in agriculture, but we did not learn from them, why? Should we continue to look for solutions for our own solutions?

1.3. Xenobiotics and biodegradation

Initially, the term xenobiotic was limited to compounds which were synthesized by man and which contained structural elements that were not supposed to occur naturally (xenobiotic = foreign to life). This definition, however, led to several problems. Some compounds synthesized in large quantities by man were called xenobiotics, although they were later also detected in natural systems. The problem is, that a compound, once synthesized and released into the environment, can no longer be distinguished from a compound synthesized by an organism.

Finally, it is not important whether a compound is synthesized by man or other organisms. The question is whether a compound, through the action of man, poses a threat to living organisms. Thereby, any toxic chemical, which occurs somewhere on earth in large quantities, is a potential xenobiotic, as it may be transported by man somewhere, where it causes a threat to nature. Although most xenobiotics are degraded in the laboratory (e.g. styrene, toluene, and benzene) by pure bacterial cultures, many of these compounds still persist in nature.

Indeed, the hazard of pollutants can be reduced by conventional technologies, which involved removal, alteration, or isolation of the pollutant. Such techniques typically consist of excavation

followed by incineration or containment. However, these technologies are expensive, and in many cases they do not destroy the contaminating compound(s), but rather transfer them from one environment or form to another. Bioremediation addresses the limitations of more conventional techniques by allowing the actual destruction of many organic contaminants at reduced cost (Fig. 1).

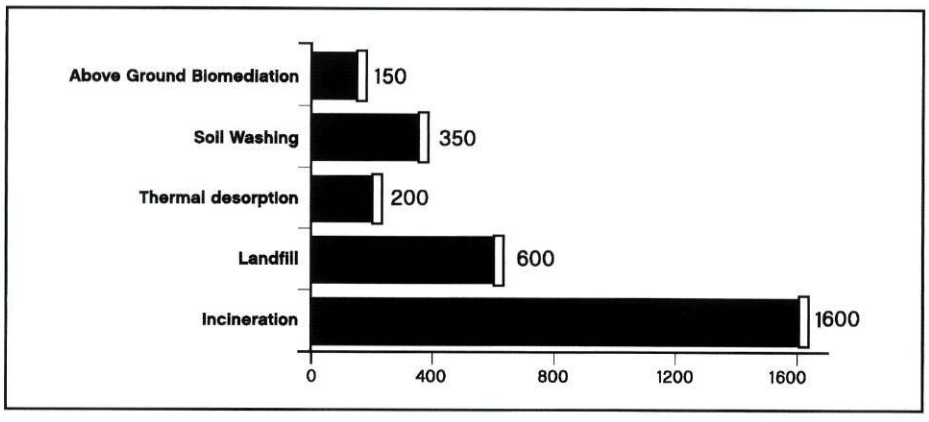


Fig. 1 Relative Cost (in dollar m^3) for various remediation technologies (adapted from Leahy and Brown, 1994)

Bioremediation is the result of the biological breakdown, or biodegradation, of contaminating compounds. While a precise definition of biodegradation is nonexistent, the process generally involves the breakdown of organic compounds by microorganisms ultimately to water, and either carbon dioxide or methane (Hoeppel et al., 1994). The extent of biodegradation and the rate at which it occurs depend on interactions between the environment, the number and type of microorganisms present, and the chemical structure of the contaminant(s) being degraded. Therefore, many factors must be taken into account for a bioremediation effort to be successful.

Research in biodegradation has demonstrated the existence of microorganisms capable of degrading chemicals such as phenolic and halophenolic compounds, polychlorinated biphenyls and nitroaromatic compounds, once thought to be recalcitrants or subject only to biotransformation. However, the metabolic pathways and environmental conditions required to achieve biodegradation of these compounds are often unique to the particular compound.

There still exists a lack of knowledge regarding the functioning of these complex microbial communities and their genetic ecology: little is known about the environmental conditions required for the functioning of an ecosystem, and about interactions between microorganisms. Until more knowledge is gained we cannot successfully exploit the biotechnological potential of microbial communities (Verstraete and Top, 1992).

1.4. **Biodiversity and** *Pseudomonas*

Prokaryotes represent a group of organisms that possess extremely diverse characteristics and live and function in quite diverse habitats (many of which seem "extreme" to those acquainted only with macrobia).

In the earlier part of the last century, *Pseudomonas* genus achieved research prominence in microbiology because of its association with human infection, and its biochemical wizardry (Holloway, 1992). In the last years the research approaches have changed and with the increasing use of genetic techniques and knowledge to understand previously intractable problems, genetic analysis has replaced physiological studies. In recent years, special emphasis has been given not only to the ability of *Pseudomonas* to cause disease in humans, but also to its role in environmental matters, particularly in plant-bacterium interactions, and in detoxification of chemical wastes.

During a routine analysis of the literature related to degradation of aromatic compounds it is possible to conclude that the major degraders of persistent pollutants are bacteria. Among bacteria, one of the frequently mentioned as active degraders are *Pseudomonas* spp. (Golovleva et al., 1992).

2 | *Metabolism of Aromatic Compounds in Pseudomonas spp.*

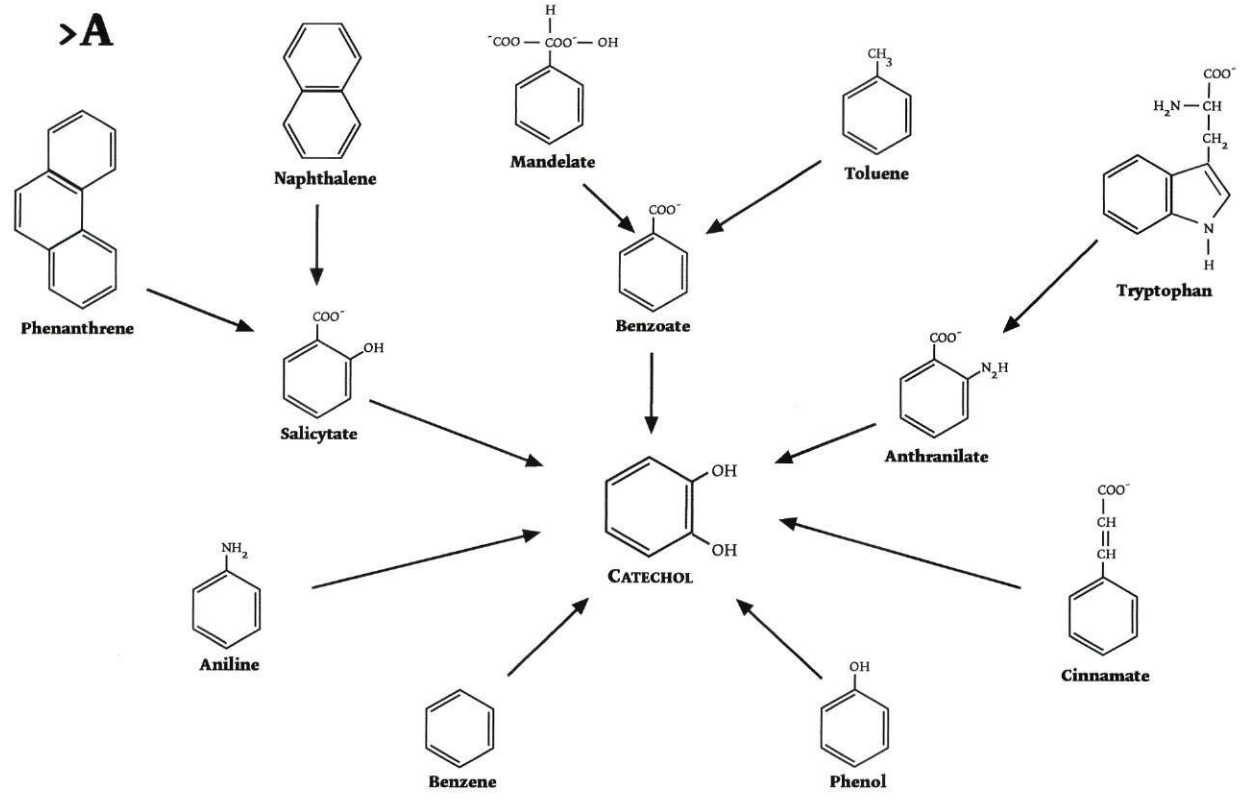
The microbial degradation of aromatic compounds has tremendous practical significance. In addition to the well-publicized problem of environmental contamination by toxic aromatic hydrocarbons, huge amounts of aromatic material are contributed to the biosphere from natural sources. The complex aromatic polymer lignin comprises about 25% of the land-based biomass on earth, and the recycling of this and other plant-derived aromatic material is a vital component of the earth's carbon cycle. The resonance energy that stabilizes the carbon-carbon bonds of aromatic rings presents microorganisms with a significant biochemical challenge (Harwood and Parales, 1996). Both aerobic and anaerobic microorganisms have been isolated that degrade aromatic compounds, but much more is known about the aerobic degradation.

In general, the aerobic degradation proceeds in two phases. First, an aromatic compound is prepared for ring cleavage by a variety of ring modification reactions leading to the formation of a dihydroxylated benzene ring (Fig. 2). The second phase of degradation includes ring fission and subsequent reactions leading to the central metabolism (formation of tricarboxylic acid cycle intermediates). Ring fission, in general, is catalyzed by dioxygenases and is termed *ortho*-cleavage, or ß-ketoadipate pathway (from the fact that ß-ketoadipate is a key intermediate of *ortho*-cleavage) when it occurs between the hydroxyl groups (intradiol cleavage) and *meta*-pathway when it occurs adjacent to one of the hydroxyls (extradiol cleavage).

A third aerobic ring cleavage reaction pathway, the gentisate pathway, is followed when the two hydroxyl groups on the aromatic ring are in *para* position to each other, and cleavage occurs between the carboxyl-substituted carbon and the adjacent hydroxylated carbon (Dagley, 1971).

Finally, it is important to notice that the biodegradation of complex organic molecules requires the concerted efforts of several different enzymes. The genes that code for the enzymes of the degradative pathways are sometimes located in the chromosomal DNA, although they are very often found on large (>50 kb) plasmids. The occurrence of plasmids in bacteria in natural environment is certainly a general phenomenon, and an important pool of genetic information residing on plasmid vehicles may flow among indigenous microorganisms. Many examples of self-transmissible plasmids that carry genes for degradation of aromatic compounds are known (Table 1) and their role in spreading these genes to other microorganisms conferring new degradative potential has been clearly demonstrated (Fulthorpe et al., 1989; 1991; Smit and van Elsas, 1990; van der Meer et al., 1992).

As a matter of fact, studies on degradative pathways often revealed the existence of biochemical blockades in natural pathways, that prevented the degradation of complex (xenobiotic) aromatic compounds, which could be overcome by the transfer of the appropriate genes (by degradative plasmids) resulting in an increased degradative potential.



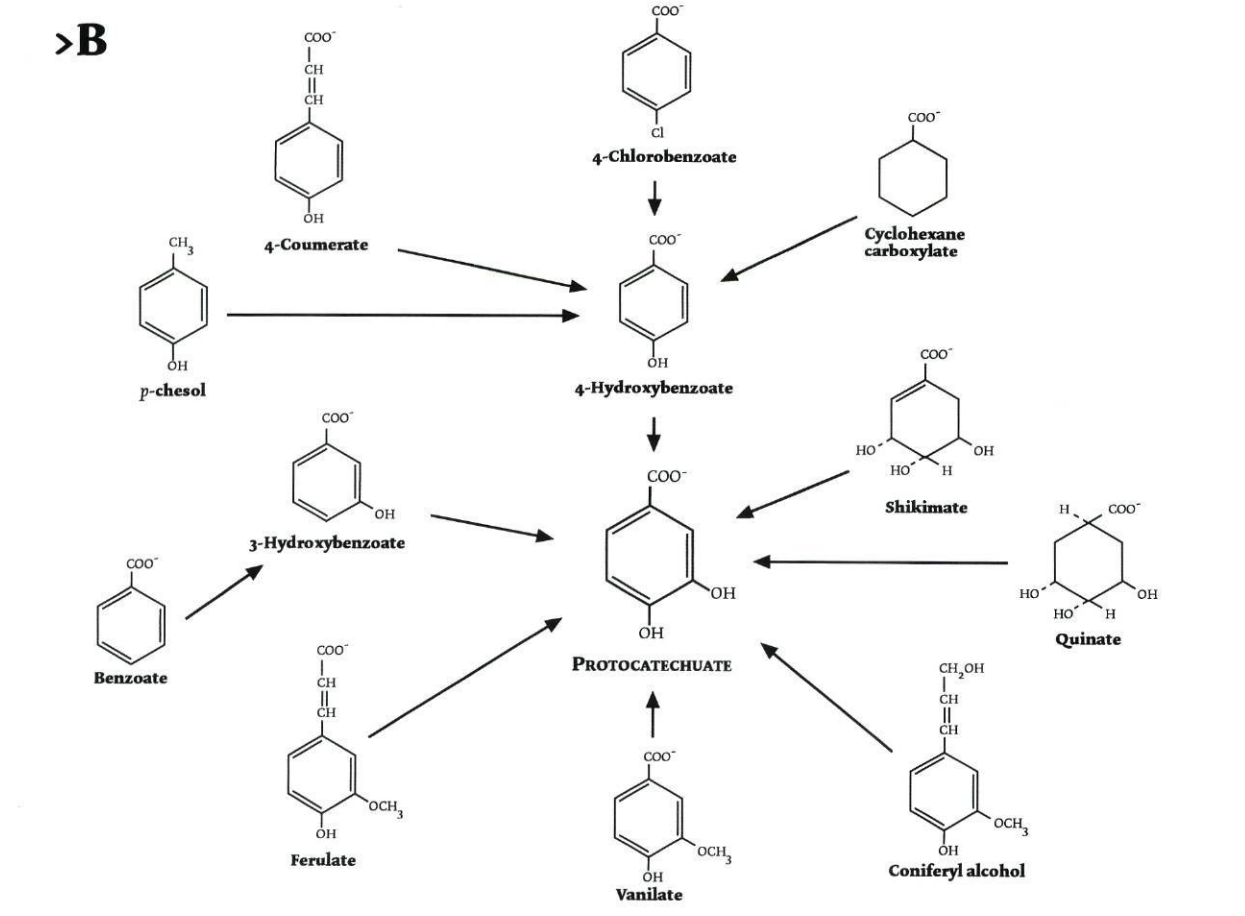


Fig. 2. Routes of convergence of aromatic compounds to catechol (A) and protocatechuate (B)

Table 1. Some examples of degradative plasmids identified in *Pseudomonas* strains

Plasmid	Size (kb)	Conjugative	Substrates	References
TOL	117	+	Xylenes, toluene, toluate	Bayley et al., 1979; Lehrbach et al., 1982
NAH7	83	+	Naphthalene via salicilate	Bayley et al., 1979; Lehrbach et al., 1983
pWW60-1	87	+	Naphthalene via salicilate	Cane and Williams, 1982
SAL1	85	+	Salicilate	Bayley et al., 1979; Lehrbach et al., 1983
pKF1	82	-	Biphenyl via benzoate	Furukawa and Chakrabarty, 1982
pWW100	~200	ND	Biphenyls via benzoate Methylbiphenyls via toluates	Lloyd-Jones et al., 1994
pCITI	100	ND	Aniline	Anson and Mackinnon, 1984
pRE4	105	ND	Isopropylbenzene	Eaton and Timmis, 1986
pWW174	200	+	Benzene	Winstanley et al., 1987
pEST100	544	ND	Phenol	Kivisaar et al., 1990
pVI150	Mega	+	Phenol, cresols, 3,4-dimethylphenol	Shingler et al., 1989
pAC25	117	+	3-Chlorobenzoate	Chatterjee et al., 1981
pJP4	77	+	3-Chlorobenzoate, 2,4-D	Don and Pemberton, 1981
pRC10	45	ND	1,2,4-Trichlorobenzene	Chaudhry and Huang, 1988
pMAB1	90	ND	2,4-D	Bhat et al., 1994

ND, not determined

Still, insertions of bacterial genes and gene clusters into other DNA contexts are often mediated by transposons, which are discrete DNA segments able to move, in the absence of genetic homology, between the donor and target sites (Berg and Howe, 1989). There are several examples of transposons containing catabolic operons for the degradation of aromatic compounds. For instance, the catabolic operons for the degradation of toluene in the TOL plasmid are part of a large transposable element Tn4651. Likewise, also other catabolic genes of plasmids such as NAH7 and Sal1 are part of transposable elements. However, in some cases transposon elements contain only the gene(s) codifying for a key enzyme such as a dioxygenase or dehalogenase (Thomas et al., 1992, van der Meer et al., 1991; 1992). Hence, these mobile elements are responsible for the insertion of defined catabolic gene cassettes into other DNA regions, thus contributing to a wide variation in the catabolic potential of bacteria.

2.1. **Enzymes of primary attack to xenobiotics**

One of the features of the *Pseudomonas* peripheral metabolism is the broad substrate specificity of the enzymes involved in the primary attack on xenobiotics, i.e., the enzymes are not only capable of converting their substrate, but also its structural analogs and sometimes even compounds with a different structure. An example is the toluene dioxygenase responsible of the conversion of toluene into cis-dihydrodiol. This enzyme oxidizes not only toluene, but also trichloroethylene, *p*-dichlorobenzene, phenol, indol, 2,5-dichlorophenol, indan and indene (Wackett and Gibson, 1988; Wackett et al., 1988; Zylstra et al., 1989b; Spain et al., 1989).

The broad substrate specificity of the primary attack enzymes is not only a characteristic of toluene dioxygenase, but also of several other enzymes (e.g. methylhydroxylases and some dehalogenases) and is a characteristic widely spread among several different degradative pathways. This fact allows these microorganisms to channel a wide range of foreign compounds into their metabolism (Golovleva et al., 1992)

2.2. Convergence of "peripheral" metabolic pathways into the central metabolites

A general comparison of the major pathways for catabolism of aromatic compounds in bacteria has revealed that initial conversion steps are carried out by different enzymes, but that the compounds are transformed into a limited number of metabolites, such as protocatechuate and catechols. These dihydroxylated intermediates are channeled into one of the ring cleavage pathways (*ortho* or *meta*). Ring cleavage pathways lead to intermediates of central metabolic routes, such as the tricarboxylic acid (TCA) cycle. This generalized scheme of catabolic pathways for aromatic compounds suggests that microorganisms have extended their substrate range by developing "peripheral" enzymes, which are able to transform initial substrates into one of the central intermediates.

2.3. *Ortho* or ß-ketoadipate pathway

The *ortho* or ß-ketoadipate pathway (Table 2) (Fig. 3) is widely distributed among taxonomically diverse eubacteria and fungi. It is almost always chromosomally encoded and plays a central role in the processing and degradation of naturally-occurring aromatic compounds derived from lignin and other plant components, as well as in the degradation of some environmental pollutants. This pathway is always present in bacterial strains that degrade chlorinated aromatic compounds by means of plasmid-encoded "modified *ortho*-cleavage" pathways, so named because the final two enzymes of the *ortho* pathway are necessary for the complete degradation of chlorinated catechols (Schlömann, 1994).

Table 2. Summary of genes, gene products, and functions of *ortho* pathways

Genes	Product	E.C. No.	Function
Catechol branch			
catA	C1,2O	1.13.11.1	Catechol 1,2-dioxygenase (type I)
catB	MLE	5.5.1.1	*Cis,cis*-muconate lactonizing enzyme
catC	MI	5.3.3.4	Muconolactone isomerase
Chlorocatechol branch			
clcA	$C1,2O_{II}$	1.13.11.1	Chlorocatechol 1,2-dioxygenase (type II)
clcB	ClcB	5.5.1.1	Chloro-*cis,cis*-muconate lactonizing enzyme
clcD	ClcD		Dienelactone hydrolase
clcE	ClcE		Maleyacetate redutase
Protocatechuate branch			
pcaGH (formerly *pcaA*)	P3,4O	1.13.11.3	Protocatechuate 4,5-dioxygenase
pcaB	CMLE	5.5.1.2	ß-Carboxy-*cis,cis*-muconate lactonizing enzyme
pcaC	CMD	4.1.1.44	γ-Carboxy- muconolactone isomerase
Common branch			
catD, pcaD	ELH	2.1.1.24	ß-ketoadipate enol-lactone hydrolase
catIJ, pcaIJ (formerly *catE, pcaE*)	TR	2.8.3.6	ß-ketoadipate succinyl-CoA transferase
catF, pcaF	TH	2.3.1.-	ß-ketoadipyl-CoA thiolase

Despite its widespread taxonomic distribution, the ß-ketoadipate pathway has been identified almost exclusively in soil microorganisms, with strong representation of bacterial groups that are found associated with plants. This makes sense in a view of large number of phenolic compounds synthesized by plants during growth.

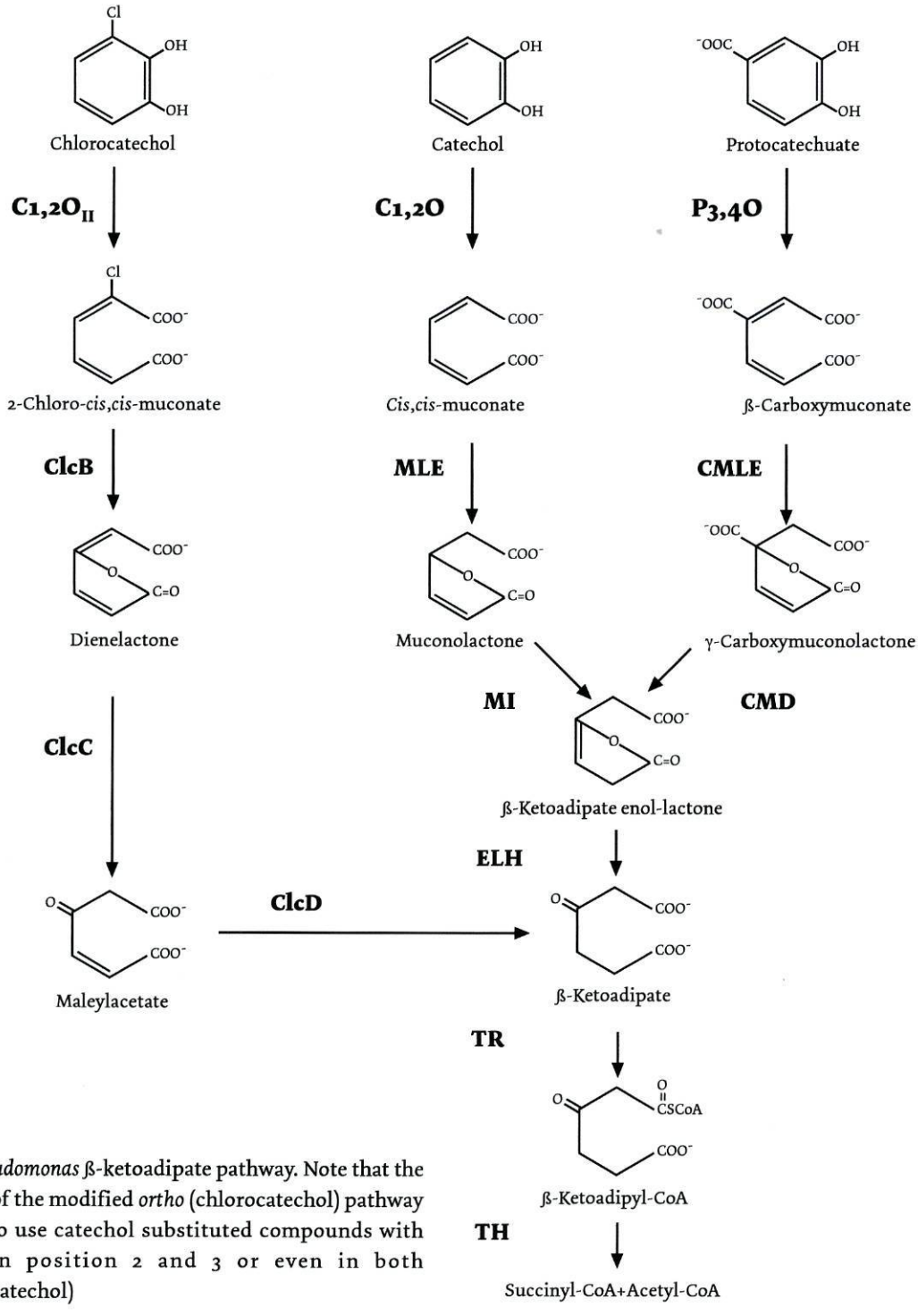


Fig. 3. *Pseudomonas* ß-ketoadipate pathway. Note that the enzymes of the modified *ortho* (chlorocatechol) pathway are able to use catechol substituted compounds with chloro in position 2 and 3 or even in both (dichlorocatechol)

2.4.*Meta* **pathway**

In general, *meta* pathways serve as non-specific routes by which aromatic rings with alkyl substituents can be metabolized without chemical modification of the substituents; by contrast, the ß-ketoadipate pathway cannot metabolize alkylcatechol.

The commonest routes for catabolism of toluene, naphthalene and biphenyls in *Pseudomonas* converge to catechol which is further metabolized by extradiol ring cleavage through *meta* pathway. Other compounds, such as vanilate and syringate, converge to protocatechuate that is also further metabolized through extradiol ring cleavage, but through a different *meta* pathway (Powlowski and Shingler, 1994).

The biochemical routes of the *meta* cleavage pathways are illustrated in Fig. 4 and the function of each enzyme is summarized in Table 3. The protocatechuate *meta* pathway has been described some decades ago but it has not been subject of many studies and the available information is only sparse. Anyway, one thing seems to be clear, in contrast to the convergence seen in the *ortho* pathway, in the *meta* pathways there is no common branch, that is catechol and protocatechuate enter into the tricarboxylic acid cycle through different intermediates. Only recently, Fukuda and co-workers (Masai et al., 1999; Masai et al., 2000; Hara et al., 2000) gave some more attention to the protocatechuate pathway and since then some genetic regulatory information emerged, but there are still too many questions to be answered to allow a discussion regarding this pathway in a detailed fashion. Because of this, from now discussions on the *meta* pathway will be referred exclusively to the case of catechol.

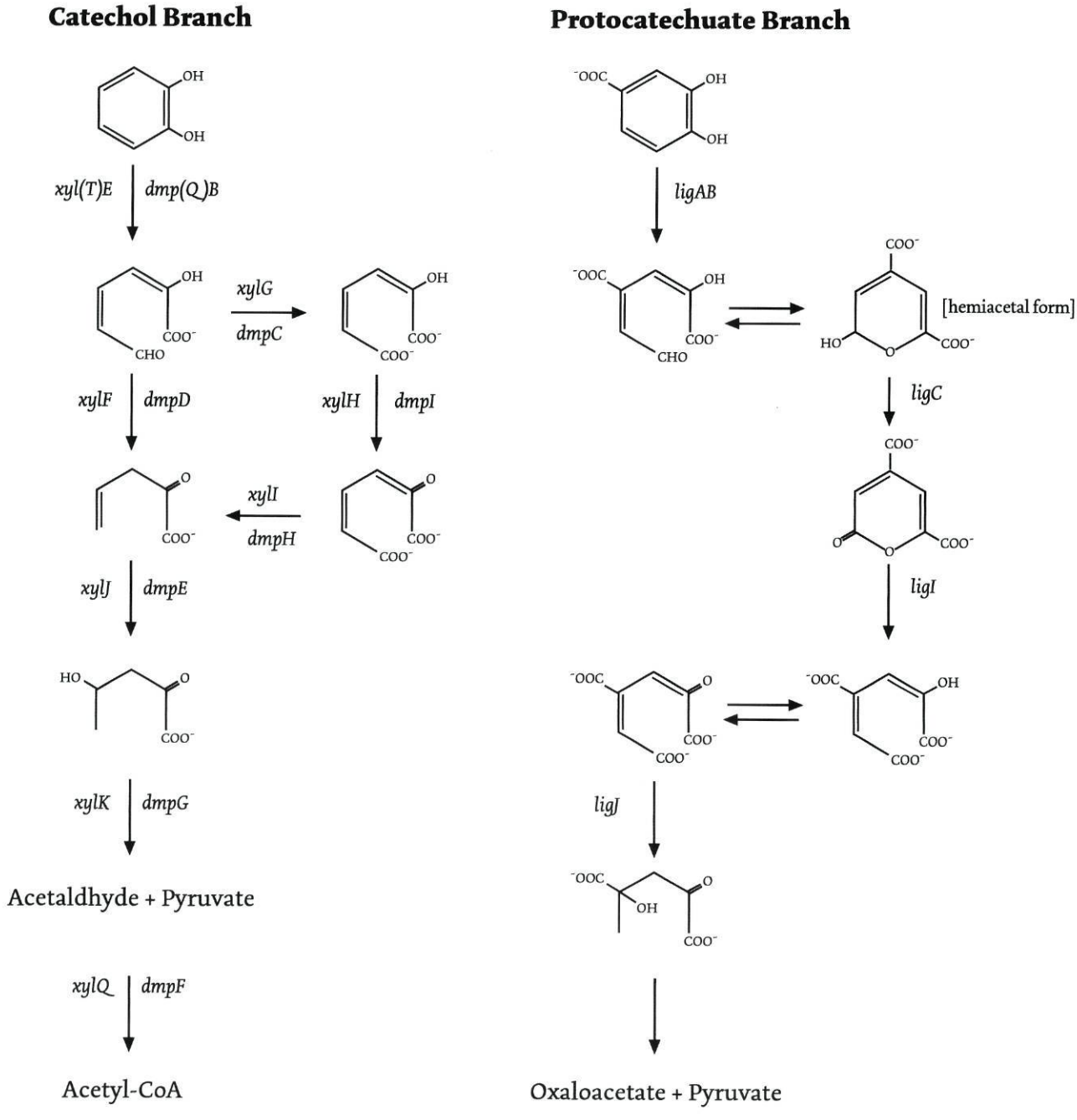


Fig. 4. Biochemical route of *meta* pathways

Table 3. Summary of genes, gene products, and functions of *meta* pathways

Genes	Product	Function
Catechol meta pathway		
dmpQ; xylT	DmpQ/XylT	Ferredoxin-like protein
dmpB; xylE; todE; bphC	C2,3O	Catechol 2,3-dioxygenase
dmpC; xylG	HMSD	2-Hydroxymuconic semialdehyde dehydrogenase
dmpD; xylF; todF; bphD	HMSH	2- Hydroxymuconic semialdehyde hydrolase
dmpE; xylJ; todG; bphE	OEH	2-Oxopent-4-dienoate hydratase
dmpF; xylQ; todI; bphG	ADA	Aldehyde dehydrogenase (acylating)
dmpG; xylK; todH; bphF	HOA	4-Hydroxy-2-oxovalerate aldolase
dmpH; xylI	4OD	4-Oxalocrotonate decarboxylase
dmpI; xylH	4OI	4-Oxalocrotonate isomerase
Protocatechuate meta pathway		
ligAB	4,5-PCD	Protocatechuate 4,5-dioxygenase
ligC	CHMSD	4-carboxy-2-hydroxymuconic semialdehyde dehydrogenase
ligI	PDCH	2-pyrone-4,6-dicarboxylate hydrolase
ligJ	OMAH	4-oxalomesaconate hydratase

Although many *meta* pathways have been described, only the two lower pathway systems of the *dmp* and *xyl* operons, have been the object of detailed genetic and biochemical analysis.

2.5. Gene organization

Analysis of DNA and the protein sequences of the enzymes that form catabolic pathways reveal that, as a rule, enzymes that catalyze similar steps within different catabolic operons tend to be highly homologous even when the initial substrates of the pathway are very different (Fig. 5) (van der Meer et al., 1992; Harayama et al., 1992). These homologies suggest a common evolutionary origin for the enzymes and even for different portions of the gene sets, which may be recruited at a given time to respond to a novel substrate.

Detailed analysis of gene structure and organization of various catabolic operons has provided further insight into the extent of their relationship. The existence of strong similarities in gene organization within the structurally and functionally heterogeneous plasmids, like TOL, NAH and pVI150 (Table 1 and Fig. 5) suggests a possible model for evolution of these catabolic pathways. It is conceivable that degradative pathways, such as that for toluene (TOL) and that for phenol (pVI150), evolved through combination in a single regulon of pre-evolved metabolic "modules" consisting, for instance, in:

(1) genes for the transformation of a hydrophobic aromatic substrate into an aromatic acid derivative (e.g. *xylCMABN*)

(2) genes for the transformation of a hydrophobic aromatic substrate directly into catechol (e.g. *dmpKLMNOP*)

(3) genes for the transformation of the aromatic acid into catechol (e.g. *xylXYZL*)

(4) genes for the ring cleavage pathway (e.g. *xylEGFJQKIH*)

Likely, co-inheritance of modules (2) and (4) or (3) and (4) in an appropriate orientation would lead to formation of an operon structure, conferring a selective advantage upon the host by facilitating growth on the respective aromatic compound. Simultaneous or subsequent recruitment of other modules would further expand the aromatic substrate range of the host. This modular gene organization is also found in chromosomally encoded catabolic systems (Fig. 5).

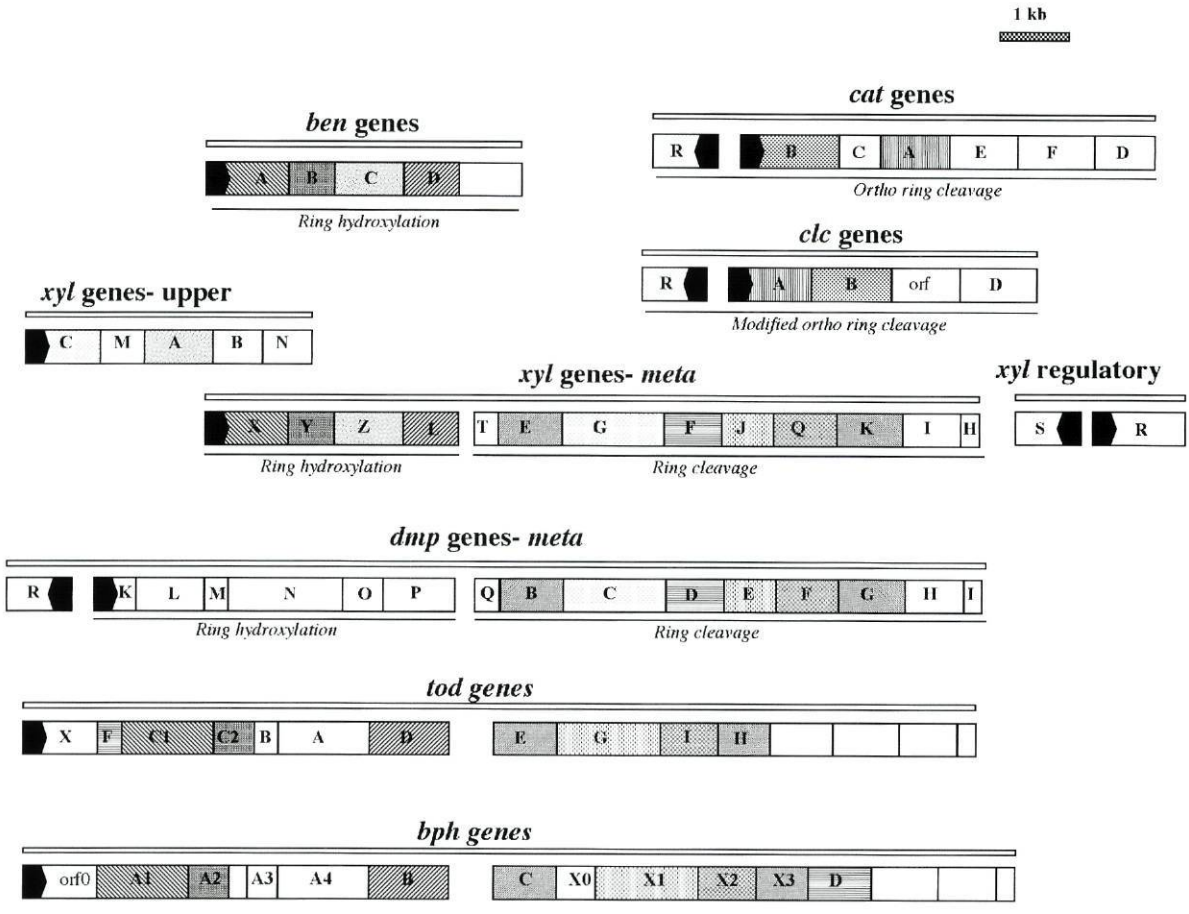


Fig. 5. Organization of different catabolic operons. The localization and sizes of the different genes are indicated by bars and drawn to scale. The direction of transcription is indicated by the black arrow. Similar patterns of hatching and shading of genes from different operons indicate significant amino acid similarity of the translated gene products or DNA sequence homology. The *cat*, *ben*, *tod* and *bph* genes are chromosomally located whereas *clc*, *xyl* and *dmp* genes are plasmid located (modified from van der Meer et al., 1992).

In fact, based on the gene organization of different pathways present in the chromosome or in plasmids, it is possible to suggest that the evolution of a certain degradative pathway is independent of its actual localization since similar modules are found in both chromosomal and/or plasmids context (e.g. *tod meta* pathway and *xyl meta* pathway, or *ben* genes and *xyl* hydroxylation unit). Indeed, the evolution of the catabolic pathways depends on gene rearrangement, recombination and transposition events where insertion elements may play a key role.

Finally, in sharp contrast to the homology among enzymes that utilize similar substrates in different pathways, the regulatory elements of the catabolic operons may be extremely different.

3. *Regulation of aromatic metabolism*

Over the years, molecular studies in *Pseudomonas* have focused on several biodegradation pathways, important virulence determinants, and characteristics of biosynthetic systems. Many of these systems are regulated in a very complex way. It is therefore not surprising that molecular details, once uncovered, fit into the emerging picture of common principles of global regulatory network and signal transmission between the environment and genes in bacteria. Pathways for catabolism of aromatic compounds that are frequently found in *Pseudomonas* provide excellent experimental systems for addressing central questions on the molecular mechanisms of adaptation during evolution. The success of a particular biodegradative pathway depends on two major steps that bacteria must overcome to adequately respond to the selective pressure imposed by the presence of novel chemical species as carbon sources. On the one hand, the right complement of genes for catabolic enzymes must be recruited to express an optimal chain of sequential transformations leading to the mineralization of the compound. On the other hand, however, only those operons that come under efficient regulatory control have a reasonable chance to prosper. Regulated promoters are the key elements that permit catabolic operons to be transcribed only when required and at levels sufficient to guarantee an adequate metabolic return when there is enough concentration of the substrate (Fernández et al., 1996).

Several families of bacterial transcriptional regulators, involved in the regulation of catabolic operons, have been recognized including two-component environmentally responsive signal transduction systems, the LysR family of regulatory elements, and the AraC/XylS group, among others. The members of the different families of bacterial regulatory proteins are widely spread among phylogenetically unrelated bacteria and share common domains, which reflect common principles of gene regulation and signal transduction. All these regulators have in common the fact that they are small DNA-binding proteins that bind the promoter regions of the operons they control, although with different mechanisms.

Many biodegradative pathways have been analyzed and their respective regulators have been characterized. Almost every family of regulatory proteins is represented among the different biodegradative regulators identified so far (Table 4).

Presumably, catabolic pathways that are very similar are more likely to have analogous regulatory systems than those that degrade different compounds by completely unrelated pathways. However, several pieces of evidences exist that often this is not the case. In fact, for example, the inducer of the *meta*-NAH pathway is salicylate, while the inducer of the meta-TOL pathway are alkylbenzoates, two compounds which are structuraly similar. But, these pathways are regulated by regulators belonging to the LysR and to the AraC families, respectively (Table 4). Each of the two families of proteins activates transcription through a totally different constellation of protein-protein and

Table 4. Regulators of aromatic catabolic pathways

Family and Proteins	Strain	Catabolic function	Reference (s)
LysR family			
BphR	*P. pseudoalcaligenes* KF707	BP-PCB	Labbé et al., 1997
CatM	*A. calcoaceticus*	Catechol	Niedle et al., 1989
CatR	*P. putida*	Catechol	Rothmel et al., 199 0
ClcR	Plasmid pAC25	Chlorocatechol	Coco et al., 1993
NahR	NAH7 plasmid of *P. putida* G7	Naphthalene-salicylate	Schell and Poser, 1989
TcbR	*Pseudomonas* sp. strain P51	Chlorocatechol	Van der Meer et al., 1991
TfdR	*A. eutrophus* plasmid JP4	2,4-Dichlorophenylacetate 2,4-D hydroxylase	Matrubutham and Harker, 1994 You and Ghosal, 1995
TfdS	*A. eutrophus* plasmid JP4	2,4-D (*tfdA* oxygenase)	Kaphammer and Olsen, 1990; You and Ghosal, 1995
PcaQ	*Agrobacterium tumefaciens*	Protocatechuate	Parke, 1996
NtrC family (σ^{54} dependent)			
DmpR	*Pseudomonas* sp. strain CF600	(Dimethyl)phenol	Shingler et al., 1993
PphR	*P. putida* P35X	(Methyl)phenol	Ng et al., 1995
TbuT	*P. pickettii* PX01	Toluene	Byrne and Olsen, 1996
TomR	*P. cepacia* G4	Toluene(*ortho* monooxygenase pathway)	Francesconi et al., 1995
XylR	*P. putida* mt-2 (pWWO)	Toluene-xylene	Inouye et al., 1988
	P.putida pWW53	Toluene-xylene	Williams et al., 1992
AraC family			
XylS	*P. putida* mt-2 (pWWO)	Toluene (*meta* pathway)	Inouye et al., 1988; Gallegos et al., 1993
XylS1	*P. putida* pWW53	Toluene (*meta* pathway)	Williams et al., 1992
BenR	*P. putida*	Benzoate (chromosomal *ortho* pathway and TOL *meta* pathway)	Cowles et al., 2000
PobR family			
PobR	*A. calcoaceticus*	*p*-hydroxybenzoate	DiMarco et al., 1993
PcaR	*P. putida*	Protocatechuate	Parales and Harwood, 1993
TetR family			
CamR	*P. putida* (CAM)	Camphor	Aramaki et al., 1995
Crp family			
AadR	*Rhodo-pseudomonas palustris*	Benzoate and p-hydroxybenzoate (anaerobic)	Dispensa et al., 1992
Two-component signal transduction family			
TodS-TodT	*P. putida* F1	Toluene (dioxygenase pathway)	Lau et al, 1997
BpdS-BpdT	*Rhodococcus sp.* strain M5	BP-PCB	Lau et al., 1996
StyS-StyR	*P. putida* Y2	Styrene	Velasco et al., 1998
	P. fluorescens ST	Styrene	Marconi et al., 1998
	P. putida CA-3	Styrene	O'Leary et al., 2001

protein-DNA contacts at the cognate promoter regions. Moreover, in some cases, the inducer of the corresponding regulator is the actual substrate to be used as carbon source, while in other cases, the inducer is an intermediate compound produced.

Additionally, in the latest years three examples of degradative pathways controlled through two-component signal transduction systems have been identified and characterized (Labbé et al., 1997; Lau et al., 1997; Velasco et al., 1998; Marconi et al., 1998; O´Leary et al., 2001). These systems were formerly associated only with complex metabolic responses to environmental changes, such

as nitrogen fixation, alginate production, nodulation process, virulence, or with stress response, and came to expand even more the diversity of regulatory circuits in aromatic degradation.

3.1. LysR family (regulation of *ortho* pathway)

Structural genes are often flanked by regulatory genes, many of which are divergently transcribed. The best studied regulatory protein in the ß-ketoadipate pathway is CatR, which positively regulates the expression of *catBC* and *catA* genes in *P. putida* in response to the inducer *cis,cis*-muconate (Rothmel et al., 1990; Houghton et al., 1995). Although *catBC* and *catA* are contiguous on the *P. putida* PRS2000 chromosome, this is not the case in other strains and *catA* is independently transcribed from *catBC* (Houghton et al., 1995). In *P. aeruginosa*, *catA* is located about 3 kb away from *catBC* (Kukor et al., 1988).

CatR is a member of the LysR family of regulators (Rothmel et al., 1990). A closely related subgroup of the family includes ClcR (Parsek et al, 1994a), TfdR (Matrubutham and Harker, 1994), and TcbR (van der Meer et al., 1991), proteins that are regulators for modified *ortho*-pathways involved in the degradation of monochlorocatechol, dichlorocatechol, and trichlorocatechol, respectively. The CatR protein has been purified, and its DNA binding properties and mechanism of *catBC* operon activation investigated (Rothmel et al., 1991; Parsek et al., 1992; Parsek et al., 1994b). An inducer-independent binding site, also referred to as a repressor binding site (RBS), has been localized to a 27 bp region between bases -79 and -53 relative to the transcription start site of *catB*. Two CatR dimers have been proposed to bind at this site, one bound to the DNA and the second stacked on the first dimer (Parsek et al., 1992). Addition of the inducer (100 μM *cis,cis*-muconate) to the system increases the affinity of CatR for *catB* promoter by about twofold, and it also stimulates the binding of CatR dimer to a site called the activator binding site (ABS) at the nucleotides -47 to -34 (Parsek et al., 1992; Parsek et al., 1994b). Parsek et al have proposed that in the presence of inducer there is one dimer bound to the RBS and one bound to the ABS (Parsek et al., 1992; Parsek et al., 1994b). DNA bending experiments show that binding of CatR to RBS in the absence of inducer results in bending of DNA. In the presence of inducer, the binding of a CatR dimer to the ABS causes a relaxation in the bending angle. These observations have led to the proposal that a relaxation in the bending of DNA in the *catB* promoter region facilitates activation of transcription, possibly by allowing direct interaction of CatR with the subunit of RNA polymerase in the -35 region. There is evidence that this general type of transcription activation mechanism may also be used by other LysR proteins (Parsek et al., 1995).

3.2. AraC/XylS family (regulation of *meta* pathway)

The best characterized AraC member in catabolism of aromatic compounds is XylS protein that positively regulates *Pm* promoter, which drives the expression of the TOL *meta*-cleavage pathway (Gallegos et al., 1997). XylS recognizes two direct repeats (operator region), located between positions -70 and -35 of the Pm promoter, that facilitate the formation of a XylS dimer (González-Pérez et al., 1999). Transcriptional activation by XylS is stimulated by alkylbenzoates (inducers) and modulated by the intracellular level of the protein. The modular structure of XylS protein has been recently shown by demonstrating that, although the carboxy-terminal domain, which comprises two potential HTH (helix-turn-helix) DNA-binding motifs, could bind to the operator region and activate transcription, deletions in the amino-terminal and central regions of the protein seriously reduced the activity of XylS and caused the loss of effector control (Kaldalu et al., 2000). Mutational analysis of the second HTH motif revealed that conservation of amino acids in the family reflects structural requirements rather than functionality in specific DNA interactions (Manzanera et al., 2000). Transcriptional initiation from *Pm* promoter is mediated by RNAP with different sigma factors, σ^{32} in the early exponential

growth phase and σ^{38} in the stationary phase. Interestingly, a second function for XylS inducers is that of triggering the heat-shock response that stabilizes σ^{32} and provides the appropriate RNAP for transcription from *Pm* during the exponential growth phase (Marqués et al., 1999).

3.3. NtrC family (regulation of the *meta* pathway)

Transcription of *dmp* operon from *Po* promoter is tightly regulated by the divergently transcribed *dmpR* gene product (Shingler et al., 1993). DmpR belongs to the σ^{54}-dependent family of regulators that control expression of genes involved in a variety of physiological processes. The activities of these regulators are themselves modulated in response to a wide variety of environmental signals (Shingler, 1996).

Members of the σ^{54}-dependent family of regulators act by binding to enhancer-like elements located 100 to 200 bp upstream from the promoters they regulate. These regulators act in concert with RNAP utilizing the alternative sigma factor σ^{54} ($E\sigma^{54}$), which recognizes a distinct class of -24/-12 bacterial promoters that differ considerably from the more usual -35/-10 type of promoters $E\sigma^{70}$-dependent. Holoenzyme $E\sigma^{54}$ forms stable closed promoter complexes but, unlike $E\sigma^{70}$, is isomerization incompetent and therefore incapable of proceeding to form open transcriptional complexes in the absence of a positive regulator. Hence, all -24/-12 promoters are regulated by $E\sigma^{54}$-dependent transcriptional activators that promote the isomerization process. ATP hydrolysis catalyzed by σ^{54}-dependent family of regulators provides the necessary energy for the isomerization process (Kustu et al., 1989). Close physical contact between the enhancer-bound regulator and the promoter-bound $E\sigma^{54}$ is facilitated by DNA-bending proteins such as integration host factor or intrinsic bends (Pérez-Martín et al., 1994).

Members of the σ^{54}-dependent family are composed of distinct functional domains involved in signal reception, transcriptional activation, and DNA binding (Shingler, 1996) (Fig. 6). The highly conserved central C domain contains a nucleotide-binding motif, and the probable roles of this domain are interaction with σ^{54}-RNA polymerase and binding and hydrolysis of ATP. A region of variable length separates the C domain and the carboxy-terminal D domain, which contains a conserved HTH DNA-binding motif found in a number of transcriptional activators and repressors. The B domain (Q linker) is a short hydrophobic region rich in Gln (Q) residues that serves as an inter-domain linker between the C domain and the amino-terminal regulatory A domain.

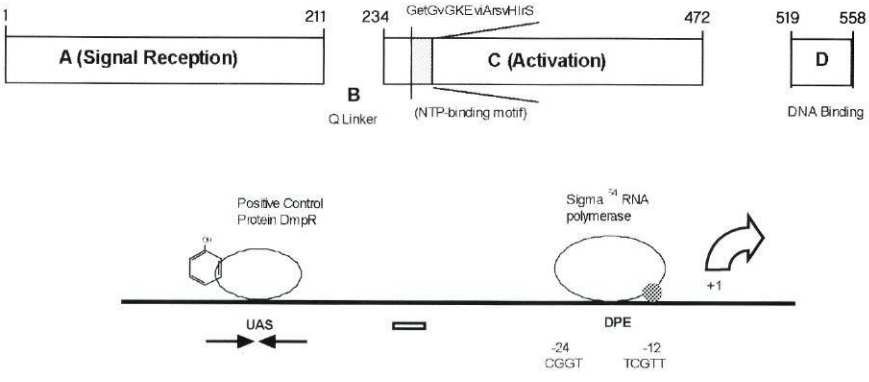


Fig. 6. Schematic representation of the domain structure of DmpR (top) and the *Po* dmp operon promoter region (bottom). Shaded box represents the location of a motif (capital letters) implicated in ATP binding and hydrolysis. Black arrows indicate the location of the upstream activation sequence (UAS), which consists of a large inverted repeat implicated in DmpR binding. The location of a integration host factor binding site is indicated by an open bar. The downstream -24/-12 promoter element (DPE) consensus sequence is indicated together with +1 transcriptional start. NTP, nucleotide triphosphate.

The regulatory A domains of this family determine the activity of the regulator and fall into different subgroups that reflect the mechanism of activation. Many members of the σ^{54}-dependent family are part of two-component regulatory systems, in which a sensor kinase activates the constitutively expressed transcriptional activator by transfer of a phosphate group to the regulatory A domain (Parkinson, 1993). The A domain of DmpR (Shingler et al., 1993) and that of XylR (Inouye et al., 1988) share 62 % identity at the amino acid level. In contrast to other σ^{54}-dependent activators, these two regulators do not require specific sensor proteins in order to respond to the presence of specific (effector) aromatic compounds in the growth medium. This specific activation is mediated by the A domain, as demonstrated with a chimeric DmpR-XylR protein in which only the A domain of DmpR has been exchanged for that of XylR. This hybrid responds to effectors of XylR, but no longer to those of DmpR (Shingler and Moore, 1994). The isolation of effector specificity mutants of DmpR (Pavel et al., 1994; Shingler and Pavel, 1995) and XylR (Delgado and Ramos, 1994), which exhibit single amino acid changes in the A domains of the respective proteins, provides additional evidence that the specificity of the effector activation of this group of regulators resides in the A domain. These observations suggest that the aromatic effectors may directly interact with the regulator. By using affinity-purified DmpR, aromatic effectors were shown to directly activate the ATPase activity of the regulator *in vitro* (Shingler and Pavel, 1995).

Effector-independent (semiconstitutive) DmpR and XylR derivatives that have alterations or single-amino-acid substitutions in their regulatory A domains, in the activation C domains, or in the short flexible B domain have been isolated (Delgado and Ramos, 1995; Fernández et al., 1995; Shingler an Pavel, 1995). These mutants, which presumably partially mimic the activated state of the protein, gave the first indication that the A domains may serve a repressive function. Indeed, deletion derivatives of both DmpR and XylR lacking the A regulatory domains have high effector-independent (constitutive) activity *in vivo*, and in the case of DmpR, the constitutive activity correlates with constitutive ATPase activity (Fernández et al., 1995; Shingler an Pavel, 1995). These observations led to a working model for regulation of the activities of DmpR and XylR (Fig. 7).

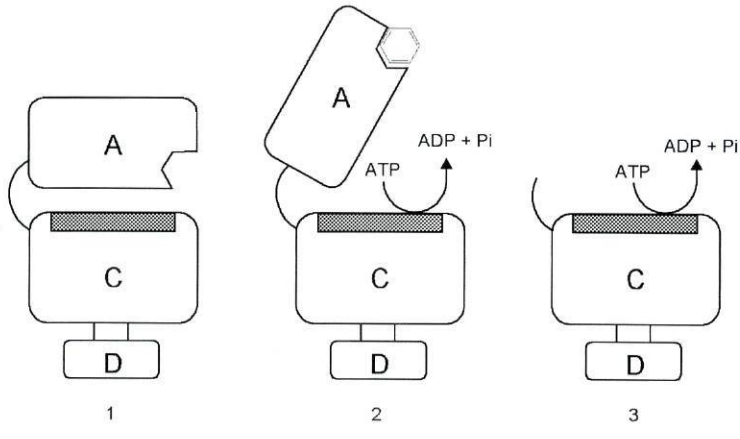


Fig. 7 Model for the activation of DmpR and XylR by their specific effectors. Domain structures are indicated as boxes. 1, The amino-acid terminal A domain, in absence of effectors, blocks the ATP binding and hydrolysis surface (indicated by color) of the C domain. 2, In the presence of aromatic effectors, the repression mediated by the A domain is relieved. 3, Removal of the A domain results in a constitutively active, effector-independent regulator.

3.4. **Two-component system family**

Regulation of gene expression in response to environmental signals is in large part controlled by phosphorylation of a superfamily of cytoplasmic proteins termed response regulators. The simplest bacterial signaling systems have two protein components: a histidine kinase (sensor), often located in the cytoplasmic membrane, that monitors some environmental parameters, and a response

regulator that mediates changes in gene expression in response to sensor signals. These signaling proteins typically communicate by means of two distinctive domains termed transmitter (T) and receiver (R). Sensors contain a carboxy-terminal transmitter domain of roughly 240 amino acids and response regulators an amino-terminal receiver domain of approximately 125 amino acids. These modules are associated with a variety of unrelated domains that function as input and output elements. Input domains of sensor proteins modulate the transmitter autokinase activity which transfers a phosphoryl group from ATP to a histidine residue, from which it is subsequently transferred to an aspartate residue in the target receiver. Receiver phosphorylation modulates the activity of its adjoining output domain (Fig. 8).

Input control and transmitter activity in sensors

Input domains in sensor proteins appear to modulate the autokinase and in some cases phosphatase activities of their adjoining transmitters to control the phosphorylation state of response regulators. It was postulated that transmitters could have two autokinase states resulting from "OFF" and "ON" conformations. The overall level of autokinase activity in a sensor population would reflect the proportion of transmitters in a certain signaling state. Stimuli would modulate autokinase activity by shifting the OFF/ON equilibrium. Transmitter modules recognize and interact with cognate receivers, emitting signals to them under input control. The only catalytic activity of transmitters is the intramolecular formation of high energy phosphohistidine. Structurally, orthodox transmitters contain short blocks of common sequence, similarly arranged but variably spaced. These blocks are block "H", containing the histidine residue that serves as the site of autophosphorylation, block "G1" and "G2" that resemble glycine rich portions of nucleotide-binding domains and block "N" and "F" whose function has not been elucidated. Sometimes transmitters lack one of these similarity regions. Finally, it is reasonable to suppose that a dimeric organization is the general rule for transmitter-containing proteins (Parkinson and Kofoid, 1992).

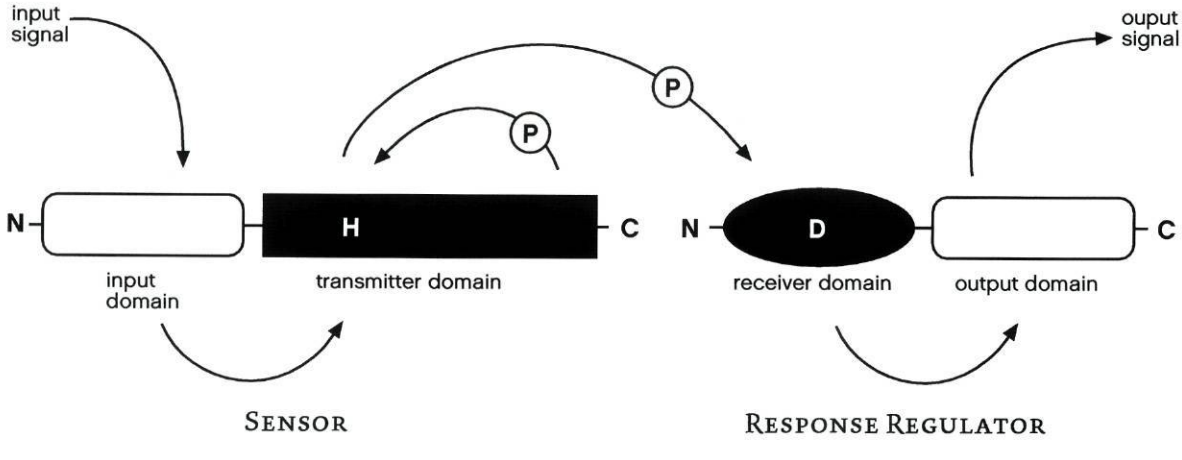


Fig. 8. The two component system for sensory signaling via communication modules. Information flows through noncovalent controls exerted by one domain upon to another (crosshatched arrows) and by phosphorylation (arrows labeled P) involving histidine (H) and aspartate (D) residues.

Receivers

Receiver modules recognize and interact with their cognate transmitters, accept signals from them, and then modulate output activity. Once the transmitter has been autophosphorylated, the transfer of phosphate from histidine to receiver is most probably catalyzed by the receiver itself rather than by the transmitter. This fact is supported by the observation that several response regulators (e.g. NtrC) can phosphorylate themselves by using acetylphosphate or other small

molecules. Thus, receivers are enzymes in their own right capable of using various phosphates sources, including appropriately presented phosphohistidines, as substrates for *in vivo* autophosphorylation. The *in vitro* half-lives of phosphorylated receivers vary greatly, from a few seconds to several hours. Receiver dephosphorylation also seems to be autocatalytic. Structurally, receivers contain four highly invariant residues, which correspond to Asp-12, Asp-13, Asp-57 and Lys-109, that play central roles in the phosphorylation and signaling activities of receiver domains (Parkinson and Kofoid, 1992).

Response regulators and output activity

Response regulators and their associated histidine kinases are common proteins. They are found in all bacterial strains that have been examined as well as in archea, and have been described in eukaryotes (Swanson et al., 1994). It has been estimated that in *Escherichia coli* there may be as many as 50 different response regulators and a comparable number of different histidine kinases. In general, response regulators that function to control transcription act via their C-terminal domains (output domains) that interact with RNA polymerase and DNA. Phosphorylation of the receiver domain activates the C-terminal DNA-binding/transcriptional regulation domain causing the repression or activation of target genes. Some receiver sequences do not follow the common structural features and may employ different modes of activation and have been referred to as unorthodox response regulators.

Depending on their associated C-terminal DNA-binding/transcriptional activation domain, the majority of response regulators fall into three distinct subfamilies designed OmpR, FixJ, and NtrC in accord with the name of their best characterized member (Da Re et al., 1994; Pao and Saier, 1995) (Fig. 9). Each member within a given subfamily controls a different set of promoters, and almost identical response regulators can function in different species to produce different responses.

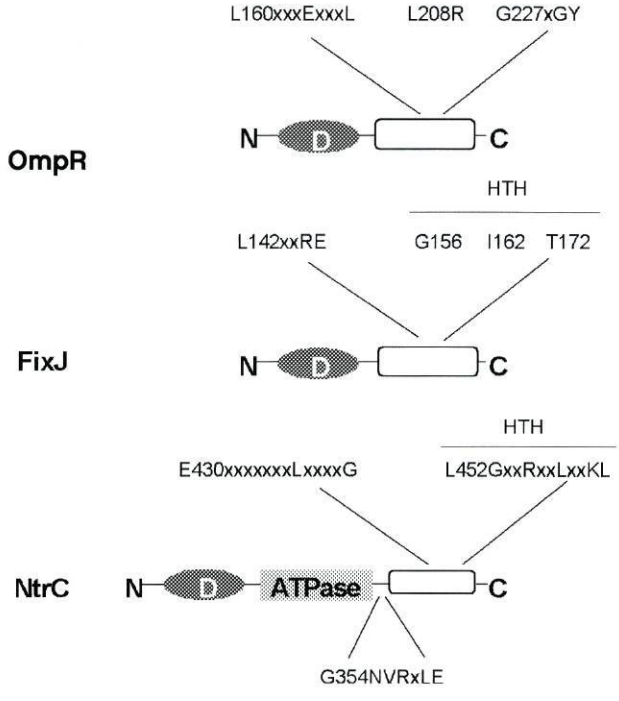


Fig. 9. Signature sequences in DNA-binding domains of the three subfamilies of response regulators. The numbering corresponds to the amino sequences of *E. coli* OmpR (239 aa in total), *Rhizobium meliloti* FixJ (204 aa in total) and, *E. coli* NtrC (469 aa in total), whose domain structures are shown schematically. D, receiver domain, HTH, putative helix-turn-helix motif.

Members of the OmpR family of response regulators contain a conserved DNA-binding domain of approximately 150 amino acids that is linked to the C terminus of a generic receiver domain. OmpR homologs can repress and/or activate target genes, depending on features of the promoters with which they interact. In general, genes that are regulated by members of the OmpR family are transcribed by σ^{70} RNA polymerase holoenzyme (Drummond et al., 1986). OmpR proteins function by making direct contact with the C-terminus of the subunit of RNA polymerase (Pratt and Silhavy, 1994). In their dephosphorylated state, the receiver domains of OmpR-like regulators appear to inhibit their DNA binding and/or transcriptional control. Phosphorylation of the receiver domain activates the associated C-terminal DNA-binding transcriptional regulation domain by relieving its inhibitory effect.

The FixJ family is characterized by a conserved C-terminal domain of approximately 100 amino acids that does not exhibit significant sequence similarity to the DNA-binding domain of OmpR family, but nevertheless appears to function similarly (Da Re et al., 1994). A putative HTH DNA-binding motif located near the C-terminals within a region homologous to the transcriptional activator domain of factors has been described (Kahn and Ditta, 1991). This class of DNA-binding domains is also shared by another large family involved in regulation of diverse physiological responses, the LuxR proteins.

Members of the NtrC family of response regulators have two domains that are linked to the N-terminal receiver domain: a C-terminal DNA-binding domain and a middle domain that functions as an ATPase. The middle domain has a glycine-rich consensus sequence termed a Walker box, the ATP-binding motif characteristic of many ATPases, and the C-terminal DNA-binding domain contains a HTH DNA-binding motif (Drummond et al., 1986). NtrC proteins control transcription from promoters that use the σ^{54} form of the RNA polymerase. Whereas activation by members of the OmpR and FixJ families seems to involve the recruitment of RNA polymerase, members of the NtrC family facilitate isomerization from closed to open transcription initiation complexes (Popham et al., 1989). The transition depends on the central domain ATPase activity, which is activated by phosphorylation of the receiver domain in the absence of DNA (Weiss et al., 1991) Unphosphorylated NtrC is a homodimer, and the ATPase of the middle domain depends on the formation of tetramers or higher order multimers (Weiss et al., 1991).

Often, sensor proteins have multiple components, not only an input and a transmitter domain as shown in Fig. 8, but also specific sensing domains, feedback loops and other complex signaling modules. Moreover, in some cases the histidine kinase and response regulator domains are part of the same proteins and form what are called hybrid histidine kinases. Hybrid kinase systems contain, in addition, a separate response regulator protein.

Many two-component systems have been identified and characterized, but until recently none of them were involved in the regulation of degradation of aromatic compounds. Indeed, the first example of a two-component system involved in a degradation pathway appeared in 1996 and was found to control toluene degradation in *Pseudomonas putida* F1, the TodS-TodT system (Lau et al., 1997) (Fig. 10). Two years later a similar system was identified in *Pseudomonas putida* Y2 and *Pseudomonas fluorescens* ST involved in the regulation of styrene degradation (Velasco et al., 1998; Marconi et al., 1998).

TodS is a hybrid histidine kinase and the predicted primary structure consists of six domains: an N-terminal basic leucine zipper (bZIP) characterized by a heptad repeat of leucines preceded by some charged residues; an input domain (input1); a transmitter domain, often also called histidine kinase domain (HK1) ; a receiver domain; a putative oxygen sensing domain (input 2) similar in sequence to a well-characterized oxygen-sensing, heme-binding domain of FixL protein; and a

duplicated transmitter domain (HK2) at the C-terminus. The presence of the bZIP is remarkable since usually has been observed only in eukaryotic transcriptional factors such as yeast GCN4 and oncogene products Jun and Fos (Kerppola and Curran, 1991). TodT, the response regulator, belongs to the FixJ family (Fig. 10).

Despite this initial characterization, up to now, a series of questions remain unanswered. No studies had been conducted that demonstrated the role of each of these domains and functional mechanisms behind these regulatory systems involved in aromatic degradation remained unknown.

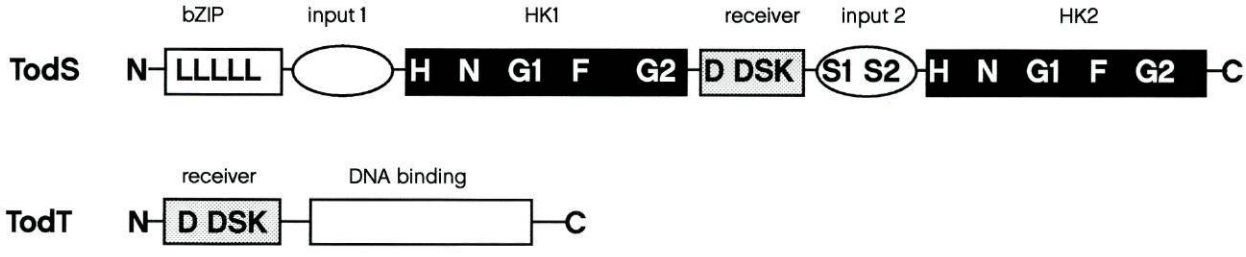


Fig. 10. Schematic domain structures of TodS and TodT. The putative leucine zipper (bZIP) is shown on the N-terminal of the sensor protein (TodS). HK1 and HK2 are the two histidine kinase domains (transmitters) characterized by the conserved amino acid blocks, and the putative histidine residues that may be phosphorylated are underlined. The receiver domains contain the conserved DDSK residues characteristic of bacterial regulators and the putative aspartate (D) residues that may be phosphorylated are underlined. The two putative sensing domains are indicated as Input1 and Input2. Input 2 contains the S1 and S2 boxes characteristic of oxygen sensing domains like in case of FixL. The DNA binding domain is characterized by the -helix-turn--helix secondary structure typical of DNA-binding domains of response regulators belonging to the FixJ family.

4. Degradation of styrene

4.1. **Styrene**

Styrene, also known as phenylethene, phenylethylene and vinylbenzene, is a simple monosubstituted aromatic compound. Its vinyl group makes it invaluable in the production of both polymers such as polystyrene and co-polymers such as styrene-butadiene rubbers. Styrene is one of the most important aromatic chemicals produced industrially, with more than 3.64 x109 kgs manufactured in 1990 in the US alone (Fu and Alexander, 1992) and an estimated industrial consumption growth rate of 5 % per year. Styrene can also be produced naturally, usually by the decarboxylation of cinnamic acid (Warhurst and Fewson, 1994). This acid has been used as an anti-bacterial agent in food products, and this has led to cases of food poisoning with styrene, due to the activity of yeasts such as Pichia carsonii (Warhurst and Fewson, 1994).

Styrene is colourless, volatile, strongly smelling and slightly soluble in water (Table 5). It is toxic in fairly low quantities, mainly due to its toxic effects on membranes (Bond, 1989) and due to the intermediates produced by enzymatic oxidation in the intoxicated cells, such as styrene 7,8-oxide (also know as styrene epoxide) that covalently binds to DNA, RNA and proteins. The release of man-made styrene into the environment can occur by a variety of routes, including factory waste-water, evaporation and the pyrolysis of polystyrene. Human exposures to styrene occurs principally by inhalation and vapor contact. High levels of styrene vapor irritate the eyes, nose and respiratory tract, and have also effects on the nervous system, liver, lungs and kidney (Sumner and Fennel, 1994). The increased production and consumption of styrene have greatly contributed to styrene pollution and exposure to man has become more likely.

Table 5. Physical properties of styrene

Molecular formula	C_8H_8
Molecular weight	104.15
Maximum solubility in water	320 mg/l
Maximum solubility in air[a]	34.2 mg/l
Log P_{ow}[b]	3.0
Water-air distribution ratio[a]	7.8
Density	0.906
Boiling point	145.5°C
Vapor pressure[c]	6.1 mmHg
Color	Colorless to yellowish

[a]- at 25°C and 760 mmHg
[b]- logarithm of the partition coefficient of styrene in a mixture of *n*-octanol and water
[c]- at 25°C

The metabolism of styrene in mammals has been extensively studied and reviewed (Bond, 1989; Sumner and Fennel, 1994), mainly due to the toxicological effects on human health of this compound and its metabolites. In mammals, styrene is metabolized in the liver via cytochrome P-450, with the formation of styrene-7,8-oxide (styrene oxide). The latter compound may follow different pathways like glutathione conjugation or glycol formation , which lead to the formation of phenylglyoxylic acid, the major urinary metabolite of styrene in mammals. While it seems clear that styrene oxide has a carcinogenic effect even in short exposure periods, other toxic effects (such as reproductive toxicants and neurotoxicants) seem to be related to long-term exposure. Moreover, pharmacokinetic models suggest that the response to styrene and styrene oxide toxicity could be species dependent (Sumner and Fennel, 1994).

Therefore, industrial strategies to avoid soil and air contamination with styrene are required in order to avoid the development of an already very serious problem. In general, styrene concentrations in industrial waste gases are low (less than 10 mg/l) and so microbial purification of these gases might be preferable to chemical or physical treatments (Cox et al., 1993).

In recent years the possible use of the styrene catabolic enzymes in the bioconversion of fine chemicals has been pointed out. This interest has been particularly directed towards the production of styrene oxide (Predragosa-Moreau et al., 1993; Wubbolts et al., 1994; Panke et al., 1998; 1999), which is an important chiral intermediate in organic synthesis, but also to several other compounds (Shirai and Hisatsuka,1979; Warhurst et al., 1994; O'Connor et al., 1997). The use of enzymes in the asymmetric synthesis offers the advantage of, often, being more effective than chemical synthesis and with lower costs.

4.2. **Microbial metabolism of styrene**

Recent research has shown that it is possible to isolate large numbers of styrene-utilizing organisms from soil. Hartmans et al., (1990) isolated 14 strains of aerobic bacteria and two fungal strains from non-polluted soils. Most research in styrene metabolism has focused on aerobic bacteria, and little investigation has been directed on fungal or anaerobic metabolism. Aerobic breakdown of styrene proceeds via attack on either aromatic ring or the side-chain.

Some bacteria able to degrade styrene through direct ring attack have been described, namely, *Xanthobacter* 124X (Hartmans et al., 1989), *Pseudomonas* MST (Bestetti et al., 1989), and *Rhodococcus rhodochrous* NCIMB 13259 (Warhurst et al., 1994). Nevertheless, only this last microorganism has been well characterized. In this strain styrene breakdown starts with the hydroxylation of the aromatic ring by a dioxygenase, leading to the formation of vinylcatechol. This molecule is then subjected to the action of both catechol 1,2-dioxygenase and catechol 2,3-dioxygenase ring cleavage enzymes. The former enzyme produces a dead-end product which accumulates in the culture broth when cells are grown on styrene. On the contrary, the catechol 2,3-dioxygenase constitutes the first step of the *meta* pathway that proceeds regularly in which the vinyl group is eliminated as acrylic acid (Warhurst and Fewson, 1994).

However, the majority of the investigated organisms have been found to degrade styrene via vinyl side-chain oxidation leading to phenylacetic acid formation. This route, also known as styrene side-chain oxidation upper pathway, is common to several well-characterized strains such as *P. putida* S12 (Nöethe and Hartmans, 1994), *P. fluorescens* ST (Baggi et al., 1983), *Rhodococcus* S5 (Hartmans et al., 1990), *P. putida* CA-3 (O'Connor et al., 1995), *Pseudomonas* sp. Y2 (Utkin et al., 1991), and *Pseudomonas* sp. VLB120 (Panke et al., 1998).

In these strains styrene degradation starts with the oxidation of the side chain double-bond with

formation of styrene oxide which is converted into phenylacetaldehyde and phenylacetic acid (Fig. 11B). Phenylacetic acid is then mineralized into TCA cycle intermediates. However, until the last three years, the phenylacetic acid catabolism had not been studied in detail .

4.3. **Styrene side-chain oxidation (styrene upper pathway)**

4.3.1. Catabolic genes

Despite the large number of strains isolated over the years for their ability to grow on styrene, only recently genetic studies have been reported in *P. fluorescens* ST (1996, 1997) and even more recently in *Pseudomonas* sp. Y2 (1998) and *Pseudomonas* sp. VLB120 (1998). The identification of the chromosomal region harboring the catabolic operon codifying for the styrene upper pathway was performed by complementation studies using a strain unable to grow on styrene, but able to grow on phenylacetic as recipient of a mobilizable gene bank of *P. fluorescens* ST (Marconi et al., 1996). Sequencing of this region showed the presence of four genes, named *styA*, *styB*, *styC* and *styD*. The *IS1162* insertion sequence, previously identified in this strain (Solinas et al., 1995), was found to be located immediately downstream of the catabolic genes. In Table 6 the most important features of the sequenced genes are summarized (Beltrametti et al., 1997).

Table 6. Some characteristics of the four *P. fluorescens* ST catabolic genes

Gene	Gene product	Length (aa)	Molecular mass (kDa)	pI
styA	StyA	415	46.4	5.5
styB	StyB	170	18.4	6.0
styC	StyC	169	18.1	8.4
styD	StyD	502	53.4	5.7

Genetic studies with the other two styrene-degradative strains revealed that the styrene catabolic genes of strains ST, Y2, and VLB120 share more than 90 per cent identity (Velasco et al., 1998; Panke et al., 1998). The question if these independently isolated strains are evolutionary related or if they have recruited the styrene degradation pathway through horizontal transfer cannot be answered yet. However the presence of very homologous catabolic pathways in strains belonging to different species or genera has been frequently reported (van der Meer et al., 1992).

The function of each single *sty* gene has been studied initially in *P. fluorescens* ST. Through biotransformation experiments with recombinant *E. coli* clones it was demonstrated that *styA* and *styB* constitute the functional styrene monooxygenase gene, *styC* codes for styrene oxide isomerase and *styD* for phenylacetaldehyde dehydrogenase (Beltrametti et al., 1997). The order of these genes in the chromosome corresponds to that of the catabolic steps. (Fig. 11).

Styrene monooxygenase (SMO)

Cloning of *styA* and *styB* genes of *P. fluorescens* ST in *E. coli* allowed the conversion of styrene into styrene oxide (Marconi et al., 1996). The same *E. coli* recombinant clone was also able to produce indigo from indole, giving rise to blue colonies. This indigo test was used to demonstrate that the products of both *styA* and *styB* genes are necessary for the styrene monooxygenase activity. In fact cloning of *styB* alone could not give any indole oxidation, and *styB* deletion resulted in a considerable reduction of indigo formation compared to the situation where both the *styA* and *styB* genes were present (Beltrametti et al., 1997).

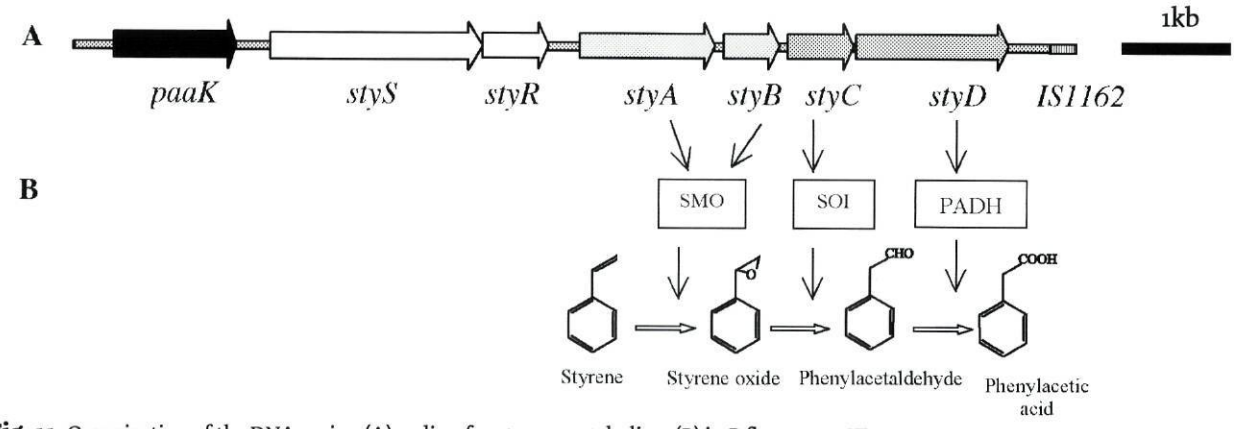


Fig. 11. Organization of the DNA region (A) coding for styrene catabolism (B) in *P. fluorescens* ST.

Similarity search with other known proteins revealed that StyA shares a degree of identity between 20 and 26 per cent with proteins with hydroxylase activity, like *p*-hydroxybenzoate hydroxylase (POBA) and salicylate hydroxylase (NAHG) (Table 7).

Table 7. Degree of identity between StyA and other known proteins

Strain	Protein	Identity (%)	Acc. No.
P. fluorescens	POBA	23.4	P00438
P. aeruginosa	POBA	23.4	P20586
Nectria haematococca	MAK1	23.7	U35892
Azotobacter Chroococcum	POBA	24.4	AF019891
Rhizobium sp.	Y4XG	26.1	P55699
Bacillus subtilis	YNFL	22.5	D87979
Acinetobacter Calcoaceticus	POBA	21.2	Q03298
P. putida	NAHG	20.7	P23262

These identity values are rather low, nevertheless a higher degree of homology is present in specific regions, like the N-terminal FAD-binding domain (Harayama et al., 1992) suggesting that also StyA, like POBA and NAHG, is a flavin-binding protein.

In Table 8 the homology of StyB from strain ST with other known proteins is summarized. Particularly interesting is the similarity with NTAB, SNAC and ACTVB proteins. The nitrilotriacetate (NTA) monoxygenase B component from *Chelatobacter heintzii* (NTAB) exhibits NTA-stimulated NADH oxidation, but is unable to hydroxylate NTA (Uetz et al., 1992). The SNAC and ACTVB proteins are NADH:riboflavin oxidoreductases, which provide the reduced form of flavin mononucleotide for the corresponding flavin-binding monooxygenases, the pristinamycin IIA synthetase (Blanc et al., 1995), and the actinorhodin synthetase (Fernandez-Moreno et al., 1992), respectively.

Table 8. Degree of identity between the *P. fluorescens* ST StyB and known proteins

Strain	Protein	Identity (%)	Acc. No.
Chelatobacter heintzii	NTAB	33.3	P54990
Streptomyces pristinaespiralis	SNAC	30.8	P54994
Mycobacterium tuberculosis	MTCY06G11.14c	30.6	Z92774
S. coelicolor	ACTVB	28.7	Q02058
Mycobacterium tuberculosis	MTCY09F9.25C	27.9	Z84498
Synechocystis sp.	SLR0001	31.4	D64000
Synechocystis sp.	SLL0550	29.5	D64003

The data reported, concerning both StyA and StyB functional analysis and their similarities with known proteins, strongly suggest that StyA catalyses the oxidation of the vinyl double-bond in the presence of molecular oxygen and reduced flavin coenzyme. StyB could be the NADH:riboflavin oxidoreductase which provide the reduced coenzyme during the oxidation process of styrene to styrene oxide by O_2. These two proteins together form the styrene monooxygenase (SMO) enzyme. Besides its key function in the mineralization of styrene, StyAB may play an important role in biotechnological production of fine chemicals. Indeed, the use of recombinant strains which overexpressed styrene monooxygenase (StyAB) showed that, in the presence of styrene, it was possible to obtain (S)-styrene-7,8-oxide at high enantiomeric excess (above 95 %) (Panke et al., 1998). This result evidenced the high degree of enantiospecificity of the conversion of styrene into styrene oxide driven by StyAB enzyme. The formation of enantiopure epoxystyrene is very interesting since this compound is known as a valuable building block in the manufacturing of optically active compounds such as some pharmaceuticals (Furuhashi, 1992).

Styrene oxide isomerase (SOI)

StyC does not show any significant homology with other proteins known for their isomerase activity. Nevertheless, the styrene oxide isomerase activity was proved to be due to the *styC* gene product by following the conversion of styrene oxide into phenylacetaldehyde in *E. coli* recombinant clones carrying only the *styC* gene (Beltrametti et al., 1997; Panke et al., 1998). This protein only shows an identity spanning from 20 to 33 % with other proteins involved in phosphate transport or with NADH oxidoreductases of mammalian mitochondrion.

Phenylacetaldehyde dehydrogenase (PADH)

Sequence similarity between the *styD* gene product and a variety of prokaryotic and eukaryotic aldehyde dehydrogenases (see Table 9) is consistent with the finding that *E. coli* cells carrying the *styD* gene from strain ST were able to transform phenylacetaldehyde into phenylacetic acid (Beltrametti et al., 1997).

The bacterial proteins listed in Table 9 are aromatic aldehyde dehydrogenases, like the CYMC protein (*p*-cumaric aldehyde dehydrogenase) of *P. putida* and the FEAB protein (phenylacetaldehyde dehydrogenase) of *Escherichia coli*. Most of the other aldehyde dehydrogenases are involved in the oxidation of retinaldehyde to retinoic acid or are liver aldehyde dehydrogenases.

Table 9. Degree of identity between StyD and known aldehyde dehydrogenases

Strain	Protein	Identity (%)	Acc. No.
P. putida	CYMC	47.1	U24215
Escherichia coli	FEAB	47.3	P80668
Zea mays	RF2	45.1	U43082
Ovis aries	ALDH1	45.5	P51977
Mus musculus	RALDH2	44.8	Q62148
Rattus norvegicus	RALDH2	44.6	Q63639
Bos taurus	ALDH1	45.2	P48644
Leishmania tarentolae	ALDH2	47.0	Q25417
Equus caballus	ALDH1	44.9	P15437

4.3.2. Regulatory genes

Sequencing of the DNA region upstream of the styrene catabolic genes in *P. fluorescens* ST has shown the presence of two open reading frames transcribed in the same direction as the sty structural genes (Marconi et al., 1998) (Fig. 11) . Analysis of the predicted amino acid sequences of these two

ORFs (*styS* and *styR*), named StyS and StyR, has indicated that they are members of a two component regulatory system (Parkinson and Kofoid, 1992; Volz, 1993), in which StyS would correspond to the sensor histidine kinase and StyR to the response regulator. Translational coupling of the two genes is suggested by an overlap of the *styR* start codon with the *styS* stop codon, in the configuration ATGA.

As for the *styABCD* structural gene products, also StyS and StyR share more than 85 % identity with those from *Pseudomonas* sp. Y2. In the case of *Pseudomonas* sp. VLB120, a StyR homolog protein was also found (still with high percentage of identity), while the presence of StyS was only in part investigated, even if data suggests that also in this case there is a StyS homolog. The high homology of the DNA region coding for styrene degradation in strains ST, Y2, and VLB120 concerning both genes organization and sequence, allows to refer to them as the *sty* genes, without making any distinction between the strains.

The styrene sensor belongs to the class of hybrid kinases, containing a duplicate histidine kinase domain and an intrinsic response regulator domain. A comparison with other histidine kinases is shown in Table 10.

Table 10. Degree of identity between the styrene sensor and other known histidine kinase proteins

Strain	Protein	Identity (%)	Acc. No.
Thauera T1	TutC	44.25	U57900
P. putida F1	TodS	41.06	U72354
B. japonicum	NodV	44.02	P15939
R. meliloti	FixL	32.4	P10955
R. capsulatus	DctS	32.8	P37739

A full-length identity of 44 and 41 per cent has been found with TutC and TodS, respectively. TutC has been proposed to play a role in the regulation of anaerobic utilization of toluene in *Thauera* sp. T1 strain (Coshigano and Young, 1997), while TodS is the sensor member of the regulation of the *tod* structural genes (Lau et al., 1997), which code for toluene degradation in *Pseudomonas putida* F1 (Lau et al., 1997; Wang et al., 1995). By analysis of their amino acid sequences, it is possible to point out that, besides the general structure, StyS shares with these two sensors other important features, mainly, a leucine zipper dimerization motif (bZIP) (Lau et al., 1997) at the N terminus, and a sequence similar to the oxygen-sensing domain of *Rhizobium meliloti* FixL (Zhulin et al., 1997). The effectiveness of the leucine zipper domain of these sensors in carrying out dimerization has been demonstrated only for TodS (Lau et al., 1997). Moreover, the TodS bZIP-domain dimer is able to bind a specific DNA sequence located upstream of the *todS* gene itself (Lau et al., 1997). If these experimental data are confirmed also for StyS and TutC, the three sensors would represent a new class of prokaryotic histidine kinases, resembling eukaryotic transcription factors. The presence of an oxygen-sensing domain in sensor kinases involved in the regulation of anaerobic (TutC), microaerobic (FixL) and aerobic (TodS and StyS) processes could mean that sensing of the same stimulus by different proteins can have different effects on their kinase activity, influencing the overall regulatory circuit. Another possibility is that these different proteins can sense different stimuli related to the cell energetic status, despite the similarity of the oxygen-sensing domain. Actually this hypothesis is supported by the finding that two boxes, named S1 and S2, which are generally considered an essential part of this domain, are also present in a large family of sensor proteins of all kingdoms, including Archea (Zhulin et al., 1997). Interesting the same boxes have been found in most of the proteins containing

he PAS domain (Kay, 1997; Crosthwaite et al., 1997; King et al., 1997), which is characteristic of proteins associated with light reception, light regulation, eukaryotic transcription regulation and clock proteins (Zhulin et al., 1997).

The highest homologies of StyR with other response regulators are reported in Table 11. These regulators have been identified as DNA binding proteins with a well conserved receiver domain (Parkinson and Kofoid, 1992). All of them belong to the FixJ family, on the basis of the sequence of their C-terminal DNA-binding domain (output domain) (Volz, 1993). The direct involvement of StyR in the positive regulation of styrene catabolic genes can be deduced from the fact that deletions of this gene abolish the expression of styrene monooxygenase, the first gene of the catabolic route, in *P. fluorescens* ST (Marconi et al., 1996; Beltrametti et al., 1997).

TodS/TodT, TutC/TutB and StyS/StyR pairs are the first examples of two component regulatory systems which regulate the catabolism of aromatic compounds in bacteria.

Table 11. Degree of identity between StyR and other known response regulators

Strain	Protein	Identity (%)	Acc. no.
Thauera T1	TutB	55.6	U57900
P. putida F1	TodT	48.9	U72354
B. japonicum	NodW	49.5	P15940
R. meliloti	FixJ	42.6	P10958
R. capsulatus	DctR	39.5	P37740

Studies on styrene-degrading strains have shown that the enzymes which convert styrene to phenylacetic acid are inducible. In particular, in styrene-grown or induced cells of both *P. putida* CA-3 and *P. putida* S12, all the enzymes of the catabolic route were induced, while cells grown on or induced by styrene oxide or phenylacetaldehyde showed very low levels of styrene monooxygenase (Hartmans et al., 1989; Hartmans et al., 1990). This finding could indicate that the two intermediates can function as inducers for the expression of the styrene oxide isomerase and phenylacetaldehyde dehydrogenase genes, but not for the styrene monooxygenase genes, even if the possibility of an enzyme inhibition cannot be ruled out.

Due to the availability of the sequence of the entire DNA region coding for styrene catabolism, is possible to speculate about the relation between these metabolic data and the styrene genes organization. It was shown that both StyA and StyB are necessary for a proper styrene monooxygenase activity (Beltrametti et al., 1997). The two genes probably belong to the same transcription unit, as no promoter-like sequences are evident in the DNA region upstream of *styB*. As already mentioned, the *styA* expression is dependent on the presence of StyR, and therefore a regulated promoter should be located upstream of *styA*. Moreover, by sequence analysis, in this region direct and inverted repeats were found, structures which are characteristic of positively regulated promoters. Interestingly one of these inverted repeats ATAAAC-4nt-GTTTAT is identical to the 6-bp inverted repeat which constitutes the specific binding site of TodT in the *todX* promoter region (Lau et al., 1997).

4.4. **Phenylacetic acid pathway (styrene lower pathway)**

Although phenylacetic acid (PA) is a common source of carbon and energy for a wide variety of microorganisms, the bacterial catabolism of this aromatic compound is still poorly understood (Ferrández et al., 1998). Some reports had suggested that aerobic PA catabolism implicated the typical initial attack by hydroxylation of the aromatic ring with the formation of the corresponding 2,5- or

,4-dihydroxyphenylacetate as intermediates (Vitovski, 1993).

Recently, has been shown that *Pseudomonas putida* U mineralizes PA aerobically through a novel catabolic pathway, which does not follow the conventional routes for the aerobic catabolism of aromatic compounds and whose first step is the activation of PA to phenylacetyl-coenzyme A (PA-CoA) by the action of a PA-CoA ligase (Miñambres et al., 1996; Olivera et al., 1998).

PA-CoA catabolon (Fig. 12) is the term used for a complex catabolic unit integrated with different degradative pathways involved in the assimilation of certain aromatic compounds (phenylacetic acid, phenylethylamine, ethylbenzene, styrene, tropic acid, trans-styrylacetic acid and *n*-phenylalkanoic acids containing an even number of carbon atoms, or C_{2n+2}PhAs) which converge into the central metabolism via PA-CoA catabolism (catabolon core or phenylacetic acid catabolic pathway).

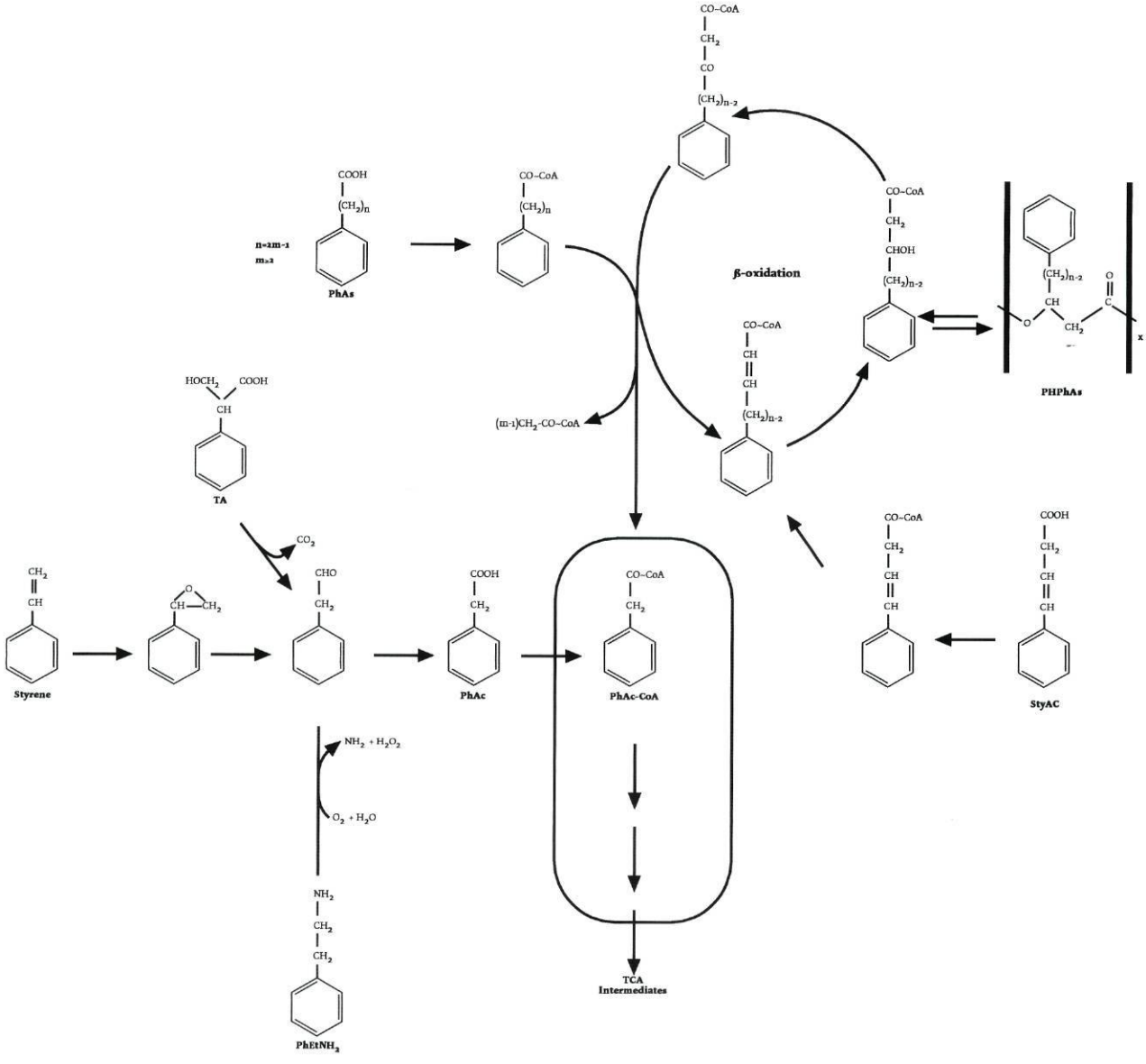


Fig. 12. The phenylacetic acid catabolon

The genetic information required for the catabolon core in *Pseudomonas putida* U is contained in a DNA region (18 kb) organized in three consecutive operons (Fig. 13).

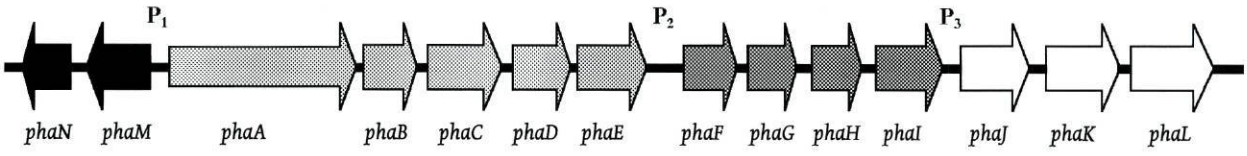


Fig. 13. The phenylacetic acid gene cluster organization

4.4.1. Transport and activation system

The genes *phaJ* and *phaK* (Table 12) encode a permease and a specific-channel-forming protein. Disruption of either of these two genes resulted in the inability of the mutants to grow in chemically defined medium containing PA as the sole carbon source. However, these mutants were able to grow on phenylalanine, 4-OH-PA, and other PA related compounds, indicating that this transport system is essential for the uptake of PA, but not for other related compounds (Olivera et al., 1998). The *phaE* gene encodes a PA-CoA ligase (phenylacetyl-CoA ligase) (Miñambres et al., 1996). Disruption of this gene prevents *P. putida* U from growing in PA, phenylethanolamine and styrene as sole carbon sources. However, these mutants are still able to grow on other related compounds such as 4-OH-PA and phenylalanine, suggesting that these compounds are not degraded via PA catabolic pathway or that *phaE* is not required . Possibly, other CoA ligase genes present in the cell may recognize these later compounds, and hence activate them to PA-CoA (phenylacetyl-CoA).

4.4.2. Hydroxylation, Ring-opening, and ß-oxidation unit

Experimental data (Olivera et al, 1998) suggest that, after activation of PA to PA-CoA, its hydroxylation (performed by PhaF, PhaG, PhaH, and PhaI) occurs originating 2-OH-PA-CoA. This information is supported by the fact that the expression of the *phaFGHI* (Table 12) operon in *E. coli* led to the accumulation of 2-OH-PA-CoA. Also the disruption of *phaL* led to the accumulation of 2-OH-PA-CoA suggesting that PhaL is involved in the hydroxylation of 2-OH-PA-CoA which is later converted into an alicyclic compound. *P. putida* U mutants in *phaA*, *phaB*, *phaC*, or *phaD* genes were unable to grow on PA and on C_{2n+2}PhAs. However, these mutants were able to grow on aliphatic compounds and C_{2n+1} PhAs suggesting that *P. putida* U has two ß-oxidation systems. One corresponding to the enzymes involved in the degradation of the aliphatic moieties of PhAs giving PA-CoA or benzoyl-CoA and the other (products of *phaABCD*) (Table 12) that catalyzes the degradation of an alicyclic PA-CoA intermediate (Olivera et al., 1998).

In other strains, the PA pathway is still poorly understood and possibly there are other routes for PA catabolism. However, in other two styrene-degrading *Pseudomonas* strains, *Pseudomonas fluorescens* ST (Marconi et al., 1998) and *Pseudomonas* sp. Y2 (Velasco et al., 1998), a *phaE* homolog has been identified. This might suggest that in these two strains the route for dissimilation of PA is similar (even if organized differently) to the other above described. However much more work must be done to clarify some key point in this route and verify if this is a common route among microorganisms or is associated only to the degradation of some unusual compounds such as styrene.

4.4.3. Regulatory elements

Three promoter regions have been identified (*P1*, *P2*, and *P3*) (Fig. 13) in the PA catabolic pathway. Furthermore, two genes (*phaN* and *phaM*) that can be involved in the transcriptional regulation of this system have been identified. Disruption of *phaN* led to the constitutive expression of *phaE* and PA-transport system (both systems are inducible in the wild type). Hence PhaN acts as a transcriptional repressor, whereas, PhaM has an unknown function since its disruption does not seem to affect the

Table 12. PA pathway genes, their products , and related gene products

			RELATED GENES	
Gene	**Gene Product** (kDa)	**Function**	**Function**	**%identity/aa**
phaA	31,4	Enoyl-CoA hydratase I	Enoyl-CoA hydratase	46
phaB	28,6	Enoyl-CoA hydratase II	Enoyl-CoA hydratase	47
phaC	53,3	3-Hydroxyacyl-CoA dehydrogenase	3-Hydroxyacyl-CoA dehydrogenase	42
phaD	52,2	Ketothiolase	Ketothiolase	45
phaE	49,3	Phenylacetyl-CoA ligase	PA-CoA ligase	86.3
phaF		Ring-oxidation complex: Protein I	4-monooxygenase subunit	14
phaG		Ring-oxidation complex: Protein II	hemoproteins	15
phaH		Ring-oxidation complex: Protein III		
phaI		Ring-oxidation complex: Protein IV	Ferredoxin consensus	
phaJ	55,1	Permease	Permease	65
phaK	46,1	Specific-channel-forming protein	Permeation of outer membrane	37
phaL	73,6	Ring-opening enzyme	Aldehyde dehydrogenase	29
phaM	21,1	Regulatory protein	CaiE, regulator	59
phaN	35,1	Repressor protein		

catabolism of PA. However it shows good homology (59%) to a regulatory protein encoded by *caiE* in *E. coli* (Eichler et al, 1994).

5. Limiting aromatic degradation

5.1. **Carbon source utilization**

Some decades ago, Monod published a milestone contribution to studies of microorganisms (Monod, 1949). The systematic description of bacterial growth (Monod, 1942) and the ideas surrounding chemostat theory (Monod, 1950) led to the notion that a limited number of growth constants define the behavior of bacterial cultures. Monod's 1949 review concluded: "There is little doubt that, as further advances are made towards a more integrated picture of cell physiology, the determination of growth constants should and will have a much greater place in the experimental arsenal of microbiology". Ironically, the desire for "constants" defining growth yields or growth affinities has come up against another of Monod's legacies, namely the uncomfortable realization that bacteria are experts in adapting to changing growth conditions (Ferenci, 1999). The studies of the Pasteur school laid the groundwork for the characterization of adaptive mechanisms far beyond simple induction/repression mechanisms, to explain complex changes under diverse environmental conditions. Many bacterial genes are regulated by growth phase, nutrition limitation and other stress stimuli (Neidhardt and Savageau, 1996; Kolter et al., 1993).

Carbon source utilization is a quite complex story that depends on several known, but also, probably, unknown mechanisms. In particular, growth yield, and growth rate can vary deeply according to different growth environments. Among these mechanisms there are some that have been studied in detail and largely contributed to the complex picture that emerged upon these findings. First, direct evidence was obtained to show that the concentration dependency of growth rate on a carbon source is related to the affinity of different transport systems to a certain substrate. Second, the permeability barrier of the outer membrane has an important influence on limiting access of nutrients and hence the nutrient affinity of bacteria (West and Page, 1984; Nikaido and Vaara, 1985). Third, the transport adaptations are significant to growth yield considerations because transporters with different modes of energization have an impact on the energetics of nutrient uptake (Muir et al., 1985). This means that, additional nutrient has to be expended on catabolic reactions rather than in biosynthesis depending on the transport mechanism. Finally, not only the type of carbon source determines a certain uptake system and hence a certain metabolic state of the cells, but also its external concentration. For example, gene expression can increase or decrease with the carbon source concentration, or can actually exhibit a maximum at intermediate dilution rates in chemostat (Ferenci, 1996).

Most of the nutrients are transported into bacteria by the action of transport proteins that is often mechanistically similar even when the transporters show variable affinity for diffeerent types of nutrients. Indeed, these mechanisms are energy-dependent and often driven by proton motive

force. Therefore, membrane transporters have specific sites for both substrate and protons. This fact leads to another relevant consequence of bacterial growth. As growth proceeds the pH of the growth medium changes, which, besides other consequences, implies changes in protein activities and affinity to the nutrient. When these changes are severe, growth diminishes and even stops leading to a starvation situation.

Hence, nutrient utilization results in changed environmental conditions and so changed gene expression and so altered physiological properties.

5.1.1. Choosing the right carbon source

For nearly a century it has been recognized that the presence of a rapidly metabolizable carbon source in the growth medium can inhibit the synthesis of enzymes involved in the metabolism of other carbon-containing compounds (Monod, 1947; Magasanik, 1970). The phenomenon was intensively studied during the first half of the 20th century, primarily from a physiological standpoint, but these studies did not lead to a mechanistic insight. As glucose was often the most effective carbohydrate causing repression of the synthesis of target enzymes, the phenomenon became known as glucose effect. Early analyses of the glucose effect led to the postulate that it occurs whenever growth conditions are such that degradation (catabolism) exceeds biosynthesis (anabolism). This postulate in turn led to a second notion, namely, that it was the accumulation of one or more cytoplasmic catabolites derived from the repressing carbohydrate, that gave rise to the glucose effect. This concept caused the term "catabolite repression" (Saier, 1996). Hence, the growth response of microorganisms presented with two carbon/energy sources is frequently biphasic (diauxic), reflecting the sequential, rather than simultaneous, utilization of the carbon/energy sources. During the first exponential phase of growth, when the preferred carbon source is being utilized, expression of genes encoding the catabolic enzymes required for assimilation of the second carbon source is repressed, despite the continued presence of the second substrate in the media. This so-called catabolite repression control (CRC) is abrogated as the preferred carbon source approaches depletion. Cell growth may lag (slow or cease) until the catabolic machinery required to assimilate the second carbon source is fully established. At this point a second distinct logarithmic growth phase is exhibited. (Collier et al., 1996).

5.1.1.1. Glucose effect

In 1965, Markman and Sutherland identified cyclic AMP (cAMP) in *E. coli* and showed that its cytoplasmic concentration varied inversely with growth rate when the carbon source was varied (Makman and Sutherland, 1965). The subsequent identification of cAMP receptor protein (CRP) and description of the phenotypic properties of mutants lacking either cAMP biosynthetic enzyme, adenylate cyclase (*cya* mutants), or CRP (*crp* mutants), led to general acceptance of the notion that cAMP, acting together with CRP, provided the principal means of effecting catabolite repression. Further studies led to the recognition of the fact that the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) in enteric bacteria could coordinately modulate both cytoplasmic inducer and cAMP levels in a physiological meaningful manner that could account for the repression of enzyme synthesis (Saier, 1989; 1991).

5.1.1.2. Back to the startpoint

Meanwhile, evidence was slowly accumulating that suggested that our picture of catabolite repression in bacteria, based solely on a cAMP-dependent mechanism, was incomplete and far from being a general feature among bacteria. In fact, cAMP-independent catabolite repression effects were

observed in several cases, even in *E.coli* (for review see Ullmann and Danchin, 1983), the specie used to develop the cAMP-dependent catabolite repression model. However, the trend had been set; interest in bacterial catabolite repression was minimal, and progress in the elucidation of the cAMP-independent repressive mechanisms was exceptionally slow. This subject has been studied mainly in enteric bacteria like *E. coli* and *S. typhimurium* and in *B. subtilis* (Saier et al., 1995; Mach et al., 1988; Saier, 1996).

The experimental data currently available establish that a single bacterium such as *E. coli* may possess numerous mechanisms for the control of carbon utilization, and that such enteric Gram-negative bacteria use regulatory mechanisms that are entirely different from those found in the low-GC Gram-positive bacteria (Saier et al., 1995). Recent studies are beginning to reveal the details of catabolite control mechanisms that occur in other evolutionarily divergent bacteria. The non-enteric Gram-negative bacterium *Haemophilus influenzae* apparently controls carbon utilization by mechanisms that parallel those of *E. coli* (Macfadyen and Redfield,1996). However, Gram-negative *Pseudomonads* (Collier et al., 1996), photosynthetic bacteria such as *Rhodobacter* (Inui et al., 1996) and high-GC Gram-positive bacteria of the genus *Streptomyces* (Paulsen, 1996) provide examples of bacteria that exhibit catabolite repression phenomena which in some aspects superficially resemble, but are probably mechanistically distinct from those found in enteric bacteria. Moreover, species of *Rhodobacter, Azopirillum, Pseudomonas, Azotobacter* and *Agrobacterium* exhibit a regulatory phenomenon termed "reverse catabolite repression" (Lynch and Franklin, 1978; Ucker and Signer, 1978; Mukherjee and Ghosh, 1987; Nautiyal et al., 1992). In this process, exogenous Krebs cycle intermediates repress the utilization of carbohydrates rather than the other way round. Although some progress has been made in identifying the protein constituents involved, the mechanistic details of these processes have yet to be elucidated.

5.1.2. Carbon utilization in *Pseudomonas*

In *Pseudomonas* spp., with rare exceptions, succinate and other TCA cycle intermediates impose the most severe level of CRC on a majority of the pathways. Many pathways can also provide nitrogen, sulphur or other compounds to the cell, and in several cases, these compounds impose an additional level of catabolite repression.

It is possible that metabolism of most CRC-active molecules generates a common effector, or one of a limited number of effectors. These might be intermediates of a metabolic pathway and/or more general indicators of the metabolic state of the cell such as the level of reducing equivalents, magnitude of the proton motive force or the availability of high energy phosphodiester bonds. The adenylate energy charge (EC) ratio, defined as

$$\mathbf{EC = (ATP + 1/2ADP)/(ATP + ADP + AMP)}$$

is one measure of the metabolic state of cells and has been found to vary with the growth phase in many different organisms (Chapman et al., 1971). It is tempting to speculate that the EC provides a unifying device through which many CRC-active substrates transduce their effects in *Pseudomonas*. According to this hypothesis, the most highly preferred carbon sources, such as TCA intermediates, would establish the highest EC ratios, the less preferred, such as mannitol or pyruvate, would generate the lowest ratios, and carbon sources of intermediate desirability, such as glucose, would establish intermediate EC ratios. Indeed, the EC ratio of *P. aeroginosa* growing in a mix of succinate and glucose is high during succinate utilization, drops prior to glucose utilization, then increases during glucose utilization, but not to the same value achieved with succinate (Wiebe and Bancroft, 1975). When cells grow only on succinate the EC drops as cells enter stationary phase, a period when CRC of some operons is partially abolished (Collier et al., 1996).

Up to now several attempts were made on the identification and characterization of the molecular mechanisms of CRC process. Some *P. aeruginosa* mutants defective in CRC (CRC$^-$) have been identified (Wolf et al, 1991) and the responsible gene (*crc*) cloned (MacGregor et al, 1991), sequenced and its protein product (Crc) purified (MacGregor et al, 1996). The phenotype of *crc* null mutants (loss of CRC) indicates that CRC acts to repress gene expression in the presence of a preferred carbon source rather than activate gene expression in the absence of preferred carbon sources. Crc exhibits no significant homology to known DNA binding regulatory proteins, contains no obvious helix-loop-helix domains that are hallmarks of such proteins, and exhibits no specific DNA binding activity (MacGregor et al, 1996). Although interpretation of negative results is perilous, these observations suggest that Crc-mediated repression is unlikely to result strictly from Crc binding to regulatory elements in CRC sensitive promoters. Crc is more likely to be a component in a regulatory cascade one or more steps before the direct interaction with CRC responsive promoters. Because Crc is constitutively expressed (MacGregor et al., 1996) and CRC is established very rapidly following addition of a preferred carbon source (Smyth and Clarke, 1975), Crc is probably reversibly activated/deactivated by phosphorylation (Collier et al., 1996)

5.1.3. Carbon utilization in the presence of aromatic compounds

Microbial degradation of aromatic compounds is an important approach to eliminating toxic pollutants from environment. Most of the catabolic pathways have generally been analyzed under conditions in which the target compound is present as the sole growth-supporting substrate. In nature, however, mixtures of potential growth substrates are often present. Hence, the utilization of an aromatic compound depends not only on the presence of its respective catabolic pathway, but also on the presence of other carbon sources. Often these other carbon sources are more favorable substrates for microorganisms and may be utilized in detriment of the aromatic compound, leading to a catabolite repression situation. As a consequence, bioremediation of aromatic pollutants in mixtures with more-favorable substrates is impaired, or occurs only after the depletion of the preferred carbon source(s).

In *Pseudomonas* species the expression of catabolic pathways for terpenes such as camphor (Hartline and Gunsalus, 1971), aromatic compounds like benzene (Mason, 1994), aniline (Helm and Reber., 1979), protocatechuate (Zylstra et al., 1989a), phenol (Müller et al, 1996), toluene (Holtel et al., 1994; Duetz et al., 1994), and chloroaromatic compounds (McFall et al., 1997), is subjected to carbon catabolite repression. However, the mechanisms involved are still unknown and seem not related to the cAMP-dependent mechanism.

5.2. Other factors affecting aromatic degradation

Up to now discussion has focused on degradation pathways and their regulation, but it is also important to consider that bacteria live in precarious environments. Nutrients and toxin levels, acidity, temperature, osmolarity, humidity, and many other conditions can change rapidly and unexpectedly. To survive, cells must constantly monitor external conditions and adjust their structure, physiology and behavior accordingly, in a series of adaptation mechanisms. So, not only their ability to use a certain compound as sole carbon-source is important, but, at least equally important is their capacity to adapt to the environmental situation. Indeed, many processes in modern biotechnology, particularly biotransformations and environmental bioremediation, are hindered by the toxic effects of organic solvents on the cells (Heipieper et al., 1994).

5.2.1. Effects of aromatic compounds in microorganisms

Organic solvents like alcohols, aromatics and phenolic compounds are classical antimicrobial agents (Hugo, 1978; Lucchini et al., 1990). Therefore, they have been widely used as disinfectants, food

preservatives, and tools for permebialization of cells (de Smet et al., 1978; Davidson and Branden, 1981; Naglak et al., 1990; Sikkema et al., 1992). The antimicrobial action of a solvent correlates with its hydrophobicity as expressed by the logarithm of the partition coefficient of the compound in a mixture of *n*-octanol and water (log P_{ow}). Organic solvents with log P_{ow} values between 1 and 5, such as toluene, styrene or phenol, are highly toxic for microorganisms. It has been established that there is a systematic relationship between values of log P_{ow} in the range between 1 and 5 and the partitioning of solvents in membrane buffer systems (Osborn et al., 1990; Sikkema et al., 1992, 1994). Hence, the log P_{ow} value is a suitable parameter that describes the accumulation of these hydrophobic compounds in membranes (Heipieper et al., 1994). To understand the mechanisms that allow microorganisms to survive in the presence of the solvents, several aspects have been taken into consideration.

First, the accumulation of organic solvents in the membrane results in its permeabilization and leakage of macromolecules such as RNA, phospholipids and proteins (Jackson and de Moss, 1965; Woldringh, 1973; Heipieper et al., 1994). Studies with bacterial and artificial membranes in the presence of solvents revealed an increase in the membrane surface area and a passive flux of protons and other ions across the membrane (Monti et al., 1987; Sikkema et al., 1992, 1994). This flux of ions dissipates the proton motive force (Δp), and affects both the proton gradient (ΔpH) and the electrical potential ($\Delta\psi$) (Sikkema et al.,1994). Therefore, a consequence of the membrane toxicity by organic solvents is a fall in the energy status of the cell (Bowles and Ellefson, 1985; Uribe et al., 1990). The accumulation of solvents into the membrane also affects the function of the proteins engaged in energy transduction and of other proteins embedded in the membrane.

Another important aspect of the membrane structure, the fluidity, which is defined as the reciprocal of viscosity, is affected by organic solvents (Sikkema et al., 1994). An increased fluidity of membranes results in changes in stability, structure, and interactions within the membrane (Yuli et al., 1981; Zeng et al., 1988). Finally, membrane-active compounds can affect the hydration characteristics of the membrane surface and the thickness of the membrane itself (Shimooka et al., 1972; Seeman, 1972).

5.2.2. Adaptation mechanisms

5.2.2.1. Transient responses

Adaptive responses in bacteria range from rapid, short-term transient changes to long-term global reorganizations of gene expression and cell morphology. Several adaptive changes in the structure of the membrane have been observed in response to the accumulation of organic solvents in order to reestablish its stability and fluidity (Heipieper et al, 1994., 1996). For instance, a mechanism for changing the fluidity of the membrane by the isomerization of the *cis* bond of an unsaturated fatty acid into *trans* configuration have been observed (Heipieper et al., 1992). This conversion is caused by an energy-dependent isomerase (Diefenbach and Keweloh, 1994; Holtwick et al., 1997).

The isomerization increases the membrane ordering and consequently decreases the membrane fluidity (Diefenbach and Keweloh, 1992; Keweloh and Heipieper, 1996). The *cis/trans* isomerization has also been reported as a response to starvation (Guckert et al., 1986), to the presence of antibiotics (Isken et al., 1997) and heavy metals (Heipieper et al., 1996). This indicates that the *cis/trans* isomerization may be part of a general stress response of microorganisms.

Another interesting way to diminish the toxic effects of an aromatic compound is its removal through active excretion. Isken and de Bont (1996) demonstrated that the amount of toluene accumulated in *P. putida* S12 cells is dependent on energy. Indeed, in cells adapted to toluene, the presence of different energy inhibitors resulted in significantly higher amounts of toluene accumulating in the cells. Since this strain is not able to degrade toluene, it was concluded that the amount of toluene in the cell is kept at a relative low level by action of an active efflux system. Genes encoding this efflux system were

identified (named *srpABC*), and showed homology with proton-dependent efflux pumps, such as those encoded by the *arcAB* operon in *E. coli* (Kieboom et al., 1998). This suggests that the solvent efflux is dependent on the proton motive force. Since then, other examples of these solvent efflux systems have been found suggesting that this adaptation mechanism might be present in many unrelated bacteria and be a general feature of solvent adaptation. It is important to notice that several other adaptative mechanisms have been reported (see Isken and de Bont, 1998 for a review) and it is obvious that only a combination of different mechanisms allows the survival of bacteria when in presence of a toxic hydrophobic compound. The regulation of such a complex response system may be connected to a general stress response. This possibility is supported by the fact that adaptation to solvents does not only enhance the resistance to other solvents (Heipieper and de Bont, 1994), but also to heavy metals (Heipieper et al., 1996) and to antibiotics (Isken et al., 1997). Such a correlation was also found in *E.coli* where the overexpression of stress response genes enhanced tolerance to various environmental factors (Aono et al., 1995; Nakajima et al., 1995; Asako et al., 1997).

5.2.2.2. Long-term (Genetic) adaptation mechanisms

If a microbial community is exposed to xenobiotic compounds for long periods, it can often adapt and give rise to a degradative-efficient community. In fact, many microorganism able to metabolize a certain xenobiotic at considerable rates have been isolated, that most probably in their original state were not able to do so. From this phenomenon emerges the fact that changes in the enzymatic systems have occurred that imply changes in genetic information. These molecular events are called genetic adaptation. The increase of knowledge about the degradative biochemical pathways of the different microorganisms may reveal the underlying principles of metabolic diversification and adaptation.

Amino acid similarities of the different aromatic pathway enzymes and the diverging organization of catabolic genes (Fig. 5) suggest that several different gene clusters (e.g. *meta* pathways) may be combined in modules, to which other peripheral genes may be added. Furthermore, it shows that many DNA rearrangements have occurred during the evolution of different pathways. This is also supported by the finding that exposure of a community to a severe environment induces gene transfer, recombination and transposition events, to confer higher survival capacity to such a community (for a review see van der Meer et al., 1992).

In summary, microorganisms possess a broad range of adaptation mechanisms, but only a complex and concerted functioning of each element will allow them to adapt to severe growth conditions. In the last decades, many efforts have been made to exploit each one of these properties targeting the development of bioremediation solutions. However, these strategies often failed most probably due to the lack of knowledge that still exists about the mechanisms that control the concerted response. In fact, for instance, many promising laboratorial results obtained with engineered strains failed on the field due to limited competitiveness, or other unclear reasons.

Therefore, scientific attempts to increase not only the breakdown capacity of microorganisms, but also their tolerance, and field survival should be put in place. Moreover, pure culture studies should be paralleled with studies on the behavior and survival of selected, known communities to allow a better understanding of microbial interactions and their effects.

Aims And Working Plan

The aim of this thesis is to contribute to a better understanding of some of the main mechanisms which control the relationship cell-environment during aromatic compounds degradation. The key control elements for aromatic compound catabolism are regulatory proteins and regulated promoters. A large number of catabolic genes and regulators have been sequenced and characterized, which has led to the acknowledgment of their importance, in general terms as well as on evolutionary aspects of bacteria adaptation. However, a deep knowledge on regulative mechanisms has been obtained only in few cases.

In general, these studies concern the activation mechanism of specific catabolic pathways upon substrate induction. Only recently attention has been focused on the relationship between aromatic compounds catabolism and the general metabolic and energetic status of the cell. It is not surprising that these studies have been first performed with already well known catabolic systems, since a fine tuning of the catabolic genes modulation is expected in different growth conditions. Nevertheless, this research topic is still in its infant phase.

Up to now, the most investigated phenomenon has been carbon catabolite repression, which controls the preferential use of one carbon source from a mixture of substrates.

Most of catabolic genes for aromatic compounds are subjected to this phenomenon. A relationship between catabolite repression and sugar transport (Cases et al., 1999) or alternative sigma factors (Cases and de Lorenzo, 2001) or the metabolic alarmone (p)ppGpp (Sze and Shingler, 1999) has been suggested, depending on the single catabolic pathway studied. However, the involved regulative mechanisms are not known.

The experimental model used in this thesis is styrene degradation in *Pseudomonas fluorescens* ST. The interest of this system concerns the fact that a two-component regulatory system controls the expression of the catabolic genes. This kind of regulation, for genes involved in aromatic hydrocarbon degradation, has been reported only in a few cases, that is toluene degradation in *P. putida* F1 and *Thauera* sp. T1 strains, and biphenyls catabolism in *Rhodococcus* sp. strain M5. As far as styrene catabolism is concerned, only three *Pseudomonas* sp. strains, besides *P. fluorescens* ST, have been studied at molecular level, and found to contain a regulatory system highly homologous to that of ST strain (Velasco et al., 1998; O´Leary et al., 2001; Panke et al., 1998).

Even if the essential role of the two regulative genes, *styS* and *styR*, which encode for the two-component system, has been demonstrated for all the styrene degradative strains, no data is available on the regulation mechanism nor on the modulation of styrene gene expression in different growth conditions.

During my thesis work, my interest was directed towards two main aspects of styrene catabolism control. The first concerned the study and characterization of the expression of structural and regulative *sty* genes in different physiological growth conditions. The second was related to the problem of aromatic hydrocarbons toxicity.

The experimental work performed on the physiological characterization of the *sty* gene cluster expression implied the construction of suitable tools for monitoring gene expression in the natural host. Moreover, for a comprehension of the molecular regulative mechanisms underlying styrene catabolism, it was necessary to perform the characterization of the promoter of the catabolic operon, in order to identify *cis* and *trans*-acting elements important for its activity.

Two different approaches were followed to study the complex problem of aromatic hydrocarbon toxicity. One concerned the identification of genes induced by styrene, but different from the genes involved in its catabolism. The second approach dealt with the activity of glutathione S-transferase in different *Pseudomonas* sp. strains in relation to phenol toxicity.

This enzyme has been often found associated to detoxification processes and its implication in aromatic compounds catabolism has been suggested.

The general structure of this thesis can be briefly summarized as follows:

{part A}

PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF STYRENE GENE CLUSTER

part A-I describes the development of new molecular tools (Gram-negative promoter probe vectors) which display several advantageous features for gene expression studies.

part A-II reports a detailed physiological characterization of the expression of the regulatory and structural genes for styrene degradation

part A-III presents data on molecular analysis of the promoter of the styrene-catabolic operon. From the results obtained a complex picture emerged, in which factors other than the specific positive regulator are involved in the catabolic promoter activation.

{part B}

MOLECULAR RESPONSES TO AROMATIC COMPOUNDS TOXICITY

part B-I deals with the identification and characterization of genes induced by the presence of styrene, which has shown the existence of a cross talk between different degradative pathways.

part B-II describes studies on the presence and possible role of glutathione S-transferases (GSTs) (identified by N-terminal sequencing) in different *Pseudomonas* strains. Results of this work have pointed out to a strict correlation between GST induction and the process of toxic compounds degradation.

Finally, concluding remarks and future perspectives are presented.

{PART A} PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF STYRENE GENE CLUSTER

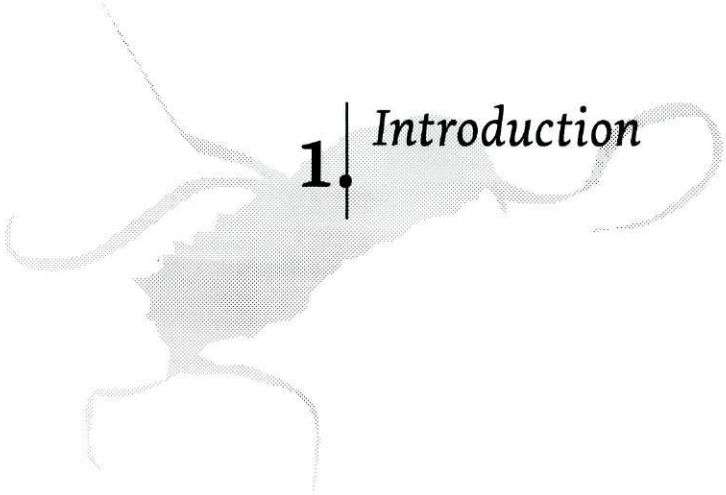
[I]

Development Of A New Family Of Promoter Probe Vectors

{adapted from FEMS Microbiology Letters, 2001. Santos, P.M., I. Di Bartolo, J. M. Blatny, S Valla, and E. Zennaro. New broad-host-range promoter probe vectors based on the plasmid RK2 replicon. 195:91-96}

...There are no such things as applied sciences, only scientific applications...

Louis Pasteur



1. Introduction

The plasmid vector systems available for application of recombinant DNA technology in bacteria have been developed to very sophisticated levels for extensively studied species such as *Escherichia coli*. The majority of these vectors are narrow in their host-range, and in general the systems available for use in most bacterial species have not been developed to a satisfactory degree. Often, one of the main disadvantages of the commonly used broad-host-range systems is the lack of information (or availability) of the DNA sequence, limiting their manipulation.

The broad-host-range RK2 plasmid replicons have been used to develop cloning and expression vectors (Blatny et al., 1997a; 1997b; Winther-Larsen et al., 2000a; 2000b). Major advantages of these tools are that their nucleotide sequences are known, that they are reasonably small, that they contain convenient restriction endonuclease sites for cloning and expression of heterologous genes, that there are means of stabilizing their maintenance, and that a broad-host-range regulated expression system has been incorporated in some of them. Another advantage of the RK2 systems is that it is possible to benefit from the many studies of the molecular biology of these replicons. RK2 replication is initiated by the plasmid-encoded TrfA protein, which binds to the iteron-containing origin of vegetative replication, *oriV* (Perri and Helinsky, 1991; Perri et al., 1991; Pinkey et al., 1988). TrfA also controls plasmid copy number, and numerous point mutations in *trfA* are known to confer different copy numbers to the replicons in which they are present. This can be applied in RK2 vectors, for example for manipulation of expression levels of cloned genes (Blatny et al., 1997b). The copy up mutants also display a similar phenotype in species other than *E. coli*, but the applications are somewhat limited by the unexplained observation that many species appear to tolerate surprisingly low copy numbers of RK2 plasmids (Haugan et al., 1995).

For many studies in microbial molecular microbiology it is of interest to clone promoters from the organism of interest or to analyze their activities by the use of reporter gene systems, most often in the host from which the promoters originated. Such vectors have not been systematically developed for RK2 replicons. Here the construction of such vectors, and the study of their properties in *E. coli* and four other bacterial species is reported.

2. Materials and Methods

2.1. DNA manipulations

The vectors were all made in *E. coli*, and transformations, restrictions, and ligations were carried out by standard procedures (Sambrook et al., 1989). Plasmid DNA was prepared by the alkaline lysis protocol (Sambrook et al., 1989) or with the QIAGEN Midi-isolation kit (Qiagen). DNA fragments were purified from agarose using Qiaquick gel extraction kit or QIAEXII kit (Qiagen). DNA 5' and 3' protruding ends were made blunt by the use of Klenow polymerase and T4 DNA polymerase, respectively. *Nco*I 5' d(pCCCATGGG), *Bgl*II 5' d(pCAGATCTG), *Sac*I 5' d(pCGAGCTCG), and *Xba*I 5' d(pCTCTAGAG) phosphorylated linkers were used for conversion of restriction sites. PCR amplifications were performed with the TaKaRa polymerase kit from TaKaRa or using the *Pfu* polymerase from Stratagene. All PCR fragments were controlled by sequencing, using the DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer) and an Applied Biosystem automated sequencer (model 373 Stretch). Both commercially available and designed primers were used. Further details of the construction procedures are indicated in Table 1.

2.2. Enzyme assays

The promoterless and *Ptrc* promoter-containing constructs were transformed into *E. coli* JM109 and assayed for Luc or ß-galactosidase activity in Luria-Bertani (LB) medium (Miller, 1972) and Mineral salts Medium (Hartmans et al., 1989) containing 0.4 % of glucose and 1 mM thiamine. Cultures to be used for enzyme assays were inoculated at 1 % in LB or 2 % in Mineral Medium from over-night grown cells under the same conditions. The cultures were then incubated further with shaking at 37°C up to an OD600 of around 0.1, after which each of them was split in two (uninduced and induced). For induction from *Ptrc* 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) was added. Aliquots were removed just before adding IPTG (T0), 1 hour after induction (T1) and 3 hours after induction (T3). The Luc assays were performed with a luciferase assay system kit from Promega, and carried out according to the manufacturers instructions. A Packard luminometer was used to measure the activities, given as relative light units (RLU). ß-galactosidase activities were measured as described by Miller (1972) and expressed as Miller units. Plasmids were transferred to *Pseudomonas fluorescens* ST, *Pseudomonas putida* KT2442, *Sphingomonas* sp., and *Burkholderia* sp. LB400 by triparental matings, using *E. coli* HB101(pRK2013) as helper strain and *E. coli* JM109(pPR9TT) or JM109(pPR9TTTrc) as donors. Enzyme assays were performed as for *E. coli*, except that the cells were grown at 30°C in LB medium only. Cells were sampled 3 hours after the OD600 had reached 0.1 (corresponding to T3). The IPTG inducer was not used since there was no *lac* repressor present in these hosts.

2.3. Nucleotide sequence accession numbers

The nucleotide sequences of pJB785TT, pJB785TTKm1 and pPR9TT have been deposited in GenBank under the accession numbers AF187995, AF320510, and AF187996, respectively.

Table 1. Bacterial strains and plasmids

Strains and plasmids	Relevant genotype	Reference or source
Strains		
E. coli HB101	*recA pro leu hsdR* St^r	Boyer et al., 1969
E. coli JM109	*endA1 hsdR17 (rk+ mk+) supE44 thi recA1 gyrA96 relA1* $\lambda^-\Delta$*(lac-proAB) (F' traD36 proAB+ lacI*^{q} *lacZ M15)*	Yanisch-Perron et al., 1985
P. fluorescens ST	Sty^+	Baggi et al., 1983
P. putida KT2442	*hsdMR*, Rif^r	Franklin et al., 1981
Sphingomonas sp.	Soil isolate	Lab collection
Burkholderia sp. strain LB400	Biphenyls degradation	Kumamaru et al., 1998
Plasmids		
pRK2013	RK2-Tra+ RK2-Mob+ Km^r ori ColE1	Figuriski and Helinski ,1979
pJB653	RK2 expression vector; Ap^r; 7.0 kb	Blatny et al., 1997a
pJB781	Deletion of the 1.7 kb *MunI/PstI* region of pJB653, followed by a second deletion of the 1.1 kb *MunI/BbvII* fragment (*MunI* was recovered after the first deletion) such thatthe *Pm/xylS* regulatory system was completely removed; Ap^r; 4.2 kb	This study
pGEM-*luc*	Contains the *luc* gene encoding firefy luciferase; Ap^r; 4.9 kb	Promega
pJB784	The *KpnI* site in pJB781 was converted to an *NcoI* site, and the *luc* gene from pGEM-luc was cloned as a 1.7 kb PCR *NcoI/BamHI* fragment into the same sites of the vector; Ap^r; 6.0 kb	This study
pJB785	The *SacI* site in pJB784 was converted to a *BglII* site; Ap^r; 6.0 kb	This study
pTrc99a	Expression vector; Ap^r; 4.2 kb	Pharmacia
pBluescriptII KS+	ColE1 replicon; Ap^r; 2.9 kb	Stratagene
pBSTrc	pBluescriptII KS+ derivative in which a 461 bp PCR blunt-ended fragment containing the *Ptrc* promoter region from pTrc99A was cloned into the *SmaI* site of the vector; Ap^r; 3.5 kb	
pJB785Trc	pJB785 derivative in which a 444 bp *BamHI/NcoI* fragment containing the *Ptrc* promoter from pBSTrc, was cloned into the *BglII/NcoI*-digested vector ; Ap^r; 6.4 kb	
pBTac1	Expression vector containing strong *rrnB* transcriptional terminators T1T2; Ap^r; 4.6 kb	Boehringer Mannheim
pBSTT2	pBluescriptII KS+ derivative in which a 412 bp PCR blunt-end fragment from pBTac1, containing *rrnBT1T2*, was cloned into the *SmaI* site of the vector; Apr; 3.3 kb	This study
pJB785TT	pJB785 derivative in which a 410 bp *BamHI/BglII* fragment from pBSTT2, containing *rrnBT1T2*, was cloned into the *BglII* site of the vector: Ap^r; 6.4 kb	This study
pJB3Km1	RK2 based cloning vector; Km^r; 5.8 kb	This study
pBSKm	pBluescriptII KS + derivative in which a 1.2 *SalI* fragment from pJB3Km1, containing Km resistance cassette, was cloned into the same site of the vector, Ap^r, Km^r; 4.1 kb	This study
pJB785TTKm1	pJB785TT derivative in which a 1.2 *HincII* fragment from pBSKm, containing Km resistance cassette, was cloned into *HindIII* site made blunt of pJB785TT, Ap^r; Km^r; 7.6 kb	This study
pJB785TTTrc	pJB785TT derivative in which a 444 bp *BamHI-NcoI* fragment containing the *Ptrc* promoter, from pBSTrc, was cloned into the *BglII/NcoI*-digested vector; Apr; 6.8 kb	This study
pHP45ΩCm	Contains an interposon with the Cm^r gene from pKT210; Ap^r; Cm^r; 5.8 kb	Fellay et al., 1987
pJBCm1	pJB785 derivative in which the Cm^r gene from pHP45ΩCm was cloned as a 3.4 kb *HindIII* fragment into the same site of the vector; Ap^r; Cm^rr; 9.4 kb	This study
pUC7Cm	ColE1 replicon, Ap^r; Cm^r; 4.1 kb	Blatny et al., 1997a
pJBCm2	pJB785 derivative in which the Cm^r gene from pUC7Cm was cloned as a 1.4 kb *BamHI* blunt-ended fragment into the *HindIII* site made blunt of pJB785, Ap^r; Cm^r; 7.4 kb	This study
pMC1871	ColE1 replicon; promoterless *lacZ* gene; Tc^r; 7.5 kb	Pharmacia
pBSlacZ	pBluescriptII KS+ derivative in which the promoterless *lacZ* gene from pMC1871 was cloned as a 3.1 kb fragment into the *BamHI* site of the polylinker of the vector; Ap^r; 6.0 kb	This study
pUC18	ColE1 replicon; Ap^r; 2.6 kb	Norrander et al., 1983
pUClacZ	pUC18 derivative in which the *lacZ* gene and polylinker of pBluescriptII KS+ were cloned as a 3.1 kb PCR blunt-ended fragment, from pBSlacZ, into the *SmaI* site of the vector; Ap^r; 5.7 kb	This study
pPR9	pJBCm2 derivative in which the *lacZ* gene from pUClacZ (containing the upstream part of the polylinker of pBluescriptII KS + from *KpnI* to *BamHI*) was cloned as a 3.1 kb *BglII* fragment into the *BamHI/BglII* sites of pJBCm2; Ap^r; Cm^r; 8.9 kb	This study
pPR9Trc	pPR9 derivative in which a 456 bp *HindIII/SmaI* fragment from pBSTrc, containing the *PTrc* promoter, was cloned into the same sites of the vector; Ap^r; Cm^r; 9.3 kb	This study
pPR9TT	pPR9 derivative in which a 410 bp *BamHI/BglII* fragment from pBSTT2, containing the *rrnBT1T2* transcriptional terminator, was cloned into the *BglII* site of pPR9; Ap^r; Cm^r; 9.3 kb	This study
pPR9TTTrc	pPR9TT derivative in which a 456 bp *HindIII/SmaI* fragment from pBSTrc, containing the *Ptrc* promoter, was cloned into the same sites of the vector; Ap^r; Cm^r; 9.7 kb	This study

Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; St^r, streptomycin resistance; Rif^r, rifampicin resistance; Km^r, kanamycin resistance; Sty^+, styrene degradation

3. Results and Discussion

3.1. Construction of promoter probe vectors based on the RK2 replicon

The previously reported expression vector pJB653 (Blatny et al., 1997a) was used as a starting point for the constructions, and further details of the procedures are outlined in Table 1. The *Pm-xylS* promoter system of pJB653 was removed, followed by insertion of the *luc* reporter gene containing an *Nco*I site at the ATG start and conversion of an upstream *Sac*I site to a *Bgl*II site, generating pJB785. This construct can be used for expression studies of promoters with a known sequence upstream of the coding part by inserting the promoter and ribosome-binding site with the ATG of the gene of interest positioned directly into the *Nco*I site of the vector. Initial expression studies of pJB785 showed that it expressed a very low, but significant level of luciferase, even though no upstream promoter had been inserted (data not shown). This was somewhat surprising since all other genes are transcribed in the opposite direction relative to *luc*. Since it could represent a significant problem for studies of weak promoters, the transcriptional terminators *rrnB*T1T2 from pBTac1 were inserted upstream of the cloning sites in pJB785, generating plasmid pJB785TT (Fig. 1).

The selection marker in pJB785TT is Ap^r which is not always convenient for use in some gram-negative bacteria. To eliminate this potential problem a pJB785TT derivative was constructed in which the Km^r resistance marker was inserted, generating plasmid pJB785TTKm1 (Fig. 1). The vector system reported here was used to study expression of the *sty* promoters in *P. fluorescens* ST (Part A-II), and for these purposes the Cm^r marker was introduced, generating plasmid pPR9. This vector is also different from pJB875 in that it is not designed mainly to insert the expression cassettes at the ATG start. Instead of *luc*, *lacZ* was used as the reporter gene in such a way that the vector allows expression of fusion proteins with ß-galactosidase. This means that one may insert promoter regions including the ribosome-binding site and the start ATG plus, if desired, the N-terminal part of the gene controlled by that promoter. The only requirement is that the inserted ATG is positioned such that it becomes in-frame with *lacZ*, generating a fusion protein. This vector can also be used for random promoter cloning, most easily by using the *Bgl*II site which allows cloning of *Sau*3A-generated fragments. Similar to pJB785, pPR9 was found to express low levels of ß-galactosidase in the absence of an upstream inserted promoter, and to eliminate this leakage (as for pJB785) the *rrnB*T1T2 transcriptional terminators were inserted, generating pPR9TT (Fig. 1). Moreover, in both promoter probe vectors systems the reporter gene can easily be replaced by another desired reporter in a single step cloning procedure.

3.2. Analysis of expression properties in *E. coli*

To study the expression properties of pJB785TT and pPR9TT with respect to their reporter genes the *Ptrc* promoter (regulated like *Plac*) was used as a test system, generating plasmids pJB875TTTrc and pPR9TTTrc, respectively. In both cases the insertion of the promoter was made such that the

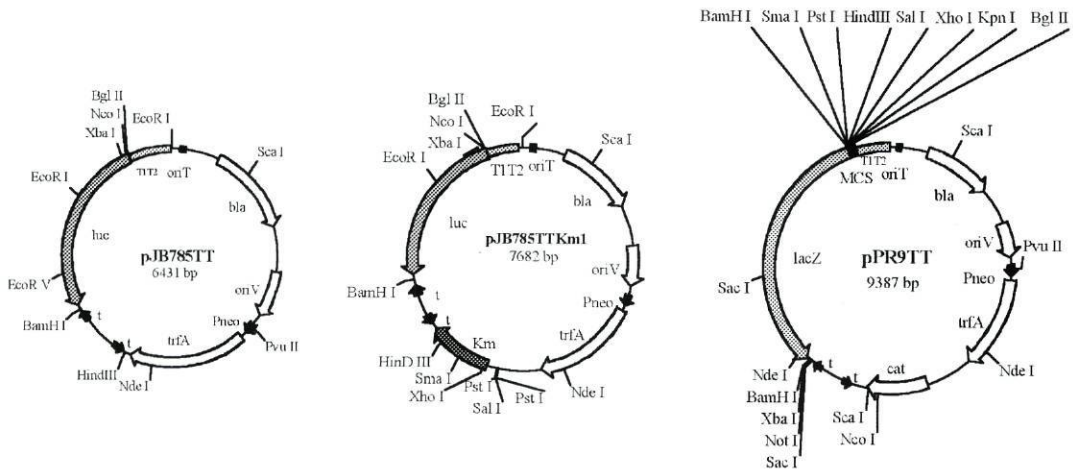


Fig. 1. Construction of broad-host-range promoter probe vectors. *oriV*, origin of vegetative replication; *oriT*, origin of conjugative transfer; *trfA*, gene encoding plasmid replication initiation protein, *bla*, ampicillin resistance gene; *cat*, chloramphenicol resistance gene; *km*, kanamycin resistance gene; *Pneo*, constitutive promoter from which *trfA* is expressed; t, transcriptional terminator; T1T2, transcriptional terminators *rrnB*T1T2. For details regarding the cloning steps, see text and Table1.

site from plasmid pTrc99A served as the translational initiation site, and the fusion protein expressed from pPR9TTTrc contains 7 amino acids added at the N-terminus of the truncated *lacZ* gene obtained from plasmid pMC1871 (see Table 1).

The Luc assays (Table 2) showed that the activity expressed from pJB785TT (lacking *Ptrc*) was very close to zero in both media (LB and Mineral medium) used, and was reduced by one to two log-factors relative to pJB785 (not shown). pJB785TTTrc, on the other hand, expressed high levels of luciferase. In the absence of IPTG the activity per cell mass unit (OD600) increased as growth proceeded. The reasons for this are not completely clear, but was observed that the Luc activities drop as cells enter stationary phase, and the low activities observed at To may therefore be a carry-over effect from the cells used for inoculation. In the presence of IPTG induction very strong stimulatory effects were observed, leading to maximal activities that were up to 36-fold higher than in the absence of inducer. The induction folds were generally higher in Mineral medium than in LB, but quantitatively the expression levels under induced conditions were higher in LB than in Mineral medium.

The ß-galactosidase fusion vector pPR9TTTrc was then subjected to a similar analysis as for the *luc* vector, and the overall expression patterns were found to be quite similar (Table 3). The basal expression levels (pPR9TT) were found to be close to detection level, as for pJB785TT. One difference from the Luc assays is that there was no drastic increase in expression in uninduced cells as growth proceeded. In accordance with the suggested explanation for the corresponding Luc results this may simply mean that ß-galactosidase activity is not affected to the same degree as luciferase by

Table 2. Expression of luc from the *Ptrc* promoter in *E. coli*.

Plasmids	**LB** [1]				**Mineral Medium** [2]			
	To	T1	T3	Induction fold (T1, T3)	To	T1	T3	Induction fold (T1, T3)
pJB785TT	0.0007	0.0006	0.0005		0.0004	0.0012	0.0008	
pJB785TTTrc -	0.24	5.2	8.8		0.43	1.2	3.9	
pJB785TTTrc +	0.25	95.4	100	18-11	0.43	43.3	100	25- 36

1- 100% corresponds to 6.74×10^6 0.21×10^6 RLUs/ OD600 unit

2 - 100% corresponds to $2.08 \times 10^{\ddagger}$ 0.09×10^6 RLUs/ OD600 unit

- without 1 mM IPTG added at To; ‡ with 1 mM IPTG added at To; T1, 1 hour after induction; T3, 3 hours after induction

stationary phase conditions. The induction folds were comparable to those observed for Luc although the maximum induction values were somewhat lower for ß-galactosidase (about 22 fold). Note also that the maximal expression level for ß-galactosidase was higher in Mineral medium than in LB.

Table 3. Expression of *lacZ* from the *Ptrc* promoter in *E. coli*

Plasmids	LB [1]				Mineral Medium [2]			
	To	T1	T3	Induction fold (T1, T3)	To	T1	T3	Induction fold (T1, T3)
pPR9TT	0.1	0.03	0.1		0.03	0.01	0.01	
pPR9TTTrc -	3.6	3.8	6.9		3.6	3.6	4.6	
pPR9TTTrc +	3.8	75	100	14-20	3.4	56	100	16-22

1- 100% corresponds to 6232± 63 Miller Units
2 - 100% corresponds to 7444 ±78 Miller Units
- without 1 mM IPTG added at To; + with 1 mM IPTG added at To; T1, 1 hour after induction; T3, 3 hours after induction.

3.3. Analysis of expression properties in other strains

Since the systems described here were thought for general use in Gram-negative bacteria it was necessary to demonstrate that the properties observed in *E. coli* were maintained in other species. To study this, pPR9TT and pPR9TTTrc were introduced into *P. fluorescens* ST, *P. putida* KT2442, *Sphingomonas* spp., and *Burkholderia* sp. LB400. Background expression was very low also in all these four new species, suggesting that *rrnB*T1T2 is functionally active. It is also clear that these organisms must lack a gene expressing ß-galactosidase, or at least express it at extremely low levels under the conditions used. Since they do not express the *lac* repressor it was irrelevant to use IPTG for induction. *Ptrc* was found to be constitutively expressed at levels similar to (*Burkholderia* sp. LB400) or significantly higher than those observed under induced conditions in *E. coli*. It could therefore be concluded that the vectors appear to function equally well in all five hosts tested (Table 4).

Table 4. Expression of *lacZ* from the *Ptrc* promoter in species other than *E. coli*

Strain	Plasmid [1]	
	pPR9TT	pPR9TTTrc
Pseudomonas fluorescens ST	0.02	92
Pseudomonas putida KT2442	0.02	100
Sphingomonas sp.	0.02	91
Burkholderia sp. strain LB400	0.01	39

1- 100% corresponds to 18378±329 Miller Units.

4. Conclusion

The RK2 replicon promoter probe vectors described here display several important advantageous features that are not all shared by other available promoter-probe systems. So far the usefulness of some of theses properties were demonstrated by analyzing promoters in the *sty* operon of *P. fluorescens* ST (Part A-II), but it seems obvious that, by taking advantage of all the available knowledge on RK2 molecular biology, numerous other applications should be possible.

[II]

Effects Of Different Carbon Sources On The Expression Of The Styrene Gene Cluster

{Adapted from Applied Environmental Microbiology, 2000. Santos, P. M., J. M. Blatny, I. Di Bartolo, S. Valla, and E. Zennaro. Physiological analysis of the expression of the styrene degradation gene cluster in Pseudomonas fluorescens ST. 66:1305-1310}

...The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' ('I found it!'), but rather 'hmm....that's funny...'"

Isaac Asimov

1. Introduction

Strains belonging to the genus *Pseudomonas* able to grow on styrene as sole carbon source have been studied both at the physiological (O'Connor et al., 1995; 1996; 1997) and the molecular level (Beltrametti et al., 1997; Marconi et al., 1996; Panke et al., 1998; Velasco et al., 1998). In these strains the catabolic genes are organized in a cluster, whose expression requires the presence of two genes, styS and styR, organized in an operon and coding for a sensor kinase and a regulatory DNA binding protein, respectively. Two-component regulatory systems for genes involved in aromatic hydrocarbon degradation have only been reported for toluene degradation in *Pseudomonas putida* F1 and *Thauera* T1 (Coschigano and Young, 1997; Lau et al., 1997) and for the degradation of biphenyls in *Rhodococcus* M5 (Labbé et al., 1997).

The strain *Pseudomonas fluorescens* ST, able to grow on styrene as the sole carbon source, has been characterized and both the regulatory genes (*styS* and *styR*) and the upper pathway (*styA*, *styB*, *styC* and *styD*), coding for the conversion of styrene into phenylacetic acid, have been sequenced (see General Introduction). Up to now studies of this catabolic systems dealt only with the molecular aspects of the gene organization. However, to understand the functioning, the limitations and optimal expression conditions of this system, the present work focused on the characterization of the regulatory system and in particular on the effects of different carbon sources on the styrene-induced expression of the regulatory and structural genes. In *Pseudomonas* spp. several examples of carbon catabolite repression on the expression of catabolic pathways for aromatic and non-aromatic compounds have been reported (Holtel et al., 1994; Müller et al., 1996; Yuste et al., 1998). However, none of these studies dealt with catabolic operons regulated by a two-component regulatory system. In this work, the effects of growth on different carbon sources on the expression of the styrene regulatory and degradative operons are reported.

2. Materials and Methods

2.1. Bacterial strains, plasmids, media and chemicals

The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas fluorescens* ST and *Escherichia coli* cells were routinely grown at 30 and 37°C, respectively, in Luria-Bertani (LB) medium (Miller, 1972) or mineral salts medium (Hartmanns et al., 1989) with different carbon sources supplied at the following concentrations: 0.2 % succinate; 0.05 % glucose; 0.1 % lactate; 0.1 % acetate. In induction studies, styrene was added via the gas phase introducing a glass tube containing 30 µl of styrene into the culture flask as previously described (Marconi et al., 1996). When necessary, cultures were supplemented with ampicillin (100 µg/ml), tetracycline (15 µg/ml), or chloramphenicol (30 µg/ml). Isopropyl-ß-D-thiogalactopyranoside (IPTG; 1 mM), 5'bromo-4'-chloro-3'-indolyl-ß-D-galactopyranoside (X-Gal, 1 mM) and 2-nitrophenyl-ß-D-galactopyranoside (ONPG, 1 mM) were added to the media when appropriate.

TABLE 1. Bacterial strains and plasmids

Strains and plasmids	Relevant genotype	Reference or source
Strains		
P. fluorescens ST	Sty$^+$	Baggi et al., 1983
E. coli DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA* (Nal^r) (*lacIZYA-argF*)*U169 deoR*(80*dlac*(*lacZ*)*M15*)	Beth. Res. Laboratories
E. coli S17.1	*recA pro thi hsdR* RP4 -2-Tc::Mu-Km::Tn7 Tra$^+$ Tp^r Str^r	Simon et al., 1983
Plasmids		
pPR9TT	*lacZ* -promoter probe vector RK2 replicon. Ap^r; Cm^r; 8.9 kb	Part A-I
pBluescriptII KS+	ColE1 replicon; Ap^r; 2.9 kb	Strategene
pTZ19R	ColE1 replicon; Ap^r; 2.9 kb	MBI Fermentas
pTE30	pTZ19R derivative containing a chromosomal fragment of *P. fluorescens* ST carrying two truncated genes encoding for *paaK* and *styS*; Ap^r; 6.0 kb	Our laboratory
pBSPs	pBluescriptII KS+ derivative in which a 409 bp PCR blunt-ended fragment containing *PstySR* was cloned into the *Hinc*II site of the vector; Ap^r; 3.3 kb	This study
pPR9Ps	pPR9 derivative in which the 411bp fragment, from pBSPs, containing *PstySR*, was cloned as a *Hin*dIII/*Pst*I fragment into the same sites of the vector; Ap^r; Cm^r; 9.4 kb	This study
pPR9TTPs	pPR9TT derivative in which a 411bp fragment, from pBSPs, containing *PstySR* was cloned as a *Hin*dIII/*Pst*I fragment into the same sites of the vector; Ap^r; Cm^r; 9.7 kb	This study
pTPE30	pTZ19R derivative containing a chromosomal fragment of *P. fluorescens* ST carrying the genes coding for *styR*, *styA* and *styB*; Ap^r; 6.0 kb	Beltrametti et al., 1997
pBSPa	pBluescriptII KS+ derivative in which a 492bp PCR blunt-ended fragment *PstyA* was cloned into the *Hinc*II site of the vector; Ap^r; 3.3 kb	This study
pPR9TTPa	pPR9TT derivative in which a 560bp *Bam*HI/*Xho*I fragment from pBSPa, containing the *PstyA* was cloned into *Bgl*II/*Xho*I sites of the vector; Ap^r; Cm^r; 10.3 kb	This study

2.2. DNA manipulation

Transformations of *E. coli*, restrictions and ligations were carried out by standard procedures (Sambrook et al., 1989). Plasmid DNA was prepared by the alkaline lysis protocol (Sambrook et al., 1989) or with QIAGEN Midi-isolation kit (Qiagen). DNA fragments were purified from agarose using Qiaquick gel extraction kit or QIAEXII kit (Qiagen). PCR amplification of the styrene monooxygenase promoter region, named *PstyA*, from plasmid pTPE30 (Marconi et al., 1996) was performed using the following custom primers: 5' GCTCTAGAATGTCAGATCTCTGGC 3' and 5' GGGGTACCTACGTAGTAGTAGTGG 3' containing a *Xba*I and a *Kpn*I site (underlined), respectively. PCR amplification of the regulatory genes promoter region, named *PstySR*, from plasmid pTE30 was performed using the following custom primers: 5' CAAGCTTGAATGCTTCATGTCGGC 3' and 5' GGAATTCCGATCCAGAATGATCCG 3' containing a *Hind*III and a *Eco*RI site (underlined), respectively. PCR amplifications were performed by standard procedures using, except otherwise specified, *Pfu* polymerase from Stratagene. All PCR fragments were controlled by sequencing with an Applied Biosystem automated sequencer (model 373 Stretch) by DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer). Both commercially available and custom primers were used for sequencing reactions.

2.3. Northern blot analysis

P. fluorescens ST cells were grown on succinate, glucose and styrene to an optical density at 600 nm (OD600) of approximately 0.3. RNA preparation and electrophoresis were performed essentially as described by Leoni et al. (1996). RNA was transferred onto nitrocellulose filters (Optitran BA-S 83, Schleicher & Schuell) as described by Sambrook et al. (1989) and heat fixed. A 1.7 kb *Xho*I-*Bgl*II DNA fragment containing 1100 bp of the *styS* gene and 600 bp of the *styR* gene, was labeled with [α-^{32}P]dATP (3.0 Ci/nmol; Amersham Corp.) by a random priming labeling kit (Boehringer), and purified through a Sephadex G-50 spin column. Filter hybridization and washing were performed following standard procedures (Sambrook et al., 1989).

2.4. Conjugative matings

Plasmids were transferred from *E. coli* S17.1 to *Pseudomonas fluorescens* ST by matings on membranes, and the mixtures were incubated on nutrient-yeast extract broth agar medium (Difco) at 30°C for 14 hours. The mating mixtures were then plated on selective media.

2.5. Induction conditions

In induction assays, *P. fluorescens* ST cells harboring pPR9TTPa, pPR9Ps or pPR9TTPs were pre-grown overnight at 30°C in mineral salts medium supplemented with succinate or lactate or glucose or acetate or with styrene. The styrene-grown cells were transferred to styrene mineral medium, while the succinate or lactate or acetate or glucose grown cells were inoculated into their corresponding mineral medium with or without styrene. Cell growth was measured by monitoring the optical density at 600 nm.

2.6. Styrene monooxygenase assay

To quantify styrene monooxygenase (SMO) activity, indigo production was assayed essentially as described by O'Connor et al. (1997). Cells were harvested in the exponential and stationary phase by centrifugation, washed with 50 mM K-phosphate buffer (pH 7.0), and resuspended in the same buffer to an OD600 of 3.0. A 100 µl volume of concentrated cells was added to 400 µl of 50 mM K-phosphate buffer (pH 7) containing 0.25 mM indole in a 1.5 ml polypropylene tube. The samples were incubated horizontally at 30°C with vigorous shaking for 30 minutes. The samples were then

centrifuged at 14000 rpm for 2 minutes and the supernatants were carefully discarded. The cell pellets were resuspended in 1 ml of DMF (dimethylformamide) by shaking for 15 minutes. The tubes were then centrifuged to remove cell debris, and the optical density of the supernatants was determined at 600 nm. The presented data correspond to the results obtained from, at least, three independent experiments with a deviation ranging from 5 to 10 %.

2.7. **ß-Galactosidase assay.**

ß-Galactosidase activity was measured as described by Miller (1972), and expressed as Miller units. The presented data corresponds to the results obtained from, at least, three independent experiments with a deviation lower than 10 %.

3. Results

3.1. Construction of *PstySR-lacZ* fusions

In order to study the activity of the promoter of the styrene regulatory operon, named *PstySR*, two newly developed promoter probe vectors, pPR9 and pPR9TT, based on RK2 replicon and containing *lacZ* as a reporter gene (Part A-I) were used. The putative promoter region, *PstySR*, was obtained by PCR as described in Materials and Methods. This region is located upstream of the *stySR* genes, encoding a sensor histidine kinase and a response regulator, respectively (Fig. 1).

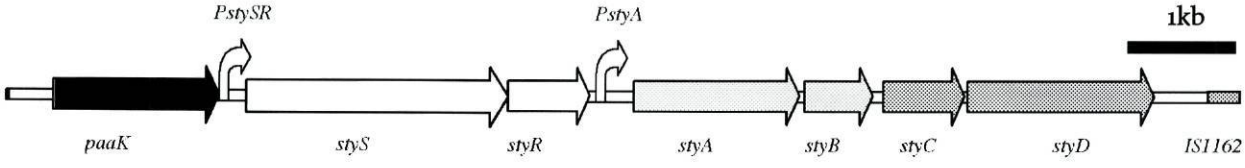


Fig. 1. Regulatory and catabolic operons of the styrene degradation in *P. fluorescens* ST. *styS*, sensor; *styR*, regulator; *styAB*, styrene monooxygenase; *styC*, styrene oxide isomerase; *styD*, phenylacetaldehyde dehydrogenase; *paaK*, phenylacetyl-coenzymeA ligase; *IS1162*, insertion sequence. Bent arrows indicate the promoter regions and the orientation of gene transcription. *PstySR*, promoter of *stySR*; *PstyA*, promoter of *styABCD*.

The 409 bp PCR product included the stop codon of the upstream gene, *paaK* (Marconi et al., 1998; Velasco et al., 1998), the intergenic region containing *PstySR*, and the first 46 codons of *styS*. *PstySR* was cloned in both pPR9 and pPR9TT generating pPR9Ps and pPR9TTPs, respectively (see below). To do this, *PstySR* was first cloned into the *Hinc*II site of pBluescriptII KS (+), generating pBSPs, and then transferred to pPR9 and pPR9TT as a 411 bp *Hind*III/*Pst*I fragment into the *Hind*III and *Pst*I sites of these vectors, in frame with the *lacZ* gene.

3.2. Activity of the *PstySR* promoter under different growth conditions

It has been previously described that a transcription termination-like sequence is present just downstream of the *paaK* stop codon (Velasco et al., 1998). However, the effectiveness of this putative terminator has not been proven. In order to assess the functioning of such a putative terminator *PstySR* was cloned in both pPR9 and pPR9TT. With the purpose to study the activity of the *PstySR* under different growth conditions in a homologous system, pPR9Ps and pPR9TTPs plasmids were transferred into strain ST, which contains the *stySR* and *styABCD* operons (Fig. 1) in the chromosome. The ß-galactosidase activity of strain ST cells harboring pPR9Ps was not dependent on the presence of styrene and was not influenced by additional carbon sources in any of the growth phases analyzed. ß-galactosidase activity of *P. fluorescens* ST (pPR9Ps) cells grown on succinate, succinate plus styrene and styrene is reported in Fig. 2.

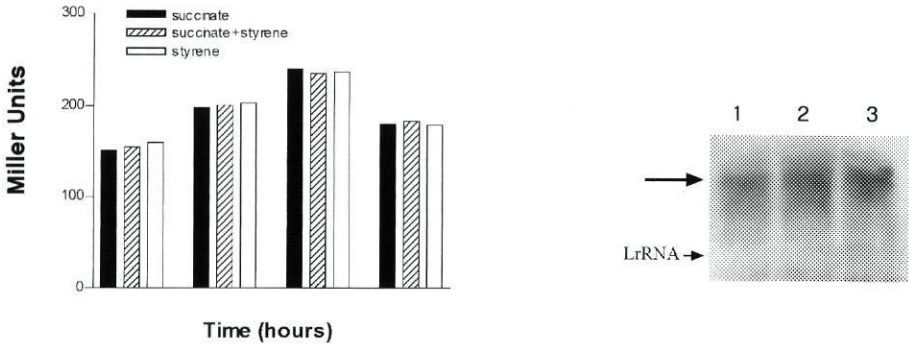


Fig. 2. Activity of the *PstySR* promoter under induced and uninduced conditions. The graph shows ß-galactosidase activity of *P. fluorescens* ST (pPR9Ps), measured at the indicated times of the growth on 0.2 % succinate, 0.2 % succinate and styrene, and styrene. The gel shows *stySR* expression as determined by Northern blot analysis. *P. fluorescens* ST cells were grown on glucose (lane 1), succinate (lane 2) and styrene (lane 3) to an OD600 of 0.3. Experimental procedures for RNA preparation and detection are described in Material and Methods. Each lane contained approximately 20 µg of total RNA (relative amount of transcripts has been confirmed by observation of EtBr dying obtained prior to blotting). The probe was a 1.7 Kb DNA fragment containing both *styS* and *styR* sequences. LrRNA, large rRNA. The arrow indicates the position of the *stySR* transcript.

Similar results were obtained on glucose, glutamate or in LB (data not shown). To confirm that these results were not due to a reading through from the vector because of the inefficiency of the putative terminator located upstream of *PstySR* , the same experiments were performed with pPR9TTPs.

The results obtained with this vector were identical to the ones obtained with pPR9Ps indicating the effectiveness of the natural terminator. However, since RK2-based vectors such as pPR9 and pPR9TT occur at 5-7 copies per chromosome (Blatny et al., 1997a), the possibility that the multicopy presence of *PstySR* could result in this apparent constitutive expression of *stySR* genes was checked. Therefore, the transcripts of *stySR* genes in *P. fluorescens* ST grown on succinate, glucose or styrene were analyzed. The results, reported in Fig. 2, show that the *stySR* transcript is present comparable amounts in the growth conditions examined, confirming the data obtained with the ß-galactosidase assay.

3.3. **Construction of** *PstyA-lacZ* **fusion**

The *PstyA* promoter (Fig. 1) is induced in the presence of styrene and is responsible for the expression of the styrene catabolic operon (Beltrametti et al., 1997, Marconi et al., 1997; Panke et al., 1998; Velasco et al., 1998). Sequence analysis of the DNA region upstream of *styA* has shown the presence of an inverted repeat located 75 bp upstream of the start codon, containing a sequence identical to the *tod-box* involved in toluene utilization in *Pseudomonas putida* F1 (Lau et al., 1997). This box has been demonstrated to be the DNA binding site of TodT, which belongs to a two component regulatory system highly homologous to StyS/StyR. To study the activity of *PstyA* a 492 bp PCR fragment including 23 C-terminal codons of the upstream *styR*, the intergenic region containing *PstyA* and the first 81 codons of the *styA* gene was cloned into the *Hinc*II site of pBluescriptII KS(+), generating pBSPa. pPR9TTPa was then constructed by cloning the 560 bp *Bam*HI/*Xho*I fragment from pBSPa into the *Bgl*II/*Xho*I sites of pPR9TT, in frame with the *lacZ* gene.

3.4. **Effect of different carbon sources on** *PstyA* **activity**

pPR9TTPa was transferred by conjugation into *P. fluorescens* ST, and cells were grown on mineral medium containing styrene as sole carbon source (Fig. 3A) and on mineral medium supplemented with different carbon sources in the presence or absence of styrene (Fig. 4A), as described in Materials and Methods. Samples were harvested at different times of the exponential and stationary phase and

into indoxyl, which spontaneously dimerizes to the blue dye indigo. Indigo formation has been extensively used to select microorganisms expressing dioxygenase or monooxygenase activities (Beltrametti et al., 1997; Eaton and Chapman, 1995). In a previous work it was demonstrated that *E. coli* expressing a DNA fragment containing *styAB*, formed indigo from indole, and styrene oxide from styrene, indicating that the two activities are the results of the same enzyme (Beltrametti et al., 1997). In this way it was possible to directly measure, the activity of the *styAB* gene product at the same time as the expression from the cloned *PstyA*.

The results obtained showed that the indigo formation was induced only in the presence of styrene (Fig. 3B and Fig. 4B). When cells were grown on organic acids or carbohydrates neither indigo formation, nor ß-galactosidase activity were detectable (Fig. 4B). When styrene was used as the sole carbon source, the indigo formation and ß-galactosidase activity could be detected in the early exponential growth phase (Fig. 3B). However, cells grown on succinate or lactate and styrene started to accumulate indigo, as well to show ß-galactosidase activity, only in during mid-exponential growth phase (Fig. 4B). This suggests that cells started to grow utilizing succinate or lactate and that the shift in substrate utilization from these organic acids to styrene occurred before the depletion of the preferred carbon sources.

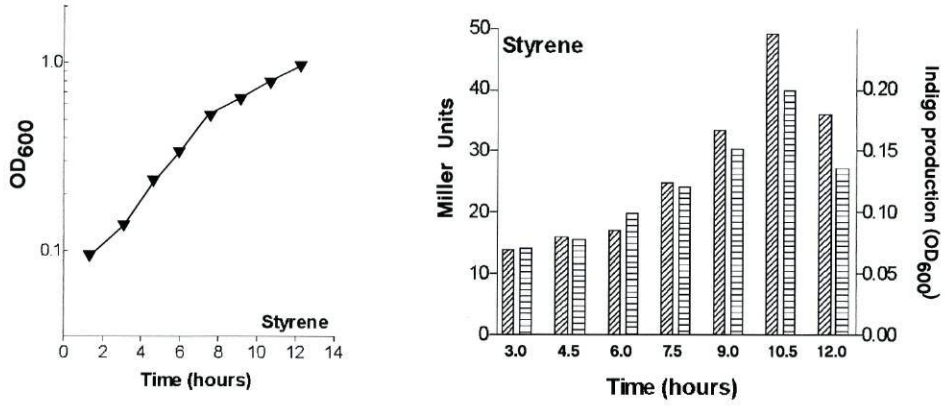


Fig. 3. Activity of the *PstyA* promoter under induced conditions. A. Growth curve of *P. fluorescens* ST harboring pPR9TTPa when styrene was the sole carbon source. B. Determination of ß-galactosidase activity (black bars) and indigo production (diagonally cross-hatched bars) at the indicated times of the growth.

The fact that, during early exponential growth phase, these organic acids exert a repression on *PstyA* induction, is confirmed by the finding that the two enzymatic activities considered could be easily detected in the early phase of the growth when glycerol was the added carbon source (Appendix 2). The influence of succinate and lactate in a concentration range from 0.05 to 0.4 % was tested. Thereby, no diauxic growth curve pattern was observed indicating that there was no mutual exclusion between the two substrates and that a threshold concentration for succinate and lactate probably exists, below which the induction of the styrene catabolic genes begins.

It has been reported that in *Pseudomonas lemoignei* the uptake of succinate is dependent on pH (Terpe et al., 1999), with an optimum at a pH range between 5.6 to 7.0. Experiments with *P. fluorescens* ST cells grown in pH 6.0 buffered mineral medium supplemented with succinate and styrene were performed, and it was found that the styrene catabolic operon was only expressed at the end of the exponential phase (Appendix 3). This higher repression level could be a consequence of an higher concentration of succinate inside the cells or to an higher activity of its transporter system. However, a diauxic growth was not obtained even at higher concentrations of succinate.

Cells grown on glucose or acetate and styrene started to accumulate indigo, as well as to show ß-galactosidase activity only after the end of exponential growth phase (Fig. 5), indicating that these carbon sources do impose a high level of catabolite repression on the expression of the styrene degradative operon.

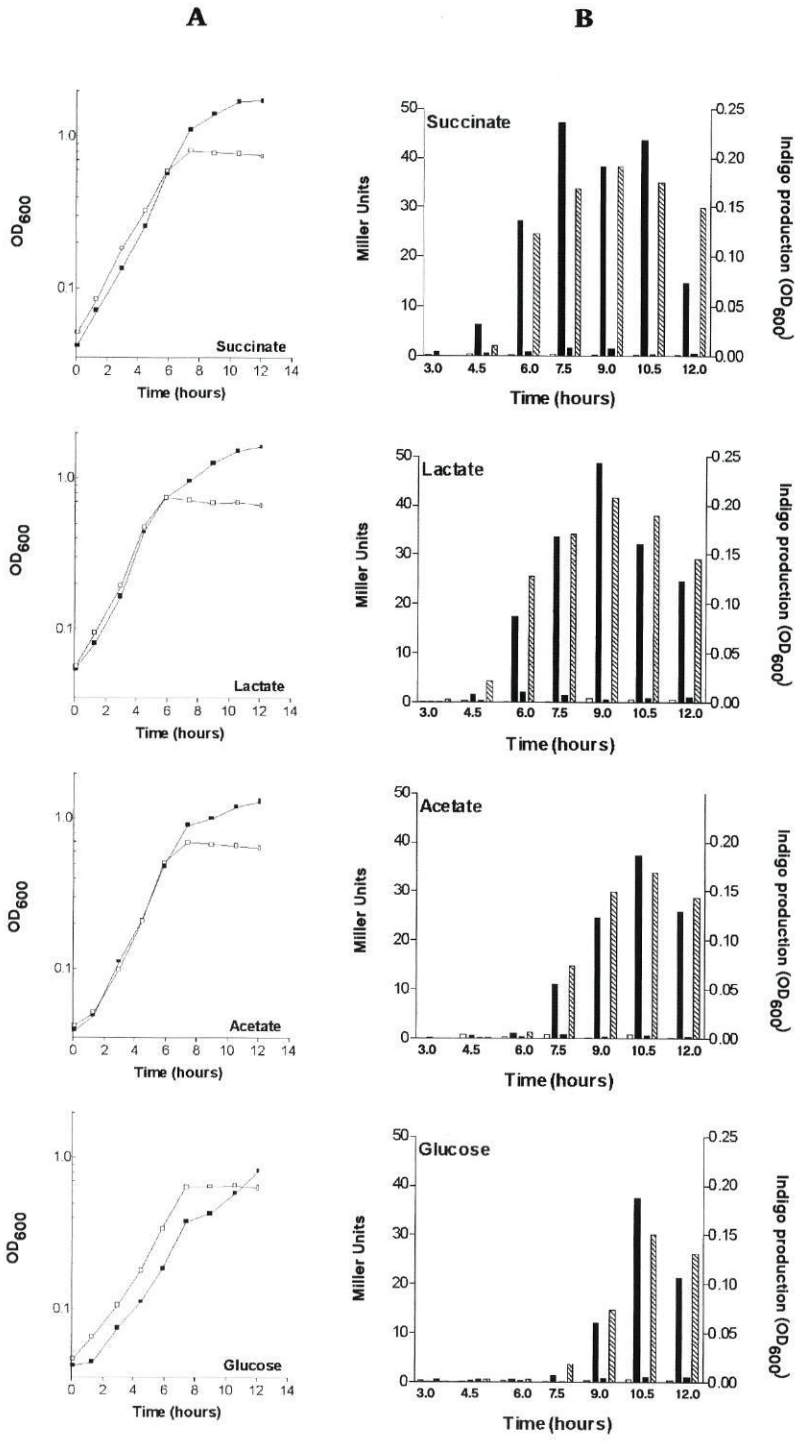


Fig. 4. Effect of different carbon sources on the *PstyA* promoter activity. (**A.**) *P. fluorescens* ST cells harboring pPR9TTPa were grown on the indicated carbon sources in the presence (solid squares) or in the absence (open squares) of styrene. (**B**) ß-galactosidase activity in the presence (solid bars) or in the absence (open bars) of styrene and indigo production in the presence (cross-hatched bars) or in the absence (stippled bars) of styrene at different times.

Furthermore, the influence of the carbon source concentration in a range of concentrations from 0.05 to 0.4 % was checked and was it observed that an increase in concentration lead to an increase in the time necessary for the shift to styrene utilization. This resulted in a prolonged second lag of the diauxic growth (Appendix 4). Among other tested substrates it was found that arginine and glycerol did not affect the *PstyA* induction, while glutamate and citrate imposed a strong repression on the *PstyA* induction (Appendix 2), as described for glucose and acetate. Finally, all assays performed in LB medium in the presence or in the absence of styrene showed that neither ß-galactosidase nor SMO are expressed in this medium. A similar repressive effect of LB medium has already been described for the majority of the aromatic or aliphatic catabolic operons up to now studied (Holtel et al., 1994; O'Connor et al. 1995; Yuste et al., 1998).

4. Discussion

The results have shown that *PstyA* expression is induced by styrene. In the presence of an additional carbon source, such as organic acids or carbohydrates, the induction by styrene was affected to different extents depending on the nature of the carbon source and on its concentration. It is known that organic acids are usually the preferred carbon sources in *Pseudomonas* spp. (Collier et al., 1996), but the mechanism of catabolite repression is not yet understood in these microorganisms. The data presented here confirm the results obtained on the SMO activity in *Pseudomonas putida* CA-3, and support the hypothesis that also in this strain catabolite repression can occur at transcriptional level (O'Connor et al., 1997).

Results obtained with pPR9Ps and with the transcript analysis showed that the expression of the *PstySR* promoter is constitutive and independent of the nature of the carbon sources. *PstySR* is the promoter of the operon coding for the two-component regulatory system, the sensor (*styS*) and the regulator (*styR*), which are necessary for *PstyA* induction (Panke et al., 1998; Velasco et al., 1998). If no control at the translation level is present, StyS and StyR are constitutively present in the cell. This suggests that some steps of the signal transduction from styrene to the *PstyA* activation is controlled by catabolite repression. The factor imposing catabolite repression can affect the kinase activity of the sensor, or inhibit the phosphorylation of the regulator or its binding to the promoter, or can directly bind to a specific sequence in the repressible promoter. However, the analysis of different promoters of aromatic and alifatic degradative operons does not show common sequences which can be the binding site for a common repressor.

In *Pseudomonas*, the presence of an organic solvent in the medium triggers a stress response which induces an overall re-adjustment of the cells through the activation of defense mechanisms, including adaptation to the solvent (Heipieper et al., 1994; Heipieper et al., 1996; for a review see Isken and de Bont, 1998). Many of these defense mechanisms are energy dependent so that growing cells in the presence of styrene will lead to an increased requirement of energy. This demand for extra energy is preferably obtained using a readily utilizable carbon source, rather than the organic solvent, whose utilization requires many steps to obtain an energy-yielding intermediate.

Finding of a two-component regulatory system is not common in the degradation of aromatic compounds. Such a system is usually associated with complex metabolic responses to environmental changes, such as nitrogen fixation, alginate production, nodulation process, virulence, or with stress response (Parkinson and Kofoid, 1992; Zhulin et al., 1997). It is possible that cells sense styrene as a stressing factor or that the styrene catabolism needs a fine regulation linked to the redox status of the cell, due to the toxicity of its catabolic intermediates styrene oxide and phenylacetaldehyde.

To my knowledge, it is not known if this kind of regulation is also associated to catabolite repression but it is possible to look at this process as a response to a specific energetic state of the cells. Recently, the involvement of IIANt^r, a protein of the PTS-like transport system, on carbon

of the σ^{54}-dependent *Pu* promoter has been reported. This protein seems to play a role in the connection between some σ^{54}-dependent promoters and nitrogen and carbon metabolism (Cases et al., 1999). This finding is in agreement with the picture emerging from the study of catabolite repression of aromatics and aliphatic degradative operons in *Pseudomonas* (Holtel et al., 1994; O'Connor et al. 1995; Yuste et al., 1998), which seems to indicate that it is a more general mechanism related to the cell metabolic state, since the same carbon source has different repressive effects depending on strain and growth conditions.

[III]

Regulation Of Styrene Gene Cluster

{Adapted from Research in Microbiology, 2002. Santos, P. M., L. Leoni, I. Di Bartolo, and E. Zennaro. Integration host factor is essential for optimal expression of styABCD operon in Pseudomonas fluorescens ST. Submitted}

...If A equals success, then the formula is: A = X+Y+Z. X is work. Y is play. Z is keep your mouth shut....

ALBERT EINSTEIN

1. Introduction

In *Pseudomonas* strains, the styrene degradation cluster has been extensively studied both at physiological (O'Connor et al., 1995; 1996; 1997; Panke et al., 1998; 1999) and molecular level (Beltrametti et al., 1997; Marconi et al., 1996; Panke et al., 1998; Velasco et al., 1998, O'Leary et al., 2001). Interestingly, in all the studied strains, the styrene gene cluster is highly homologous and organized in a similar way (Marconi et al., 1998; Panke et al., 1998; Velasco et al., 1998, O'Leary et al., 2001) suggesting that all these strains received this genetic trait by horizontal transfer from a common ancestral bacterium. Up to now little attention has been given to the regulatory mechanisms that govern the expression of this cluster. The regulatory-associated operon is located immediately upstream of the catabolic one and is constituted by two co-transcribed genes, *styS* and *styR*, coding for a sensor kinase (StyS) and a regulatory DNA-binding protein (StyR), respectively. The involvement of a two-component regulatory system is not common in the degradation of aromatic compounds. Such a system is usually associated with complex metabolic responses to environmental changes, such as nitrogen fixation, alginate production, nodulation process, virulence, or with stress response (Parkinson and Kofoid, 1992; Zhulin et al., 1997). Two-component regulatory systems for genes involved in aromatic hydrocarbon degradation have only been reported for toluene degradation in *Pseudomonas putida* F1 and *Thauera* T1 (Lau et al., 1997; Coschigano and Young, 1997) and for the degradation of biphenyls in Rhodococcus M5 (Labbé et al., 1997) (see chapter 3 of the General Introduction for more detailed information on this subject).

Different mechanisms of transcription control by regulator phosphorylation have been described (Parkinson and Kofoid, 1992, Parkinson, 1993; Pao and Saier, 1995). Phosphorylation can promote binding to DNA, or activation of an already bound regulator, as well as activation by dimerization. Finally, these processes can either activate or repress transcription (Parkinson and Kofoid, 1992).

Northern blot analysis of *styS/styR* transcripts has shown that the two genes are co-transcribed and constitutively expressed (Part A-II). The styrene sensor belongs to the class of hybrid kinases, containing duplicate histidine kinase domains, two input domains, and an intrinsic receiver domain (Velasco et al., 1998; Marconi et al., 1998). How the functions of these domains cooperate in the regulation of styrene catabolic genes has not yet been established.

Up to now, the only molecular analysis performed on the promoter of the *sty* catabolic operon (*styABCD*) concerns the identification of a palindromic sequence (named *sty box*) that could constitute the putative binding site of StyR. In fact, the same box is present in the promoters of all the studied styrene catabolic operons, as well as in the promoter of toluene catabolic operon in *P. putida* F1. In this last case, it was demonstrated that this box is the binding site of TodT, a positive regulator which shares 49 % identity with StyR (Lau et al., 1997; see General introduction, chapter 4). However,

the molecular mechanisms by which these promoters are regulated and binding properties of the corresponding regulators are not know. Moreover, the complexity of the hybrid histidine kinase StyS and the demonstrated different levels of *styABCD* genes expression in different growth conditions (Part A-II), indicate that *styABCD* genes are subjected to a very strict control, in which probably other factors are involved.

In this chapter, the characterization of this regulative system was analyzed following different approaches. In the first part, the work focused on the expression and binding capability of the regulator protein, StyR. The second part, concerns the identification of DNA sub-regions within the promoter of catabolic operon (*PstyA*) which constitute the target sites for protein interaction.

2. Materials and Methods

2.1. Bacterial strains, plasmids, media and chemicals

The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* and *Escherichia coli* cells were routinely grown at 30 and 37°C, respectively, in Luria-Bertani (LB) medium (Miller, 1972) or mineral salts medium (Hartmanns et al., 1989) with 0.1 % lactate. In induction studies styrene was added via the gas phase as previously described (Marconi et al., 1996). When necessary, cultures were supplemented with ampicillin (100 μg/ml), tetracycline (20 μg/ml), kanamycin (50 μg/ml) or chloramphenicol (30 to 200 μg/ml). Isopropyl-ß-D-thiogalactopyranoside (IPTG; 1 mM), 5'bromo-4'-chloro-3'-indolyl-ß-D-galactopyranoside (X-Gal, 1 mM), *m*-toluate (5 mM) and 2-nitrophenyl-ß-D-galactopyranoside (ONPG, 1 mM) were added to the media when appropriate.

2.2. DNA manipulation

Transformations of *E. coli*, restrictions and ligations were carried out by standard procedures (Sambrook et al., 1989). Plasmid DNA was prepared by the alkaline lysis protocol (Sambrook et al., 1989) or with QIAGEN Midi-isolation kit (Qiagen). DNA fragments were purified from agarose using Qiaquick gel extraction kit or QIAEXII kit (Qiagen). To clone *styR* into pQE60Flag, PCR amplification of a 620 bp DNA fragment containing the StyR coding sequence from pTE50 (Beltrametti et al., 1997) was performed by using the following custom primers: FwStyR (5' TGCCATGGCCGCAAAGCCCAC 3') and RwStyR (5' CGGGATCCAACTCACCCCCCT 3') containing a *Nco*I and a *Bam*HI site (underlined), respectively. With respect to the wild-type coding region, the amplified fragment contained two codon changes: the first due to the introduction of the *Nco*I site (second codon Thr Ala); the second to the fusion of StyR protein with Flag octapeptide (stop codon Trp). This fragment was first cloned into pBluescriptII KS+, for sequence control, generating pBStyR1 and then cloned into pQE60Flag (for details see Table 1).

PCR amplification of a 201 bp DNA fragment containing the catabolic genes promoter region (*PstyA*) was performed using the following custom primers: 5' CGGAATTCCGTTGACTGCTTCGGG 3' and 5' CGGAATTCCAACAATACCGATACG 3' both containing a *Eco*RI site (underlined). All PCR amplifications were performed by standard procedures using *Pfu* polymerase from Stratagene. PCR fragments were controlled by sequencing with an Applied Biosystem automated sequencer (model 373 Stretch) and a DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer).

2.3. Conjugative matings

Plasmids were transferred from *E. coli* S17.1 to *Pseudomonas* cells by matings on membranes, and the mixtures were incubated on nutrient-yeast extract broth agar medium (Difco) at 30°C for 14 hours. The mating mixtures were then plated on selective media.

Table 1. Bacterial strains and plasmids

Strains and plasmids	Relevant genotype	Reference or source
Strains		
P. fluorescens ST	Sty$^+$	Baggi et al., 1983
P. putida KT2442	*hdsR*; Rif$^+$ derivative of KT2440	Franklin et al., 1981
P. putida A8759	KT2442 *ihfA*::Km^r	Calb et al., 1996
P. putida RT31	A8759 *ihfA* and *ihfB* under the control of *Ptac* promoter and *lacIq* repressor; Rif^r, St^r, Tc^r, Km^r	Teras et al., 2000
E. coli DH5	*endA1 hsdR17 supE44 thi-1 recA1 gyrA (Nal^r) (lacIZYA-argF)U169 deoR (80dlac(lacZ)M15)*	Bethesda Res. Laboratories
E. coli S17.1	*recA pro thi hsdR* RP4 -2-Tc::Mu-Km::Tn7 Tra+ Tpr Str^r	Simon et al., 1983
E. coli M15	*lacI^q ara-14 galK2 mtl-1* F-	Stuber et al., 1990
Plasmids		
pBluescriptII KS+	Cloning vector; Ap^r; 2.9 kb	Strategene
pQE6	Expression vector under the control of *PT5 lacO'* promoter-operator element, Ap^r; 5.9 kb	Qiagen
pDMI,1	Repressor plasmid; *lacIq* ; Km^r	Certa et al., 1986
pQE60Flag	pQE60 derivative in which 6-His epitope was replaced by Flag- octapeptide; Ap^r; 5.0 kb	Our laboratory
pTE50	pTZ19R derivative containing a chromosomal fragment of *Pseudomonas fluorescens* ST carrying the genes coding for *styR*, *styA* and *styB*; Ap^r; 6.0 kb	Beltrametti et al., 1997
pBStyR1	pBluescriptII KS+ derivative in which a 621bp PCR blunt-ended fragment containing *styR* was cloned into the *Hinc*II site of the vector; Ap^r; 3.5 kb	This study
pQStyR	pQE60Flag derivative in which a 620 bp *Nco*I-*Bam*HI fragment from pBStyR1 containing *styR* was cloned in the same sites of the vector; Ap^r; 5.6 kb	This study
pBSPa200	pBluescriptII KS+ derivative in which a 201 bp PCR fragment digested with *Eco*RI containing *PstyA* was cloned into the *Eco*RI site of the vector; Ap^r; 3.2 kb	This study
pBSPa2	pBluescriptII KS+ derivative in which a 162 bp PCR blunt endfragment a containing *PstyA* portion was cloned into the *Hinc*II site of the vector; Ap^r; 3.1 kb	This study
pBSPa3	pBluescriptII KS+ derivative in which a 146 bp PCR blunt end fragment a containing *PstyA* portion was cloned into the *Hinc*II site of the vector; Ap^r; 3.1 kb	This study
pBSPa4	pBluescriptII KS+ derivative in which a 127 bp PCR blunt end fragment a containing *PstyA* portion was cloned into the *Hinc*II site of the vector; Ap^r; 3.1 kb	This study
pBSPa6	pBluescriptII KS+ derivative in which a 108 bp PCR blunt end fragment a containing *PstyA* portion was cloned into the *Hinc*II site of the vector; Ap^r; 3.1 kb	This study
pPR9TT	*lacZ* promoter probe vector; Ap^r; Cm^r; 9.3 kb	Part A-I
pPR9TTPa0	*Pa0::lacZ* promoter probe vector; Ap^r; Cm^r; 9.5 kb	This study
pPR9TTPa2	*Pa2::lacZ* promoter probe vector; Ap^r; Cm^r; 9.5 kb	This study
pPR9TTPa3	*Pa3::lacZ* promoter probe vector; Ap^r; Cm^r; 9.5 kb	This study
pPR9TTPa4	*Pa4::lacZ* promoter probe vector; Ap^r; Cm^r; 9.5 kb	This study
pPR9TTPa6	*Pa6::lacZ* promoter probe vector; Ap^r; Cm^r; 9.5 kb	This study

2.4. Expression and purification of StyR-Flag

An overnight culture of *E. coli* M15 harboring pDMI,1 and pQstyR in TSY (tryptic soy broth, DIFCO) medium was diluted 100 fold and grown at 37°C up to an OD600 of 0.5, after which 1mM IPTG was added and growth continued for further 3 hours before collecting the cells.

Cells, from 400 ml cultures, were harvested by centrifugation (7,000 g for 10 min. at 4°C), resuspended in 40 ml of buffer A (50 mM TrisHCl, pH 8.0, 0.5 mM EDTA) containing 0.5 mg/ml lysozyme and incubated for 30 min. at 37°C. 4 ml of buffer B (0.1 M $CaCl_2$, 1.5 M NaCl, 0.1 M $MgCl_2$) containing 40 U/ml DNAse I were added to the suspension and incubated for further 20 min. at 37°C.

The resulting cell lysate was centrifuged for 1h at 17,000 g. The insoluble cell fraction was recovered and incubated for 12 h in detergent mix (20 mM TrisHCl pH 7.5, 0.2 NaCl, 1 % Na deoxycolate, 2 mM

EDTA) with stirring and then centrifuged for 40 min. at 17,000 g. The obtained pellet was resuspended in 4 % B-Per reagent (Pierce) and re-centrifuged at 17,000 g. The insoluble cell fraction was treated with different concentrations of Triton X100 (0.5 to 5 %) in 1mM EDTA with stirring, resuspended in wash buffer (100 mM TrisHCl pH 7.0, 2 M Urea, 2 % Triton X100, 1 mM EDTA) and centrifuged. Purified inclusion bodies originated from this procedure were resuspended in extraction buffer (5 mM TrisHCl pH 7.0, 5 mM EDTA, 8 mM guanidine-HCl, 5 mM DTT) and incubated at 37°C for 14 h with stirring. Upon centrifugation at 100,000 g for 1h the supernatant was recovered and dialyzed against MilliQ ultra-pure water (Millipore) and concentrated with an Amicon YM10 device. The sample, containing 90 % of StyR-Flag was loaded on a SDS-Page and electro-eluted generating a 100 % pure StyR-Flag sample. It is important to notice that all the purification steps were followed by Western-blotting analysis (see below) using anti-Flag (Kodak). Amino terminal sequence of StyR-Flag purified protein was confirmed by sequencing (see below).

2.5. **Polyclonal antibodies production**

Balb/C mice were immunized with 20 μg of StyR-Flag protein in 100 μl of complete Freund's adjuvant and after two weeks a second boost, with 10 μg of StyR-Flag in incomplete Freund's adjuvant, was made. Four weeks later, the mice were sacrificed and the serum was collected.

2.6. **Induction conditions**

In induction assays, *P. fluorescens* ST cells harboring different promoter fusions, were pre-grown overnight at 30°C in mineral salts medium supplemented with lactate or with styrene. The styrene-grown cells were transferred mineral medium containing styrene as sole C-source, while the lactate grown cells were inoculated into their corresponding mineral medium with or without styrene. Cell growth was measured by monitoring the optical density at 600 nm. Cell samples were collected at OD600 of 0.5 and of 1.0.

2.7. *Pseudomonas* **protein crude extracts preparation**

Cells cultivated in the different conditions were harvested by centrifugation (7,000 g for 20 min. at 4°C), resuspended in sonication buffer (50 mM TrisHCl pH 7.5, 100 mM KCl, 1 mM EDTA, 10 % glycerol), and disrupted by sonication (6 cycles of 15 sec. burst with 15 sec. of interval) in ice. The particulate material was removed by centrifugation at 17,000 g at 0°C. Protein crude extracts were aliquoted and stored at -80°C until their use.

2.8. **Protein electrophoresis**

SDS-PAGEs was performed in 12.5 % polyacrylamide according to the method described by Laemmli (1970).

2.9. **Western Blotting analysis**

Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters and analyzed by Western blot using mouse anti-StyR or anti-Flag M2 monoclonal antibodies (Kodak), anti-mouse IgG alkaline phosphatase conjugate (Promega) and a chromogenic substrate (BCIP/NBT; Boehringer Manheim) for visualization of the StyR bands. Gels were scanned using Fluor-S Imaging system (Biorad) and relative readings of each band were obtained.

2.10. **ß-Galactosidase assay**

ß-Galactosidase activity was measured as described by Miller (1972), and expressed as Miller units. The presented data corresponds to the results obtained from, at least, three independent experiments with a deviation not higher than 10 %.

2.11. Gel mobility shift assay

Proteins were prepared as referred above, quantified and normalized (using sonication buffer) to a crude protein extract concentration of 10 mg/ml (re-confirmed by SDS-PAGE). In order to generate the DNA probe, a 201 bp DNA fragment containing *PstyA* was obtained by *Eco*RI digestion of pBSPa200. This fragment was labeled with [α-^{32}P]dATP by fill-in with Klenow enzyme. The radiolabeled DNA fragment was purified by gel filtration with a sepharose G50 spin column followed by phenol:chloroform extraction and ethanol precipitation. Upon resuspension in bi-distilled water the DNA probe was controlled for purity. Promoter fragments with different 5' deletions, used as competitors in mobility shift experiments, were generated as indicated in Table 2.

Table 2. Primers used for 5' deletions of *PstyA* promoter, generated fragments and amplified regions

Primer [a]	**Name of generated fragment**	*PstyA* **amplified region**[b]
5' CGGAATTCCGTTGACTGCTTCGGG 3'	*Pa0*	-174 to 1
5' GCTCTAGAGTTTATGATTTTTAATATATA 3'	*Pa2*	-132 to 1
5' GCTCTAGACTATTTTTAAGGATATTTTT 3'	*Pa3*	-110 to 1
5' GCTCTAGATATACCGCATAAACCAC 3'	*Pa4*	-91 to 1
5' GCTCTAGATTTATTTCCTTTTTTGCTG 3'	*Pa6*	-72 to 1

a-all primers were paired with the primer 5' GGGGTACCTACGTAGTAGTAGTGG 3' to generate the different DNA fragments
b-in nucleotides, relatively to ATG of *PstyA* promoter

The fragments obtained were also cloned into a promoter-probe system, pPR9TT (Table 1). The (5'-GAATTCATTTTAAGTCTCGTATAGCTAGC-3') and (5'-GAATTCATCAAATCGACAGGTGGTTATGC -3') primers were used for amplification of a 190 bp DNA fragment containing the integration host factor (IHF) binding site upstream of the *Pu* promoter of *xyl* genes in TOL plasmid The binding reactions were carried out in a volume of 20 µl. DNA probes (2,000 cpm) were incubated at 4°C for 30 min. with different protein crude extracts in binding buffer (10 mM TrisHCl pH 8.0, 50 mM NaCl, 2.5 mM MgCl2, 0.025 % NP-40, 125 µg/ml sonicated salmon sperm DNA, 5 % glycerol). It is important to notice that the binding buffer was determined upon binding assays performed in several salt and pH conditions (reported in the Results section). When non-labeled DNA competitors were used, protein crude extracts were added last to the binding mixture. After incubation, the reaction mixtures were loaded (10 µl) onto a 30 min. pre-run 6 % continuous nondenaturing polyacrylamide gel. The electophoresis was carried out at 4°C in 0.5 Tris-borate-EDTA buffer (pH 8.0) at 10V/cm for 2.5 h. After this, gels were dried and exposed.

2.12. South-Western experiments

Two identical samples of protein crude extracts (30 µg) from cells grown in different conditions were specularly loaded on SDS-PAGE, and transferred into nitrocellulose filters. These filters were divided in two equal parts in order to perform Western blotting (as described above) and DNA probe hybridization. It is important to refer that each part of the filter contained equal extract conditions. Filters for DNA probe hybridization were incubated in renaturation buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM DTT, 2.5 % NP-40, 2 % BSA, 10 % glycerol) for 16 hours at 4°C with gentle shaking. After renaturation, filters were washed with binding buffer (same used in gel mobility shift assays) and incubated in the binding buffer containing 10 mg/ml salmon sperm dsDNA and α^{32}P-labeled *PstyA* DNA probe (100,000 cpm/ml), for 4 hours a 25°C. Filters were washed (3-4 times) with binding buffer to remove unbound probe and then exposed.

3. Results

3.1. StyR-Flag expression and purification

The two-component system, StyS-StyR, is responsible for the regulation of the styrene degradation cluster. Hence, the purification of StyR protein is very important to study the regulation of the styrene catabolic operon. In order to purify this protein, we used a protein fusion expression system, pQE-Flag (see methods). This system allowed the expression of StyR protein fused with the Flag octapeptide (Fig. 1). The choice of this system was due to the commercial availability of anti-Flag monoclonal antibodies, which permitted to easily follow the purification process by identifying the fused protein through Western blot.

After the construction of the expression system (pQStyR-Flag) the growth conditions for an optimal expression of the fused protein were tested. However, in all the conditions tested more than 90 % of StyR-Flag protein was found in the insoluble cell fraction (Fig. 1).

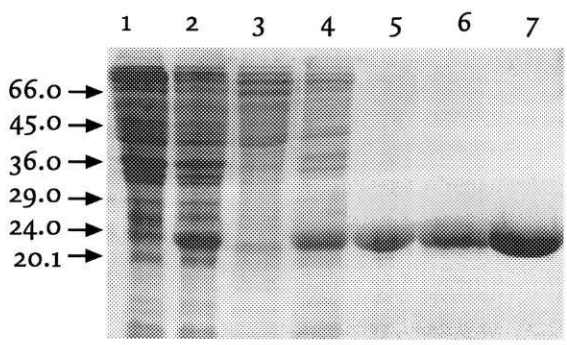


Fig.1. Expression and purification of StyR-Flag. Protein crude extracts from *E. coli* M15 cells harboring both pDMI,1 and pQstyR were prepared. In lane 1 whole cell extract from cultures without IPTG, in lane 2 whole cell extract from cultures with IPTG, in lane 3 soluble cell extract fraction from cultures with IPTG, and in lane 4 insoluble cell extract fraction from cultures with IPTG. The fraction from lane 4 was chosen to purify StyR-Flag. The insoluble fraction (inclusion bodies) was treated with different detergents, first with B-Per (lane 5) and then with Triton X100 (lane 6). Finally, a 100 % pure StyR-Flag fraction, electro-eluted from SDS-PAGE gel, was obtained (lane 7).

For this reason we decided to purify the inclusion bodies (Fig. 1) to produce polyclonal antibodies. Polyclonal antibodies obtained from mouse were able to specifically recognize, with the same affinity, both StyR-Flag and StyR (data not shown). At the moment different expression systems are being tested in order to produce soluble StyR.

3.2. StyR expression under different conditions

In Part A-II it was demonstrated that the expression of the catabolic operon is inducible by styrene and repressed to different extents when a preferred carbon source is present in the medium. At the same time it was also shown that the transcription of *stySR* operon was constitutive. In fact, the

corresponding transcript was present also in the absence of styrene and only a little increase (about 2-4 times) was observed in styrene grown cells. Since the activity of *PstyA* promoter depends on StyR regulator (Beltrametti et al., 1997), a modulation of StyR amount or of StyR activation by phosphorylation is necessary for *PstyA* regulation.

StyR expression levels and *PstyA* activities were followed in cells grown on styrene, lactate, and lactate plus styrene, taking advantage of the StyR antibodies produced and using a *lacZ* reporter system, respectively. Fig. 2 shows SDS-PAGE, Western blot, and ß-galactosidase activities from cells grown on the indicated substrates and collected at OD 0.5 and 1.0.

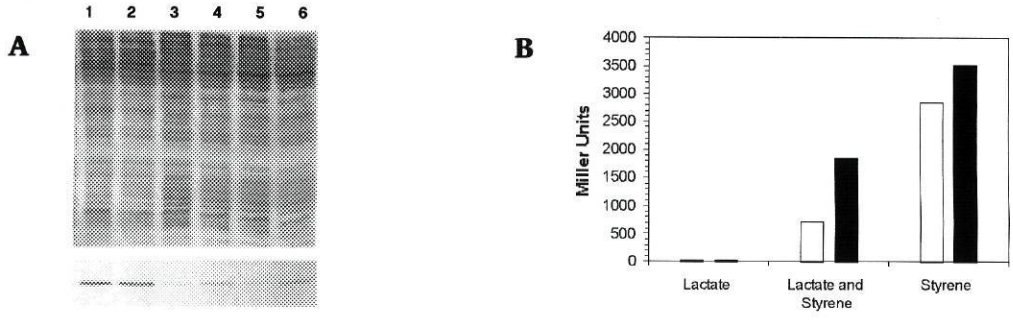


Fig. 2. Analysis of *Pseudomonas fluorescens* ST cells harboring pPR9TTPao grown in different conditions. **A.** Imunodetection of StyR protein crude extracts fromcells grown on lactate with (lane 4 and 6) or without (lane 3 and 5) styrene or in styrene (lane 1 and 2) as sole carbon source, and harvested at an OD600 of 0.5 (lanes 1, 3, 4) and of 1.0 (lanes 2, 5, 6). **B.** Expression of *PstyA* promoter using *lacZ* reporter

The data clearly show a modulation of StyR content in the different conditions. Even if StyR is always present, its amount is about ten fold higher in the induced conditions (compare lane 1 and 3 in Fig. 2A). This means that StyR is subjected to post-transcriptional control. The activity of *PstyA* promoter correlates with the amount of StyR in the different growth conditions, indicating that this regulator is the main element for *styABCD* expression. A possible explanation is that StyR is degraded in the absence of styrene and that its stability depends on its active (phosphorylated) form.

3.3. StyR DNA binding properties

In order to verify the DNA binding properties of StyR protein a novel strategy was developed. In recent literature the characterization of DNA and RNA-binding proteins using South-Western analysis has been reported. However, these experiments were performed with highly expressed proteins or partially purified ones. This technique was adapted to protein crude extracts with the advantage of using anti StyR serum for StyR identification in Western blots. In Fig. 3 the results of South-Western of protein crude extracts from strain ST grown on styrene, lactate, and lactate plus styrene to and OD 0.5 are shown.

The analysis of the results revealed several new features. The prevalent binding factor to the *PstyA* promoter region is not StyR, but rather an unknown factor (indicated with X). This factor binds constitutively to the promoter region, however the corresponding band is much stronger when StyR is present in lower amount. Since the amount of StyR correlates directly with the induction levels of the *styABCD* operon, as observed in Fig. 2, it is possible that this factor may function as a repressing factor or as a StyR DNA-binding competitor. Moreover, it is also possible that this low-molecular-weight factor is a nucleoid protein, such as IHF, HU, and H-NS, whose roles in transcription modulation, both as repressors and activators, have been extensively documented (McLeod and

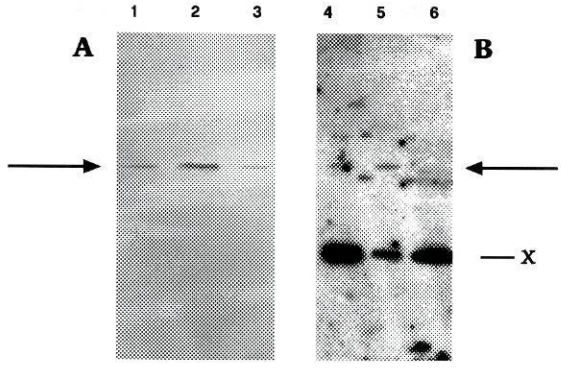


Fig 3. Study of StyR binding properties in South-Western experiments. In this experiment, Western blotting analysis (A) using a anti-StyR serum was coupled with a protein-DNA interaction assay (B) using radiolabeled *PstyA* as probe. 30 μg of strain ST crude extracts from cells grown on lactate (lane 3 and 6), lactate plus styrene (lane 1 and 4), or styrene (lane 2 and 5) were used. All samples were separated in the same SDS-PAGE, and transferred into a nitrocellulose filter. The filter was splite in order to perform both assays. StyR position is indicated by the black arrow. X denote an unknown binding factor.

Johnson, 2001). However, these hypothesis must be further investigated to understand the role of this factor in the expression of this system.

StyR binding to *PstyA* promoter was only observed in crude extracts from cells grown on styrene and lactate plus styrene. This observation can indicate that StyR only binds to *PstyA* promoter region upon activation, when cells are grown in the presence of styrene or that its amount in the absence of styrene is too low to be detected in these experimental conditions.

3.4. DNA–protein interactions within *PstyA* promoter region

The results obtained suggest that the study of protein-DNA interactions within *PstyA* promoter region may reveal interesting features related to the regulation of styrene gene cluster. In order to investigate the protein-DNA interactions that occur in the different growth conditions, the protein crude extracts above characterized were used in bandshift experiments. This option had the advantage that cell growth conditions and corresponding protein crude extracts were already defined. Studies on protein-DNA interactions employ an accurate set up of the assay conditions since many factors, such as ionic strength of the buffers, pH and temperature, play an important role. We set up the experimental conditions with cell extracts from strain ST grown on styrene. As shown in Fig. 4, three main complexes were obtained in the different binding conditions.

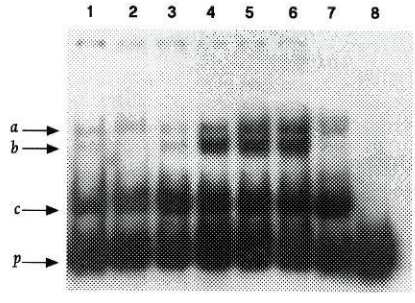


Fig. 4. Determination of gel mobility shift conditions. All reaction mixtures contained 2.5 mM $MgCl_2$,, 5 % glicerol, 0.025 % NP-40, and 125 ng/μl of sonicated salmon sperm DNA, but different concentrations of NaCl and TrisHCl buffer. In lane 1 reaction mixture contained 20 mM TrisHCl (pH 7.5), 100 mM NaCl; in lane 2, 40 mM TrisHCl (pH 8.0), 200 mM NaCl; in lane 3, 20 mM TrisHCl (pH 8.0), 100 mM NaCl; in lane 4 10 mM TrisHCl (pH 8.0), 50 mM NaCl; in lane 5, as lane 4 but with 100 μg/ml Bovine serum albumin (BSA); in lane 6, as lane 4 but with 5 mM EDTA; in lane 7, as lane 4 but with 5 mM DTT. In all reactions, except for lane 8 that did not contain protein extract, 20 μg of protein extract from strain ST cells grown on styrene as sole carbon source were used. *a*, *b*, and *c* indicate the position of the three main complexes observed almost in all conditions. *p*, unbound *PstyA* probe.

Complex *c* seems the most stable since its intensity remains almost invariable independently of the reaction condition, whereas the complex *b* seems the less stable. In general, in lower ionic strength, interactions were favored as observed in lane 4. Moreover, BSA and EDTA had a positive effect on the complex stability of the two heavier bands whereas DTT, lower pH, and higher ionic strength had a negative effect.

The positive effect of BSA is not surprising since it has been previously described as an inducer of some protein-DNA interactions (Kozmik et al., 1990). Also the effect of EDTA was expected since it can function as a nuclease inhibitor and oxidative damage protector of both DNA and proteins. DTT keeps the reaction mixture in the reduced state and may avoid oxidative damage. However, it might cause the reduction of the multimeric state of some DNA-binding factors and possibly lead to a reduced, or absence, interaction of these factors with the DNA. All the experiments described below were performed using the conditions corresponding to lane 4 of Fig. 4.

Upon definition of assay conditions, the banshift patterns of the *PstyA* DNA region with cell extracts from strain ST grown under the above-described conditions, that is styrene, lactate, and lactate plus styrene were examined (Fig. 5).

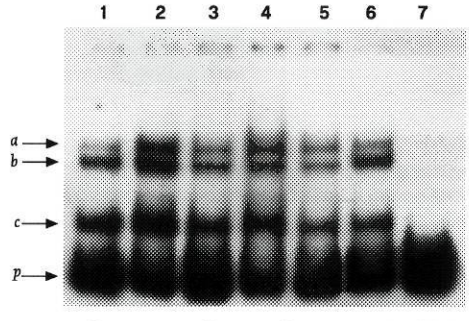


Fig. 5. *Pseudomonas fluorescens* ST protein crude extracts from cells grown in different conditions, up to an OD600 of 0.5, were used to study protein interactions with *PstyA* promoter region (gel mobility shift assay). Cells were grown on lactate (lane 5 and 6), lactate plus styrene (lane 3 and 4), and in styrene as sole c-source (lane 1 and 2). 10 μg (lane 1, 3, and 5) or 20 μg (lane 2, 4, and 6) of protein crude extracts were used in each reaction mixture. Lane 7, only *PstyA* probe.

The first evidence that comes out by the observation of the patterns obtained is that the three main complexes are present in all the studied conditions. However, the protein-DNA interactions are strongly influenced by the activation of the catabolic operon (induced condition). In fact, lysates from cells grown on styrene as sole carbon source interacted with the promoter region *PstyA* in a much more efficient way, generating three strong retarded bands. In the other conditions, all the corresponding bands appeared less intense indicating that minor protein-DNA interactions occurred.

This can be due to a lesser amount of the involved binding factors or, more probably, to the fact that some factors present or activated only in the presence of styrene are necessary for the efficient recruitment of key components of the active complex. Actually, even if the migration of each complex of the retardation pattern seems to be similar, this does not imply that each complex is made up by the same protein-DNA interaction in the different growth conditions. Moreover, it is also possible that each band is constituted by more than one single protein-DNA complex, due to the similar molecular weight of many different transcription factors.

In any case, purified protein components will be necessary to identify the complexes corresponding to the bands obtained in the gel mobility shift assays.

Meanwhile, the attention was focused on the promoter *cis* elements which can be target sites for protein binding. In Fig. 6 the sequence of the *PstyA* promoter is shown. This region spans from the stop codon of the upstream gene to the start codon of *styA*, the first gene of the *styABCD* catabolic operon, which codes for the large subunit of the styrene monooxygenase.

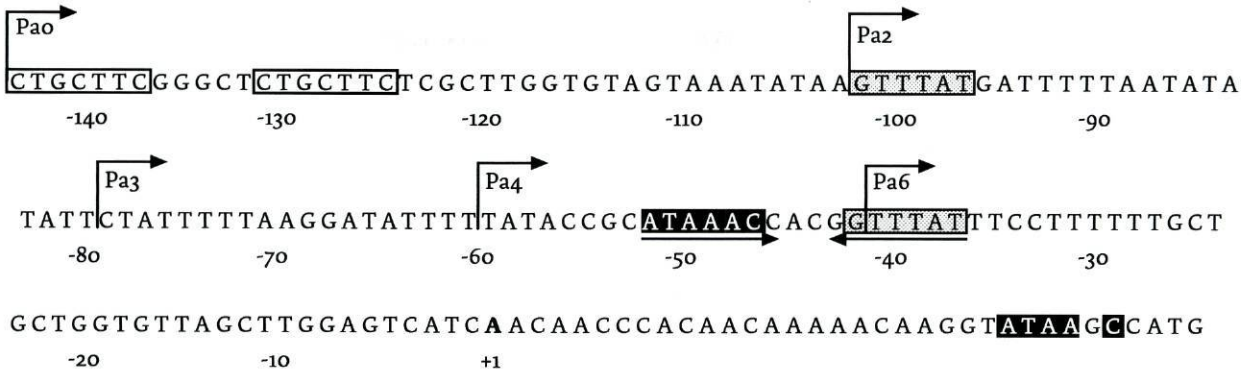


Fig. 6. Identification of possible regulatory elements in *PstyA* promoter region. Together with basic transcription elements, ribosome binding site (SD, Shine Dalgarno) and -10 regions (underlined) and the putative transcription starting point (in bold), other putative regulatory elements were found. The StyR putative binding site, named *sty box* and indicated by white arrows, is characterized by the presence of a inverted repeat. Furthermore, one direct repeat (boxed) in the early 5' of the promoter region, and a triple repeat AT-rich region was identified. *Pao*, *Pa2*, *Pa3*, *Pa4*, *Pa6*, correspond to 5' deletions DNA fragments of *PstyA* used to investigate the role of each of the putative regulatory elements.

The putative StyR binding site (*sty box*) is a palindromic sequence located from -52 to -37 and each half-palindrome is present also upstream and downstream of the *sty box* (see Fig. 6). The same situation is also observed in the catabolic promoters of *sty* genes of the other studied strains (accession numbers, AJ000330 and AF031161). This conserved structure is probably important for promoter regulation. Often, activators bind multiple sites, which can be high or low-affinity sites, and the different binding positions can affect promoter structure and therefore promoter function (Dai and Rothman-Denes, 1999). Regulative *cis* elements located downstream of the transcription origin have been found in many promoters and it was demonstrated that they act as repressive elements (Rojo, 2001). Other important features of *PstyA* promoter are the presence of a direct repeat at the 5' end and AT-rich region (spanning from -95 to -58) typical of HU or integration host factor (IHF) binding sites (McLeod and Johnson, 2001). Moreover, a canonical -35 was not found, but only an "extended" -10 , as often happens in positively regulated promoters.

In order to investigate the possible roles of each one of these elements several DNA fragments containing different 5' deletions of *PstyA* promoter were prepared (see Material and Methods and Fig. 6, indicated as *Pao - Pa6*). These fragments were used as specific cold DNA competitors (in 200 fold excess) in the binding reactions. If a DNA binding factor interacts within a DNA region encompassed by the specific competitor, this factor is sequestered by the competitor and is no longer available for binding to the full length promoter. The results of these assays are presented in Fig. 7. As expected, competition with *Pao* DNA fragment (lane 3) resulted in an absence of a retardation pattern since *Pao* was added in a 200 fold excess in respect to the radiolabeled probe and contains all the *PstyA* promoter region. The competition with *Pa2* and *Pa3* DNA fragments (lane 4 and 5) gave rise to a very faint band with a migration similar to that of the complex *a*. One could speculate that a factor binding to the 5' direct repeat could contribute to complex *a* formation.

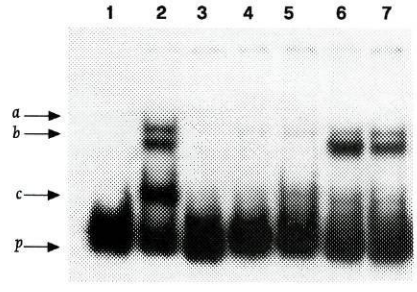


Fig. 7. Competition experiments with different 5' deletions of *PstyA* promoter. All reaction mixtures, except in lane 1 (only *Pa* probe, p), contained 20 μg protein crude extracts, from *Pseudomonas fluorescens* ST cells grown on styrene as sole carbon source and 100 ng (200 fold excess with respect to the probe amount) of specific competitor (lane 3, *Pao*; lane 4, *Pa2*; lane 5, *Pa3*; lane 6, *Pa4*; lane 7, *Pa6*). In lane 2 there is no competitor (control). Reaction mixtures were prepared as described in Materials and Methods.

An additional band with a mobility similar to complex *c* appeared when *Pa3* was the competitor (lane 5). The DNA region between *Pa2* and *Pa3* contains a half-palindrome repeat of the *sty box* and an AT-rich region which could be the binding site for StyR and/or for nucleoid proteins, respectively.

Competition with *Pa4 (lane 6)* resulted in the localization of the DNA region where the DNA-binding factor responsible for the formation of complex *b* interacts. Finally, competition with *Pa6* (lane 7) resulted a pattern very similar to that obtained with *Pa4*. In these two last patterns, bands corresponding to complexes *a* and *c* were less intense than the ones obtained when no DNA competitor was present. It is possible that the formation of stable complexes require the cooperative binding of factors which bind to the DNA region encompassed by *Pa6* or, that bands *a* and *c* are actually constituted by more than one complex originated by different protein-DNA interactions.

The DNA region between *Pa3* and *Pa4* is the target site for a factor that originates the formation of complex *b*. This region (Fig. 6) resembles the consensus for integration host factor (IHF) binding WATCAANNNNTTR, where W is A or T and R is A or G (Craig and Nash, 1984; Goodrich et al., 1990). The sequence TTTTAAGGATATTT has four mismatches in respect to the consensus. Nevertheless, IHF interaction with other sequences not perfectly matching the consensus was experimentally demonstrated (Marques and Gober, 1995; Thompson and Mosig, 1988). Hence, the possible IHF-*PstyA* interaction was investigated by using a specific DNA competitor containing a demonstrated IHF-binding region, within *Pu* promoter region (de Lorenzo, et al., 1991), as well as a *Pseudomonas putida* devoided of IHF (*ihfA* gene was deleted) (Calb et al., 1996; Teras et al., 2000). In Fig. 8 the results of these experiments are reported.

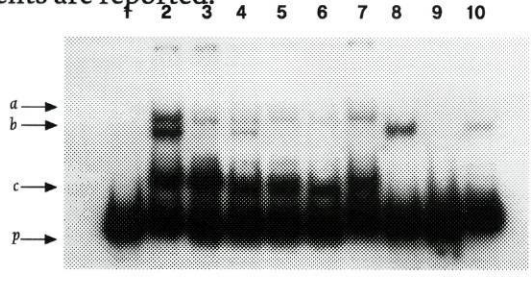


Fig. 8. IHF interaction with *PstyA* promoter region. All reaction mixtures, except in lane 1 (only *Pa* probe, p), contained 20 μg protein crude extracts, from *Pseudomonas fluorescens* ST cells grown on styrene as sole carbon source (lane 2 and 3) or from *Pseudomonas putida* KT2442 cells (lane 4 and 5), or from *Pseudomonas putida* A8759 cells (lane 6), or from *Pseudomonas putida* RT31 cells (lane 7, 8, 9, and 10) grown on LB. *Pseudomonas putida* RT31 cells were grown in the presence (lane 8, 9, and 10), or in the absence of 1mM IPTG (lane 7), after reaching OD600 of 0.3. In the reaction mixtures shown in lane 3 and 5 100 ng of IHF specific competitor (*Pu* partial fragment) were added. In the reaction mixtures of lane 9 and 10 100 ng of *Pa3* and *Pa4*, respectively, were added. See Materials and Methods for further details.

These results confirm the interaction of IHF with *PstyA* promoter region. In fact, specific competition with *Pu* DNA fragment resulted in the absence of complex *b* in both *Pseudomonas fluorescens* ST and *Pseudomonas putida* KT2442 (lane 3 and 5, respectively). The retardation pattern when the reaction mixture contained protein crude extracts from the *ihfA* mutant, *P. putida* A8759, confirmed the previous observation since no retardation band corresponding to complex *b* was observed (lane 6). Furthermore, the IPTG-controlled expression of IHF in *Pseudomonas putida* RT31 (see Table 1) (lane 7 and 8) revealed that IHF expression led to the formation of a retardation band correspondent to complex *b*. This was also confirmed by competition experiments with Pa3 and Pa4 (lane 9 and 10). Interestingly, strain KT2442 showed a retardation similar to that of strain ST. This fact supports the previous hypothesis that each of the bands observed is originated by more than one protein-DNA interactions. Probably, the retardation pattern observed with strain ST is the sum of the interactions of general (also present in other *Pseudomonas* spp.) and specific transcriptional factors for the expression of *styABCD* operon. Alternatively, aspecific DNA binding proteins can bind *PstyA* promoter in the absence of specific regulators.

In order to establish the functional consequences of *PstyA* 5' deletions and a possible correlation with *PstyA cis* elements identified by sequence analysis and retardation patterns, *Pa0*, *P2*, *P3*, *P4* and *Pa6* DNA fragments were cloned into the promoter probe vector, pPR9TT (Part A-I). These constructs were transferred, by conjugation, to *Pseudomonas fluorescens* ST. Cultures of strain ST harboring each single construct were tested for ß-galactosidase activity in cells grown on styrene or lactate as sole carbon sources. In Fig. 9 the results of these experiments are reported.

The analysis of the ß-galactosidase activities of the different 5' deletions permitted a correlation between the putative *cis* elements identified within the promoter and their functional effect on the regulation of the *styABCD* expression.

The comparison between the ß-galactosidase activities of *Pa0* and *Pa2* fusions showed a 40 % increase when *Pa2* was used. The analysis of the promoter region (Fig. 6) identified one direct repeat in the deleted region, and gel mobility shift assays using *Pa2* as specific competitor (Fig. 7) showed the appearance of a faint band, indicating that a DNA-binding factor would interact with *PstyA* in that region. This evidence suggest that in the early 5' of the promoter region there is a regulatory element that causes a certain level of repression on the expression of *styABCD*.

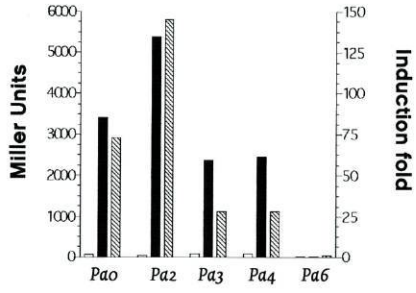


Fig. 9. ß-galactosidase activities measured in *Pseudomonas fluorescens* strain ST harboring different *PstyA::lacZ* fusions. All cells were grown on lactate (white bars) or on styrene (black bars) as sole carbon source to an OD 0.5. Induction folds (cross-hatched bars) are also indicated. See Fig. 6 and Materials and Methods for the details of each promoter region.

There is a 55 % reduction of the promoter activity, corresponding to 75 % reduction of the induction level with *Pa3* or *Pa4* deletions. To the reduction of the induction level also contributes a higher level of basal expression, indicating that a less stringent control on the promoter silencing in non-induced conditions occurred. The promoter region between *Pa2* and *Pa3* contains a putative StyR binding site and that between *Pa3* and *Pa4* constitutes the target site for IHF binding, as demonstrated by bandshift experiments (Fig. 8). These two factors exert a positive effect on the promoter activity and their absence results in a much lower expression and induction level of the reporter gene. Since the effect of IHF-mediated bend is to bring distal regions closer (Paul et al., 2001), it is not surprising that similar levels of ß-galactosidase expression were found with *Pa3* and Pa4 deletions. In fact, IHF binds to a DNA region close to the beginning of *Pa3* deletion and its positive effect can only be observed when upstream sequences are present, as in Pa2 fusion. Whether the positive effect exerted by the DNA region between *Pa2* and *Pa3* is due to the presence of a putative StyR binding site or, it simply has a structural function, still needs to be established. However, it was hypothesized that, upon IHF binding, the upstream StyR putative binding site and the *sty box* would be brought closer in an overlapping position (see below, Fig. 10).

Finally, the deletion of *sty box* and the all upstream region of *PstyA* promoter region led to promoter silencing. This fact demonstrates that *sty box* plays a key role in the regulation of the *styABCD* operon expression.

4. Discussion

The expression of the catabolic operon, *styABCD*, for the degradation of styrene is complexly regulated. Upstream of this operon two co-transcribed genes, *styS* and *styR*, organized in an operon, have been identified (Velasco et al., 1998; Marconi et al., 1998). In *Pseudomonas fluorescens* ST these two genes are expressed also in the absence of styrene in the growth medium in a range between 30-50 % of the full induced condition (Part A-II). Yet, in this work, great differences in the amount of StyR protein, depending also on the presence, or the absence, of styrene have been demonstrated. Possibly, in our system the absence of styrene in the growth medium determines a higher protein turnover or a translational control leading to a decrease in the amount of protein inside the cells.

In contrast with our data, only a inducible expression of these genes in *P. putida* CA-3 has been reported (O`Leary et al., 2001), suggesting a different regulatory control.

Using Southern-Western analysis, StyR interaction with *PstyA* promoter was demonstrated. The promoter region contains multiple putative StyR-binding sites. Experiments performed with several 5' *PstyA* deletions fused with the *lacZ* reporter gene clearly showed that *sty box* is essential for promoter activity. Most probably also the upstream putative StyR-binding site has an important role in *PstyA* activation. In fact, deletion of a fragment containing this sequence led to 55 % reduction of -galactosidase activity, corresponding to a 75 % reduction of the induction level. The same effect was observed when also the downstream IHF binding site was deleted. This suggests that IHF role is to bring closer the *sty* boxes located upstream and downstream of the IHF-mediated DNA bent, and that the complex formed enhances transcription.

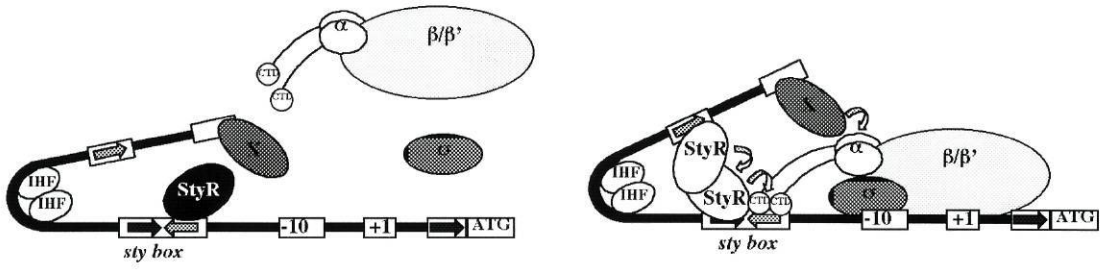
In bandshift experiments it was not possible to identify the DNA complex(es) corresponding to StyR binding, even using crude extracts from cells grown in different conditions. For this reason, presently StyR protein is being purified. This will allow to perform bandshift experiments with the single identified protein components.

PstyA expression is also subjected to negative control. Deletion of the first 41 nt at the 5' end in the *Pa2* construct led to an increase in the expression level of the ß-galactosidase in styrene-grown cells and to a two fold increase in the induction level. In bandshift experiments a faint band corresponding to complex *a* appeared in the presence of *Pa2* competitor. Moreover, the Southern-Western analysis had shown a very strong hybridization band, whose intensity was inversely proportional to that of StyR suggesting that this band corresponds to a repressing factor.

In general, apart from complex *b*, corresponding to IHF binding, strong bands were only obtained in the absence of competitor DNA. This suggests that for the formation of stable complexes important *cis* elements are distributed along the entire DNA region. These elements can have a structural function on the DNA molecule or can represent specific binding sites for regulative factors.

A vast literature is available on the regulatory mechanisms which control catabolism of aromatic compounds (Cases and de Lorenzo, 2001; Powlowski and Shingler, 1994; Díaz and Prieto, 2001).

However, little is known about the control of catabolic genes expression by two-component regulatory systems. Here, a first contribution on this subject is provided. Interestingly, IHF-dependent promoters have been frequently found in one-component regulatory systems (Goosen and Putte, 1995; Pérez-Martín et al., 1994; Friedman, 1988), but apart from NtrC response regulator, IHF involvement in two-component systems regulation has been only described for promoters controlled by EnvZ/OmpR (Goosen and Putte, 1995), and NarX/NarL (Browning et al., 2000). The new data presented here gave origin to a first working model on the possible mechanism that regulates *PstyA* activity (Fig. 10).



NOT INDUCED INDUCED

Fig. 10. Model for the regulation of *PstyA* activity. The different *cis* elements are boxed and the putative StyR binding sites indicated with arrows. X represents the putative repressor. Upon phosphorylation, two molecules of StyR interact in order to open transcription complex and sequester RNAP core (α/α', ß/ß' subunits) by interaction with carboxy-terminal domain (CTD) of the RNAP subunits, and allow transcription. Factor X interacts with RNAP core and modulates the expression level. An increase of DNA-binding affinity of StyR, upon phosphorylation, is suggested. Non-phosphorylated StyR in black and phosphorylated in white. Factor X may interact with subunits of the RNAP leading to a decrease of transcription efficiency due to unknown modulation mechanism. Bent arrows indicate possible protein-protein interactions.

An IHF-mediated interaction between StyR proteins bound at *sty box* and at the upstream half-palindromic sequence would be necessary for both full *PstyA* expression in induced conditions and a stringent promoter silencing in non-induced conditions. Possibly, the above mentioned StyR binding sites constitute high and low affinity sites. Under non-induced conditions, due to a low level of StyR and/or to a low level of phosphorylated StyR, no binding or binding only to high affinity site(s), can determine the formation of a closed complex. IHF contributes to the achievement of a closed structure by DNA bending as been suggested by the fact that deletion of the entire DNA promoter region upstream of the bending site lead to an increase of the basal activity of *PstyA* activity of around four fold.

It has been suggested that DNA bending prevents the aspecific binding of factors other than the specific activator(s). Other repressing factors can contribute to the promoter silencing . In fact, South-Western experiments have revealed that a low-molecular-weight DNA-binding factor is present in high amount in non-induced conditions. This factor could be a histone-like protein like the global regulators HU or H-NS.

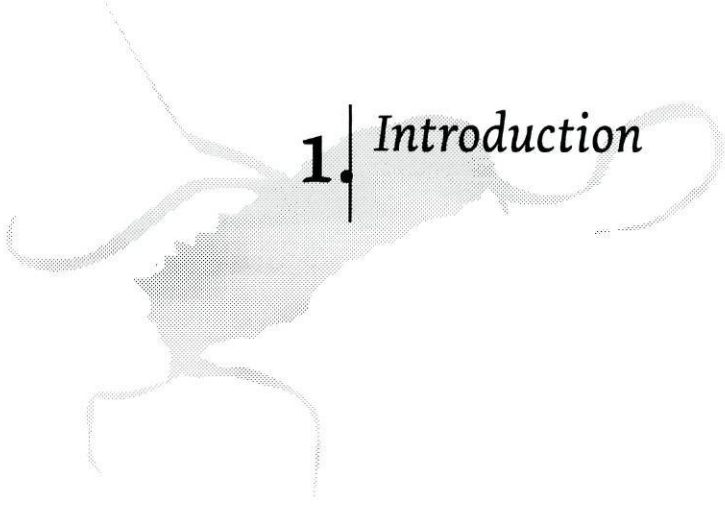
In induced conditions, also low-affinity site(s) can be occupied by StyR with the consequence that StyR-StyR interaction can occur. This new promoter structure can diminish the DNA bend allowing RNAP to access to the promoter and make the right contact with the activator. This model is probably simplistic and there are still many question to be answered before an accurate description of the functioning of this system can be achieved.

{PART B} MOLECULAR RESPONSES TO AROMATIC COMPOUNDS TOXICITY

[I]

Styrene AS A Stress Factor

...Science is always wrong. It never solves a problem without creating ten more..."
George Bernard Shaw



1. Introduction

The presence of an aromatic compound in the growth medium imposes several toxic effects to bacteria. On the one hand, the accumulation of aromatic compounds in the membrane results in membrane permeabilization (Jackson and de Moss, 1965; Woldringh, 1973; Heipieper et al., 1994), dissipation of proton motive force (Monti et al., 1987; Sikkema et al., 1992, 1994), change of membrane thickness and its surface hydration characteristics (Shimooka et al., 1972; Seeman, 1972), and membrane fluidity (Yuli et al., 1981; Zeng et al., 1988). In part, to contrast these effects, cells have developed several adaptation mechanisms that can respond transiently. These mechanisms belong to the general process of stress response which involve global regulators, such as sigma factors, chaperones, and DNA-repair enzymes.

On the other hand, exposition for long periods to aromatic compounds can give rise to degradative-efficient bacteria which, besides the specific catabolic genes, have acquired other genetic traits or mutations that lead to a better survival and improve their ecological fitness. The more extensively studied aromatic detoxification processes involve the participation of membrane active proteins such as Cit (*cis/trans* isomerase), SrpABC (solvent-resistant pump) system and TtgABC (toluene tolerance genes) system (Isken and de Bont, 1999; Duque et al., 2001). Still, it is also possible that other genes related to aromatic stress response may be triggered in the presence of aromatic compounds.

On this basis the styrene degradative strain *Pseudomonas fluorescens* ST was used to identify and characterize non-catabolic genes that are induced by styrene and that, possibly, are involved in adaptation mechanisms due to the stress imposed by the presence of styrene in the growth medium.

2. Materials and Methods

2.1. Bacterial strains, plasmids, media and chemicals

The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas fluorescens* and *Escherichia coli* cells were routinely grown at 30 and 37°C, respectively, in Luria-Bertani (LB) medium (Miller, 1972) or mineral salts medium (Hartmanns et al., 1989) supplied with 0.1% lactate. In induction studies, 0.1 % phenylacetate or 0.1 % benzoate were added while styrene was added via the gas phase as previously described (Marconi et al., 1996). When necessary, cultures were supplemented with ampicillin (Ap) (100 μg/ml), kanamycin (Km) (25 μg/ml), Isopropyl-ß-D-thiogalactopyranoside (IPTG; 1 mM), 5'bromo-4'-chloro-3'-indolyl-ß-D-galactopyranoside (X-Gal, 1 mM) and 2-nitrophenyl-ß-D-galactopyranoside (ONPG, 1 mM).

Table 1. Bacterial strains and plasmids

Strains and plasmids	Relevant genotype	Reference or source
Strains		
P. fluorescens ST	Sty+	Baggi et al., 1983
P. fluorescens IS1	mini-Tn5::*lacZ2* mutant of strain ST	This study
P. fluorescens IS3	mini-Tn5::*lacZ2* mutant of strain ST	This study
P. fluorescens IS4	mini-Tn5::*lacZ2* mutant of strain ST	This study
P. fluorescens IS5	mini-Tn5::*lacZ2* mutant of strain ST	This study
P. fluorescens IS6	mini-Tn5::*lacZ2* mutant of strain ST	This study
P. fluorescens IS7	mini-Tn5::*lacZ2* mutant of strain ST	This study
P. fluorescens IS8	mini-Tn5::*lacZ2* mutant of strain ST	This study
P fluorescens IS10	mini-Tn5::*lacZ2* mutant of strain ST	This study
E. coli DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA* (Nal^r) *(lacIZYA-argF)U169 deoR(80dlac(lacZ)M15)*	Bethesda Res. Labs
E. coli S17.1λpir	RecA pro *thi hsdR* RP4 -2-Tc::Mu-Km::Tn7 Tra^+ Tp^r Str^r	Miller and Mekalanos, 1988
Plasmids		
pBluescriptII KS+	ColE1 replicon; Ap^r; 2.9 kb	Strategene
pTZ19R	ColE1 replicon; Ap^r; 2.9 kb	MBI Fermentas
pUTmini-Tn5::*lacZ2*	R6K origin; suicide plasmid containing miniTn5 ::*lacZ2*; Ap^r; Km^r;	de Lorenzo et al.,1990
pT6Sal	pTZ19R derivative in which a 7500 bps *SalI* chromosomal DNA fragment from IS6 strain containing Km cassette was cloned into the same site of the vector; Km^r, Ap^r; 10.4 kb	This study
pT4Sal	pTZ19R derivative in which a 9500 bps *SalI* chromosomal DNA fragment from IS4 strain containing Km cassette was cloned into the same site of the vector; Km^r, Ap^r; 12.4 kb	This study
pB10Sal	pBluescriptII KS+ derivative in which a 7800 bps *SacI* DNA fragment from IS10 straincontaining Km cassette was cloned into the same site of the vector; Km^r, Ap^r; 10.7 kb	This study

2.2. Transposon mutagenesis

Mating experiments between donor (*E. coli* S17.1 λpir (pUT mini-Tn5::*lacZ*2)) and recipient strain (*Pseudomonas fluorescens* ST) were performed according to de Lorenzo and Timmis (1994). The mating mixtures were then plated on LB Km and replicated on selective media.

2.3. DNA manipulation.

Transformations of *E. coli*, restrictions and ligations were carried out by standard procedures (Sambrook et al., 1989). Plasmid DNA was prepared by the alkaline lysis protocol (Sambrook et al., 1989) or with QIAGEN Midi-isolation kit (Qiagen). DNA fragments were purified from agarose using Qiaquick gel extraction kit or QIAEXII kit (Qiagen).

2.4. Identification of genes interrupted by the transposon

Genomic DNA from strain ST and selected mutants was digested with several enzymes and subjected to Southern blotting analysis according to standard procedures (Sambrook et. al.,1989) using the Km-resistance gene DNA cassette as probe. Digestions that gave rise to single hybridization bands were chosen to clone the DNA fragments containing the transposon and its flanking regions. Fragments of corresponding size were isolated from 0.8 % agarose gels and ligated with pBluescriptII KS+ or pTZ19R digested with the same enzyme. Ligation mixtures were used to transform *E. coli* DH5α. Transformants were selected in LB Ap-Km agar plates. In order to identify the DNA regions flanking the transposon, the cloned fragments were sequenced with an Applied Biosystem automated sequencer (model 373 Stretch) by DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer). Both commercially available and custom primers were used for sequencing reactions.

2.5. Induction conditions

In induction assays, cells from *P. fluorescens* ST or selected mutants were pre-grown overnight at 30°C in mineral salts medium supplemented with lactate or with styrene or with phenylacetate or with benzoate or with benzoate and lactate. The styrene-grown cells were transferred to mineral medium containing styrene as sole C-source, while the lactate or benzoate or phenylacetate or benzoate and lactate-grown cells were inoculated into their corresponding mineral medium with or without styrene. Cell growth was measured by monitoring the optical density at 600 nm.

2.6. Enzymatic assays

To quantify styrene monooxygenase (SMO) activity, indigo production was assayed essentially as described by O'Connor et al. (1997). Cells were harvested in the exponential or stationary phase by centrifugation, washed with 50 mM K-phosphate buffer (pH 7.0), and resuspended in the same buffer to an OD600 of 3.0. A 100 µl volume of concentrated cells was added to 400 µl of 50 mM K-phosphate buffer (pH 7) containing 0.25 mM indole in a 1.5 ml polypropylene tube. The samples were incubated horizontally at 30°C with vigorous shaking for 30 minutes. The samples were then centrifuged at 14000 rpm for 2 minutes and the supernatants were carefully discarded. The cell pellets were resuspended in 1 ml of dimethylformamide (DMF) by shaking for 15 minutes. The tubes were then centrifuged to remove cell debris, and the optical density of the supernatants was determined at 600 nm.

The activity of catechol 1,2-dioxygenase (C1,2O) was measured by determining the formation of *cis,cis*-muconate at 260 nm ($\varepsilon_{cis,cis\text{-muconate}}$ = 16,800 liters $mol^{-1}cm^{-1}$) as previously described by Nakazawa and Nakazawa (1970). Reaction mixtures contained (in 1 ml) 100 mM potassium phosphate buffer pH 7.5 and crude extract (0.02 to 0.2 mg).Reactions were initiated by adding 5 µmol of catechol. ß-Galactosidase activity was measured as described by Miller (1972), and expressed as Miller units.

The presented data correspond to the results obtained from, at least, three independent experiments with a deviation ranging from 5 to 10 %.

3. Results

3.1. **Screening of the** *Pseudomonas fluorescens* **ST mutants**

In order to identify genes or operons induced by styrene but not involved in its degradation random mutagenesis with the mini-Tn5::*lacZ2* system was performed. Random insertion of this transposon into the chromosome can originate translational fusions between the *lacZ* gene (which lacks the first 8 codons) and the interrupted gene. If this insertion occurs in frame, the expression of the interrupted gene is measurable by determining the ß-Galactosidase activity (de Lorenzo et al., 1990). Hence, to screen for styrene-inducible genes the suicide vector pUTminiTn5::*lacz2* was transferred from *E. coli* S17.1λpir to strain ST cells and exconjugants were selected on LB Km plates. The clones obtained were transferred with a toothpick into microtiter-plate wells containing 0.9 % NaCl sterile solution and replicated onto mineral medium (Hartmans et al., 1989) agar containing X-Gal, 0.1 % lactate or 0.1 % lactate plus styrene or styrene. The mutants that showed ability to form blue color colonies on the mineral medium plates only when these plates contained lactate plus styrene or just styrene as C-sources were selected for further characterization. 28 clones, out of 6720 screened were not able to grow on mineral medium containing glucose or lactate as sole carbon sources, whereas 18 showed the desired phenotype (blue color formation in mineral medium containing styrene and no blue color when styrene was not present in the growth medium). Here, the characterization of some of these mutants is presented.

3.2. **Southern blotting analysis of the mutants**

In order to identify the insertion point of the transposon, the chromosomal DNA of the selected mutants was digested with *SalI* restriction enzyme, which does not cut inside the transposon and with *SacI* which cuts inside the *lacZ* gene, but not within the Km^r marker. The km resistance gene was used as hybridization probe. In this way, *SalI* hybridization bands contained genomic regions flanking the transposon on both sides, while the *SacI* hybridization bands contained only the chromosomal region adjacent to the Km^r marker. In Fig. 1 the hybridization patterns obtained are reported.

Comparison between the two patterns showed that only one transposition event had occurred in each mutant. Moreover, mutants IS6, IS7, and possibly IS1, showed the same *SalI* and *SacI* patterns indicating that the transposon localization is the same in these mutants. IS8 probably underwent

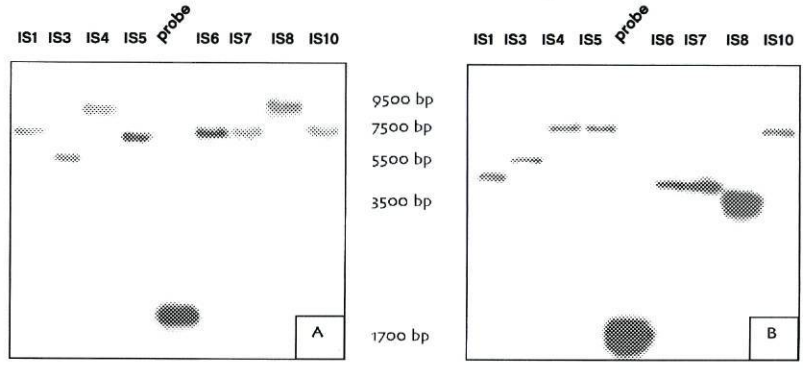


Fig. 1. Southern-blotting analysis of the transposon-mutants of strain ST. In A chromosomal DNA was digested with *SalI* and in B with *SacI*. A 1700 bp DNA fragment containing Km resistance gene was used as probe. 10 µg of chromosomal DNA was load in each lane.

a rearrangement upon transposition, since two *Sac*I bands were found in this hybridization pattern. Similarly, IS5 and IS10 mutants also seem to have the transposon localized in the same DNA region.

3.3. Identification of the insertion points of the transposon in strain ST mutants

Mutant IS3 was discarded due to the fact that a more accurate phenotypic analysis revealed that the interrupted gene was probably a constitutive gene rather than a styrene-inducible one. Mutants IS4, IS6 and IS10 were chosen for further characterization. Transposon containing fragments for *Sal*I-digested DNA from mutants IS4 and IS6 and *Sac*I-digested DNA from mutant IS10 were selected and cloned as described in Materials and Methods. Sequencing of the DNA regions flanking the transposon allowed the identification of the genes interrupted(Table 2). Interestingly, all the interrupted genes belong to aromatic degradation operons.

Table 2. Localization of the insertion point in the studied ST mutants

Mutant	Highest homology of the interrupted gene		
	Gene	Identity aa(%)	Strain
IS6	*phaA*	90	*Pseudomonas putida* U
IS8	*benA*	97	*Pseudomonas putida*
IS10	*benC*	96	*Pseudomonas putida*

In IS6 the interruped gene corresponds to *phaA* gene which codes for one of the subunits of an enoyl-CoA hydratase involved in the phenylacetate degradative pathway (Kanazawa et al., 1993) (Fig. 2A) .

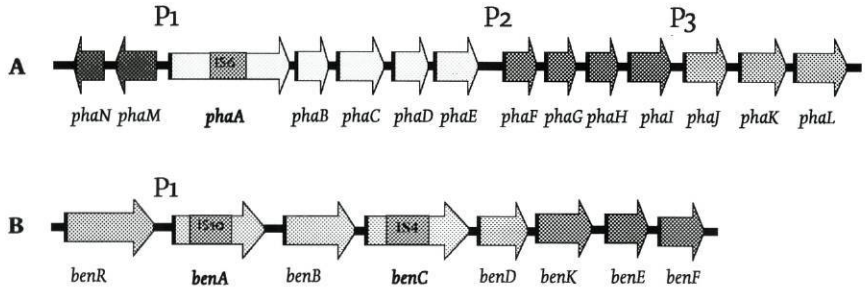


Fig. 2. Organization of the gene clusters that were interrupted by random mutagenesis. **A.** Phenylacetate degradative pathway organization (for further details see General Introduction): **B.** Benzoate degradative pathway. *P1, P2, P3, Po* and *P1* correspond to the promoter regions of the single operons. In bold the interrupted gene and the corresponding mutant is indicated.

The upper pathway of styrene degradation converts styrene into phenylacetate which, via the phenylacetate degradative pathway, is converted to TCA intermediates. Therefore, the activation of *pha* genes by styrene is not surprising. However, Olivera and co-workers (Olivera et al., 1998) demonstrated that the disruption of *phaA* gene resulted in non-functional phenylacetate degradative pathway. Nevertheless, IS6 is still able to degrade both styrene and phenylacetate, which may indicate that this gene is not essential for the degradation of phenylacetate in this strain, and that its function may be replaced by another functionally active enoyl-CoA hydratase, for example, connected to fatty acid catabolism.

Finally, IS4 and IS10 have the benzoate operon interrupted, in *benA*, and *benC*, respectively (Fig. 2B). These genes encode the subunit of benzoate dioxygenase (*benA*) and ferredoxin reductase (*benC*) which functions as the reductase component of the benzoate dioxygenase enzyme. Both enzymes have been shown to be essential for the conversion of benzoate into catechol, and, accordingly mutants IS4 and IS10 were unable to grow on benzoate. This fact suggests that somehow, styrene, or one of its intermediates may function as an inducer of the benzoate operon.

3.4. Enzymatic activities of ST mutants in the presence of different aromatic inducers

In order to understand the relationship between the aromatic effectors and expression of the different catabolic pathways (Fig. 3) enzymatic assays representative of each catabolic operon were performed. Styrene monooxygenase activity was measured following the conversion of indole into indigo (Beltrametti et al., 1997). Catechol 1,2-dioxygenase activity encoded by the *catBCA* was measured as *cis,cis*-muconate formation (see General Introduction). The expression of *pha* and *ben* operons was measured as ß-galactosidase activity encoded on *lacZ2* inserted in mutant IS6, and in mutants IS4 and IS10, respectively. All strains were grown in the presence of different aromatic inducers.

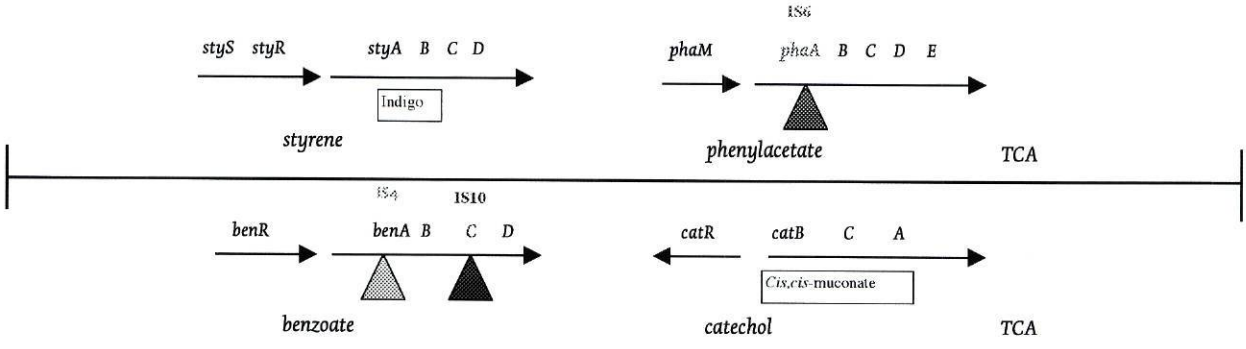


Fig. 3. Organization of the different catabolic operons involved in induction studies. *sty* genes encode the upper pathway for styrene degradation; *pha* genes encode for the phenylacetate mineralization into TCA intermediates; *ben* genes encode for benzoate conversion into catechol that is mineralized through the action of *cat* gene products into TCA intermediates (for further details see General Introduction). Localization of transposon insertions in strain ST mutants is shown with triangles. Expression of *sty* and *cat* genes was followed measuring indigo and *cis,cis*-muconate production, respectively. The substrates of each catabolic operon are also shown. Arrows indicate the transcription direction.

The results (Fig. 4) showed that the upper pathway of styrene degradation (*styABCD*) is only induced in the presence of its natural inducer (styrene) (Fig 4c). The *catBCA* operon is induced in the presence of benzoate (Fig 4b). Even if *cis,cis*-muconate (CCM) is the effector of the CatR, it has been demonstrated that also benzoate is able to induce this operon even in the absence of CCM (Rothmel et al., 1991).

In contrast to this narrow specificity of induction, the *ben* and *pha* operons were induced not only by their own natural substrates, benzoate and phenylacetate, respectively, but also by other inducers. In particular the ben operon was induced also by styrene (mutants IS4 and IS10 in Fig.4a). The regulator of the *ben* operon, BenR, belongs to the AraC family of regulators, which are activated upon recognition of the specific effector.

BenR, and other transcriptional regulators belonging to the different families, show a certain degree of sensitivity towards other related compounds, which can act as effectors. This phenomenon is known as "gratuitous induction" and lead to the activation of the regulated system, although the structural analogous of the natural effector are not themselves substrates of the induced catabolic operons (de Lorenzo and Pérez-Martín, 1996).

However, styrene is not a structural analogue of benzoate, having very different physico-chemical properties. On this basis, phenylacetate, the styrene derivative, could be a good inducer candidate, but no phenylacetate-dependent ß-galactosidase activity could be detected in IS4 and IS10 mutants, indicating that this compound is not an inducer of the *ben* operon.

The possibility that StyR can act as positive regulator of the *ben* operon is unlike since StyR and BenR mechanisms of transcription activation are completely different. It is possible to postulate that another unknown regulator, belonging to the same family of regulators as BenR, can recognize styrene as effector and act as activator of the *ben* operon. The *phaABCDE* operon resulted to be induced

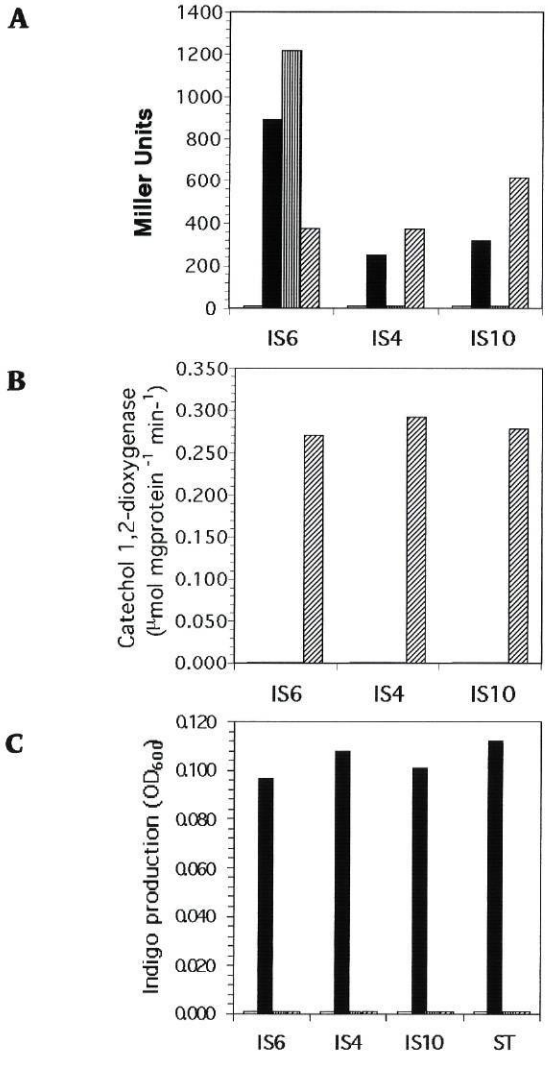


Fig. 4. ß-galactosidase, catechol 1,2-dioxygenase activites and indigo production in *Pseudomonas fluorescens* ST and in the indicated mutants. Strains were grown on lactate (open bars), on lactate plus styrene (solid bars), on lactate plus phenylacetate (vertically cross-hatched bars), and on lactate plus benzoate (diagonally cross-hatched bars) to an OD600 of 0.5.

not only by phenylacetate, but also by styrene and benzoate (mutant IS6, Fig.4a). Phenylacetate is an intermediate of styrene catabolism. Therefore, it can be expected that also this compound indirectly functions as and inducer of the *phaABCDE*. In fact, the differences between the ß-galactosidase activities with either substrates may reflect a variation on the amount of operon inducer (phenylacetate) rather than a different effect of the inducer. The *phaABCDE* operon is also induced by benzoate. It was recently demonstrated that the real inducer of phenylacetate degradation operon is phenylacetyl-CoA. However, only related compounds, such as hydroxylated derivatives or *n*-phenylalkanoates were tested as inducers (García et al., 2000). The regulator of this operon has not been characterized and it is possible that it could be activated by benzoate. Furthermore, it is also possible that BenR could activate the *pha* operon. Accordingly, this regulator has been shown to control other aromatic degradative pathways, like *xyl meta* pathway, and *pca* operon (Cowles et al., 2000). This fact suggests that BenR may participate in the modulation of different pathways that converge into central metabolism.

4. Discussion

The scope of this work was the identification of genes codifying enzymes that function as protective agents or are part of protective mechanisms against styrene toxicity. However, this kind of genes were not obtained probably due to the screening strategy. In fact, other screening procedures like aiming the study of the strain ST adaptation phase to styrene (Appendix 1) need to be setup. Probably, these other methodologies will permit to achieve new insights on the styrene-toxicity adaptation process in strain ST. Nevertheless, the performed screening allowed the isolation of some interesting mutants discussed here, and other mutants are presently under study. The results obtained in this study revealed several interesting features about styrene presence within the cells.

Styrene is a toxic, hydrophobic aromatic compound and, when present in the growth medium, it triggers not only its own degradative pathway (*styABCD* operon and phenylacetate pathway), but also, at least, another pathway (*ben* operon). Furthermore, styrene induces *cis/trans* isomerization as an adaptive response (Weber et al., 1993). Cross-talk between catabolic pathways is not uncommon (Cowles et al., 2000; de Lorenzo and Pérez-Martín, 1996) however, it often occurs when members of the same family of regulators are involved. In this case, the involved regulators belong to different families. The regulator of styrene upper pathway (StyR) belongs to the two-component FixJ family (activated by phosphorylation), whereas BenR belongs to AraC/XylS family (activated by direct interaction with the inducer). CatR belongs to the LysR family (activated by direct interaction with the inducer in a different way with regard to BenR), whereas PhaM has been suggested as the *phaABCDE* regulator, but it has not yet been characterized (García et al., 2000).

Actually, the cross-inductions presented here have no simple explanation and are not within the record of cases up to now described.

Studies on the evolution of catabolic operons and their regulatory systems have put in evidence interesting features, which can be summarized as follows: (1) structural and regulatory genes evolve relatively independently; (2) transcriptional control systems can evolve because of the specificity of pre-existing promoters and regulators; (3) recruitment of a suboptimal transcriptional control is followed by suppression of useless elements and fine-tuning of the regulator to respond predominantly to the new signal.

These concepts have led to the notion that there seem to be a sort of "regulative noise" among prokaryotic promoters (de Lorenzo and Pérez-Martín, 1996). Interestingly, the examples which support this notion mainly concern aromatic compounds degradation and refer to bacteria belonging to the *Pseudomonas* genus. This is because many of the pathways are directed to catabolism of compounds which have only recently been released to the biosphere, or which, even if present in nature for long time, only recently have reached concentrations high enough to be useful as carbon sources.

Concerning styrene degradation, *Pseudomonas* strains up to now studied (Velasco et al., 1998; Beltrametti et al., 1997; Panke et al., 1998, O'Leary et al., 2991) contain styrene catabolic operons and

the corresponding regulatory operons which are more than 90 % identical. This means that this genetic trait has only recently been recruited by the single strains. However, styrene is also produced naturally and has been present for a long time in the environment. Due to its toxicity it is possible that, apart from its degradation, cells should have evolved enzymes and/or regulators able to recognize this substrate. It is known that enzymes like toluene dioxygenase are able to convert styrene into a related derivative. Moreover, AlkS, the regulator of the *alk* operon recognizes styrene as inducer (Panke et al., 1999). These examples show that styrene can be bio-transformed also in strains which are not able to utilize this compound for growth. The induction of the *ben* operon by styrene can be due to the intervention of an unknown regulator activated by styrene. Alternatively, styrene biotransformation process can lead to the formation of a compound recognized by BenR.

[II]

Ocurrence and possible role of glutathione S-transferases (GSTs) in *Pseudomonas*

{adapted from Research in Microbiology, 2002. Santos,P. M., G. Mignogna, H. J. Heipieper, and E. Zennaro. Ocurrence and properties of glutathione S-transferases in phenol-degrading Pseudomonas strains. 153:89-98}

...Discovery consists of seeing what everybody has seen and thinking what nobody has thought..."

Albert Szent-Gyögyi

1. Introduction

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of heterogeneous, multi-functional dimeric proteins which are found in animals, plants and microorganisms. They catalyze the conjugation of glutathione (GSH) to a number of hydrophobic electrophilic substrates, as well as glutathione-dependent isomerizations. Eukaryotic GSTs are believed to be involved in cellular detoxification of substrates of xenobiotic origin forming glutathione-adducts which are then further metabolized or even transported out of the cell (Hayes and Pulford, 1995).

In contrast to the well-studied eukaryotic GSTs, bacterial GSTs only recently attracted the interest of microbiologists and molecular biologists. Several bacterial GSTs have been identified through DNA sequence analysis (Hofer et al., 1994; Van Hylckama Vlieg et al., 1998; Masai et al., 1993; Zhou et al., 2001) or protein purification techniques (Di Ilio et al., 1988; 1991; 1993; Favaloro et al., 1998a; Arca et al., 1990). The high sequence variability found among bacterial GSTs is probably linked to their functional versatility. Even though the involvement of GSTs in many different processes has been reported, the best investigated examples of GSTs using GSH as co-enzyme and/or substrate are those involved in degradation reactions such as epoxide hydrolase (van Hylckama Vlieg et al., 1998; 1999), dehalogenase (Chesney et al., 1996; McCarthy et al., 1996), and lignin etherase (Masai et al., 1993).

In the latest years several bacterial GSTs genes have been found to be located within gene clusters coding for aerobic degradation of different aromatic compound such as biphenyls (Taira et al., 1992; Hofer et al., 1993; 1994; Erickson and Mondello, 1992), polyaromatic hydrocarbons (Zhou et al., 2001), 2,4,5-trichlorophenoxyacetate (Daubaras et al., 1996), and dibenzodioxin (Armengaud and Timmis, 1997). Belonging to the same transcription unit, both GST and catabolic genes are induced in the presence of these aromatic compounds. However the physiological role of these GSTs remain to be established. In fact, GST-coding genes are not always present within the same (or highly homologous) catabolic gene clusters harbored by different strains (Bartels et al., 1999). Moreover, the absence of *bphK*, a GST-coding gene of the biphenyl operon, apparently does not affect growth on biphenyls as sole carbon source (Hofer et al.,1994; Bartels et al., 1999). Recently, it was described that in the atrazine-degrading bacterium *Ochrobactrum anthropi* organic substrates, like atrazine, phenol and chlorophenols caused an increase in the expression of a constitutively present and monocistronically transcribed GST (Favaloro et al., 1998b; 2000). On the contrary, aromatic compounds not degradable by this bacterium did not have any effect on GST activity basal level. Even if the localization of the GST-coding gene is not known, it is known that it does not belong to the transcription unit of the catabolic genes. Therefore, in this case, the induction of GST activity is not directly dependent on the activation of the catabolic operon by the aromatic substrate. These results suggested a possible metabolic relationship between GST expression and the catabolism of phenols and chlorophenols in this bacterium (Favaloro et al., 2000).

In order to verify if analogous situations can also occur in *Pseudomonas* spp. degradative strains, GSTs from four *Pseudomonas* strains able to grow on phenol as sole carbon source were purified. In this work N-terminal sequences of the purified GSTs and their activities in the presence of different substrates and under different growth conditions are reported.

2. Materials and Methods

2.1. Microorganisms and culture conditions

The bacterial strains, all belonging to the genus *Pseudomonas*, used in this study are listed in Table 1. Strains were cultivated in mineral salts medium as described by Hartmans et al. (1989) with 0.2 % succinate or 0.4 % pyruvate or 0.2 % lactate or with different phenol concentrations as sole carbon sources. Cells were grown in 150-ml cultures with shaking at 30°C.

2.2. Preparation of cell crude extracts

Pseudomonas cells cultivated in the different conditions were harvested by centrifugation (7,000g for 20 min. at 4°C), resuspended in sonication buffer (10 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 10 % glycerol), and disrupted by sonication (6 cycles of 15 sec. burst with 15 sec. of interval) in ice. The particulate material was removed by centrifugation at 17,000 g at 0°C. Crude extracts were stored at –80°C until their use.

2.3. GST purification

To obtain a large amount of purified protein, cells (around 20-25 g wet weight) from cultures were harvested by centrifugation (7,000 g for 20 min. at 4°C), resuspended at 20 % (w/v) in sonication buffer (10 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 10 % glycerol), and disrupted by sonication (8 cycles of 15 sec. burst with 15 sec. of interval) in ice. Cell debris was removed by centrifugation at 12 000 g at 0°C. The supernatant was filtered with a 0.22 μm filter device. A Glutathione Sepharose 4B column was prepared and equilibrated using the Bulk GST Purification Module from Amersham Pharmacia Biotech according to the manufacturer's instructions, and then crude cell extract was applied to this system. The column was extensively washed with PBS buffer (140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, pH 7.3) and washed with two bed volumes of 50 mM Tris-HCl buffer (pH 9.6). The enzyme was eluted with 50 mM Tris-HCl buffer (pH 9.6) containing 10 mM GSH. The GST fractions were dialyzed against sonication buffer, concentrated using a Centricon YM10 (Amicon) device, and stored at –80°C. This procedure was also applied to purify small amounts of GST protein but with some modifications. Briefly, cells from 400 ml of culture were re-suspended in 4 ml of sonication buffer, sonicated, centrifuged, and the cell debris discarded. 50 μl of glutathione sepharose 4B resin equilibrated in PBS buffer was added to the crude extract and incubated for 2 h with gentle shaking at 4°C . The suspension was centrifuged and the supernatant discarded. The resin was washed three times with 500 μl of PBS and once with 50 mM Tris-HCl (pH 9.6).

The enzymes were eluted with 100 μl of 50 mM Tris-HCl buffer (pH 9.6) containing 10 mM GSH. The GST fractions were dialyzed against sonication buffer, concentrated using a Centricon YM10 (Amicon) device, and stored at –80°C.

2.4. **Electrophoresis**

SDS-PAGEs were performed in 12.5 % polyacrylamide according to the method described by Laemmli (1970). Gels were scanned using Fluor-S Imaging system (Biorad) and relative amounts of each protein were obtained. Native-PAGEs were carried out in a non-denaturating 6 % polyacrylamide gel at 4°C in 1 Tris-glycine (pH 8.9) buffer at 10V/cm for 2 h.

2.5. **N-terminal amino acid sequencing**

The N-terminal sequence of purified protein was determined by automated Edman degradation of samples (2-5 μg) electroblotted on PVDF (Problott membranes Applied Biosystems) after SDS-PAGE. The protein bands were excised and sequenced in a gas phase sequencer (Applied Biosystems, Model 476A).

2.6. **Protein quantification**

Protein concentration of cell crude extracts and purified proteins were determined by the bicinchoninic acid assay (BCA, Pierce), using bovine serum albumin (BSA) as a standard. The concentration determined of the purified proteins was compared with densitometry analysis of the patterns obtained by SDS-PAGE, using BSA as standard, since upon GST purification procedures it is possible to have GSH contamination (normally removed by dialysis) and its presence in the assay sample may interfere with the BCA method.

2.7. **N-terminal sequence accession numbers**

The amino acid sequences of the determined N-terminals of GST_85, GST_CF600, GST_F1, GST_M1A, GST_M1B, and GST_ST have been deposited in the SWISS-PROT protein sequence database under the accession number P82996, P82997, P82998, P82999, P83000, and P83001, respectively.

2.8. **GST activity assays**

The GST activity of protein crude extracts and purified proteins were assayed by the spectrophotometric standard methods described by Habig and Jakoby (1980). For cell crude extracts, reaction mixtures contained (in 1 ml) 100 mM potassium phosphate buffer, pH 6.5, 1mM GSH, and crude extract (0.2 to 2 mg). Reactions were initiated by adding 1 mmol of 1-chloro-2,4-dinitrobenzene (CDNB). The activity was measured by determining the formation of CDNB-GSH conjugate at 340 nm ($\varepsilon_{CDNB\text{-}GSH}$ = 9600 liters mol^{-1} cm^{-1}).

In the case of the purified proteins, activity assays were performed towards different standard substrates. Reaction mixtures to determine their activity towards CDNB contained (in 1 ml) 100 mM potassium phosphate buffer, pH 6.5, 1mM GSH, and purified protein (7.5 to 40 μg). Reactions were initiated by adding 1 mmol of CDNB. The activity was measured by determining the formation of CDNB-GSH conjugate at 340 nm ($\varepsilon_{CDNB\text{-}GSH}$ = 9600 liters mol^{-1} cm^{-1}). Reaction mixtures to determine their activity towards DCNB contained (in 1 ml) 100 mM potassium phosphate buffer, pH 7.5, 5mM GSH, and purified protein (10 to 50 μg). Reactions were initiated by adding 1 mmol of DCNB. The activity was measured by determining the formation of DCNB-GSH conjugate at 345 nm ($\varepsilon_{DCNB\text{-}GSH}$ = 8500 liters mol^{-1} cm^{-1}). Reaction mixtures to determine their activity towards 1,2-Epoxy-3(4-nitrophenoxy)propane (ENPOP) contained (in 1 ml) 100 mM potassium phosphate buffer, pH 6.5, 5 mM GSH, and purified protein (10 to 60 μg). Reactions were initiated by adding 0.5 mmol of ENPOP. The activity was measured by determining the formation of ENPOP-GSH conjugate at 360 nm ($\varepsilon_{ENPOP\text{-}GSH}$ = 500 liters mol^{-1} cm^{-1}). Reaction mixtures to determine their activity towards *p*-nitrophenyl

acetate (NPA) contained (in 1 ml) 100 mM potassium phosphate buffer, pH 7.0, 0.5 mM GSH, and purified protein (10 to 50 μg). Reactions were initiated by adding 0.2 mmol of NPA. The activity was measured by determining the formation of *p*-nitrophenol at 400 nm ($\varepsilon_{p\text{-nitrophenolate}}$ = 9600 liters mol^{-1} cm^{-1}). The data presented below are the results obtained from at least three identical experiments with a deviation ranging 5 to 10 %.

2.9. **Dioxygenase activity assays**

The activity of catechol 1,2-dioxygenase (C1,2O) was measured by determining the formation of *cis,cis*-muconate at 260 nm ($\varepsilon_{cis,cis\ muconate}$ = 16,800 liters mol^{-1} cm^{-1}) as previously described by Nakazawa and Nakazawa (1970). Reaction mixtures contained (in 1 ml) 100 mM potassium phosphate buffer, pH 7.5 and crude extract (0.02 to 0.2 mg), and reactions were initiated by adding 5 mol of catechol.

The activity of catechol 2,3-dioxygenase (C2,3O) was measured by determining the formation of α-hydroxymuconic-ε-semialdehyde at 375 nm ($\varepsilon_{\alpha-\ hydroxymuconic\text{-}\varepsilon\text{-}semialdehyde}$= 36,000 liters mol^{-1} cm^{-1}) as described previously by Nozaki (1970). Reaction mixtures contained (in 1 ml) 50 mM potassium phosphate buffer, pH 7.5 and crude extract (0.02 to 0.2 mg), and reactions were initiated by adding 5 mol of catechol. The data presented below are the results obtained from at least three identical experiments with a deviation lower than 10%.

2.11. **Measurement of growth and growth inhibition**

Overnight pre-cultures were transferred to fresh mineral medium always contained the same carbon source to be used in the final culture. For growth experiments using phenol as sole carbon and energy source, cultures were started at an optical density (O.D.) of 0.05 from an overnight culture grown on 250 mg/l phenol. As both, growth rates and yields differed depending on the phenol concentration and to make sure that all sampled cells were growing exponentially, samples were taken when the cultures had reached an O.D. of 0.35-0.5. In induction studies, aromatic compounds were added after 3 h of exponential growth and growth was followed for further 3 h before sampling. Cell growth was measured by monitoring the turbidity (O.D.) at 600 nm of each cell suspension using a Perkin-Elmer EZ201 spectrophotometer. Growth inhibition caused by aromatic compounds was determined by comparing growth rates $\mu(h^{-1})$ between the supplemented and control cultures and expressed as percentage of the control growth rate as described by Heipieper et al. (1995). The average results of three identical experiments are shown in which the deviation was less than 5 %.

3. Results

3.1. Main characteristics of the chosen strains

The *Pseudomonas* strains used in this study are reported in Table 1. All of these strains, except strain ST , are able to grow on phenol as sole carbon source through different catabolic pathways. CF600 is a well characterized strain which degrades phenol via *meta* pathway (Powlowski and Shingler, 1994). The plasmid-encoded phenol catabolic operon has been completely sequenced and do not contain GST-coding genes (Shingler et al., 1992). Phenol degradation in F1 strain has been reported to occur via the *meta* pathway encoded by the *tod* genes (Spain et al., 1989) and also in this case no GST-coding genes have been found in the corresponding operon (Zylstra and Gibson, 1989; Wang et al., 1995). *P. putida* PaW85(*pheBA*) is a toluene negative derivative of strain mt-2 (pWWO) (Bayley et al., 1977) containing pAT1140 plasmid, carrying the *pheBA* genes, coding for catechol 1,2-dioxygenase and phenol monooxygenase, respectively, which makes this strain able to utilize phenol through the *ortho* cleavage pathway located in the chromosome (Kasak et al., 1993). Strain M1, isolated as a ß-myrcene degradative strain (Iurescia et al., 1999), is also able to grow on phenol and benzoate. However the localization of these catabolic genes and the degradative pathways utilized by this bacterium are not known. For this reason the catechol 1,2- and 2,3-dioxygenase activities in strain M1 cells grown on both these aromatic compounds were checked. Data obtained showed that only catechol 1,2-dioxygenase activity was present, indicating that this strain utilizes phenol and benzoate via *ortho* pathway (data not shown).

While in strain F1 and strain CF600 the *meta* pathway operons have been sequenced and do not contain GST-coding genes, M1 and PaW85 utilize phenol via *ortho* pathway (*cat* genes). Only few *cat* operons have been sequenced in different strains, therefore it is not possible to exclude that a *gst* gene is present within this catabolic operon in these strains. However, up to now no GST-coding genes have been found in the sequenced *cat* operons. The presence of GST protein(s) was also tested in another strain, *P. fluorescens* ST, which grows on styrene as sole carbon source (Baggi et al., 1983). The sequence of styrene catabolic genes has shown that no GST-coding genes are located in this operon (Beltrametti et al., 1997).

Table 1. *Pseudomonas* strains used and their specialized aromatic substrates

Pseudomonas **strains** (reference)	**Aromatic substrates**
strain M1 (Iurescia et al., 1999)	Phenol
strain CF600 (Shingler et al., 1989)	Phenol Cresols
putida F1 (Gibson et al., 1968)	Toluene; Benzene Phenol
fluorescens ST (Baggi et al., 1983)	Styrene
putida PaW85 (*pheBA*) (Kasak et al., 1993)	Phenol

3.2. GST purification and N-terminal sequence

For GST purification all the strains listed in table 1 were grown on succinate and GSTs were purified from each crude extract by affinity chromatography. SDS-PAGE analysis revealed the presence of at least one purified protein in each strain (Fig. 1).

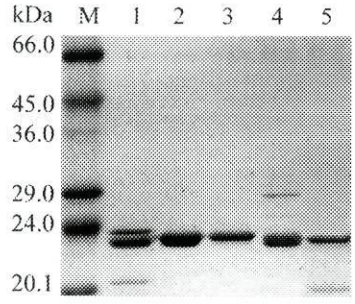


Fig. 1. SDS-PAGE of the affinity chromatography-purified GSTs. M, molecular weight marker; 1-5, protein products after purification of cell crude extracts from *Pseudomonas* sp. M1 (1), *Pseudomonas* sp. CF600 (2), *Pseudomonas putida* F1 (3) *Pseudomonas putida* PaW85(*pheBA*) (4) and *Pseudomonas fluorescens ST* (5).

The molecular weight of all these proteins was approximately 22-23 kDa, which corresponds to standard sizes previously described for GST proteins (EC 2.5.1.18). Only in two strains, *P. putida* F1 and *P.* sp. strain CF600, the purification step originated a single protein product. The less intensive low molecular weight proteins (around 20 kDa, in *P. fluorescens* ST and in *P.* sp. M1) as well as the heavier one (around 29 kDa in *P. putida* PaW85(*pheAB*)) can represent proteins that have affinity to glutathione (GSH), or that strictly interact with the putative GSTs. In the case of *P.* sp. M1 two proteins with a very similar molecular weight, matching the standard molecular weight of GSTs, were obtained (Fig. 1). The quantification of both total protein crude extract (obtained by cells sonication and cell debris discard) and purified proteins showed that the amount of each protein represented from 0.01 to 0.03 % of the total cell proteins.

The amino terminal (N-terminal) sequence of each protein with a molecular weight of ca. 22-23 kDa (Table 2) was determined. No significant homology was found when the obtained N-terminal sequences were compared with those present in the protein databases. However, GSTs are characterized by the presence of one or two tyrosine residues in the first eight amino acids, whose function, in case of GSTs belonging to the theta class and other bacterial GSTs, is substituted by a serine residue located near the N-terminus of the protein (Vuilleumier, 1997). These features are present in all purified proteins (Table 2).

Table 2. Alignment of the N-terminals of the new purified GSTs with previously reported GSTs from other bacteria

Strains	Protein name	N-terminal sequence	Accession number
Pseudomonas sp. M1	GST_M1A	M D **Y** L I T F **Y** H **S** P Q T N S	This study
Pseudomonas sp. M1	GST_M1B	M L L V I G **S** K N L **S S** T N M	This study
Pseudomonas sp. CF600	GST_CF600	M L K L H G F **S** V **S** N **Y Y** N M V K L A L L E K G	This study
P. putida F1	GST_F1	M L K L H G F **S** V **S** N **Y Y** N M V K L A L L	This study
P. fluorescens ST	GST_ST	M L H L I G F D K L **Y S Y** N M V K L A L L E K G V P F T	This study
Synechocystis sp.	GST	M L E L H Q F E L **S** Q **Y S** E K V R L I L D F K G L D Y	P74665
P. putida PaW85	GST_85	M Y H **Y Y S** P D A L R T X N	This study
Burkholderia sp. LB400	BphK	M K L **Y Y S** P G A C **S** L **S** P H I A L R E A G L N F E L	X76500
Ochrobactrum antropi	OaGST	M K L **Y Y** K V G A C **S** L A P H I I L S E A G L P Y E L	P81065
Sphingomonas sp. DJ77	PhnC	M K L F I **S** P G A C **S** L A P H I A L R E T G A A F D A	AAB66314
Ralstonia sp. U2	NagJ	M K L **Y Y S** P G A C **S S S** P H I I L R E G G F D F Q L	AAD12618
Sphingomonas paucimobilis	LigF	M T L K L **Y S** F G P G A N **S** L K P L A T L Y E K G L E F E Q	P30347
AMINO ACID POSITION		1 5 10 15 20 25	

Even if the N-terminal sequence determined for PaW85(*pheBA*) GST (GST_85) is short, this protein seems to be similar to the ones isolated in other proteobacteria species with two tyrosine residues at position 4 and 5 (Table 2). Identical N-terminal sequences were obtained with GST_CF600 and GST_F1; although they showed a different electrophoretic mobility in the native form (Appendix 5). The N-termini of these two proteins share 50 % of identity with a GST protein from *Synechocystis* sp. strain PCC 6803 (Kaneko et al., 1996), with aligned serine 10 and tyrosine 12 (Table 2). Results obtained with the two bands of strain M1, named GST_M1A and GST_M1B, do not permit to identify whether either of them, or both, correspond to putative GSTs since their N-terminal sequences do not match with other bacterial GSTs. Other examples of the presence of GST isoenzymes have been reported (Di Ilio et al., 1988), and the analysis of the complete genome sequence of both *E. coli* and *Pseudomonas aeruginosa* revealed the existence of three different putative GSTs in each of these strains. GST_M1A contains two tyrosine residues at position 3 and 8 and a serine residue at position 10. In GST_M1B no tyrosine residues are present, but serine residues are located at positions 7, 11 and 12. The presence and the location of these key residues are compatible with the possibility that these proteins purified from strain M1 have a GST activity. As far as GST_ST is concerned, its sequence from position 14 to 25 is identical to the ones of CF600 and F1, while they seem to diverge in the first 13 amino acids. Still, also in this GST serine (position 12) and tyrosine (positions 11 and 13) residues are present in the same region as the other GSTs.

3.3. **Substrate range of the purified GSTs**

In order to verify if the differences observed in the sequence of the N-termini of the purified GST corresponded to different activities towards standard substrates, enzymatic assays were performed, according to the methods described by Habig and Jakoby (1981). In Table 3 the enzymatic activities of the purified GSTs and the different substrates analyzed are shown.

Table 3. Specific enzymatic activities of the newly purified GSTs using several standard substrates

Strains	**Proteins**			**GST specific activity**[1]			
	isozymes	Conc. (mg/ml)[4]	(%)[3]	CDNB	DCNB	NPA	ENPOP
P. sp. M1	GST_M1[2]	0.681 (19.8)	0.038	560	18.6	81.2	118
P. sp. CF600	GST_CF600	0.478 (16.8)	0.033	175	12.3	269	96.3
P. putida F1	GST_F1	0.344 (20.2)	0.011	268	24.8	217	107
P. putida PaW85 (*phe*BA)	GST_85	0.305 (15.2)	0.027	1560	39.5	63.7	33.6
P. fluorescens ST	GST_ST	0.355 (17.8)	0.025	105	45.2	156	110

1 expressed in nmol min^{-1} mg $protein^{-1}$

2 considered the sum of GST_M1A and GST_M1B in terms of amount of protein

3 relative abundance in respect to the total amount of protein crude extract starting material

4 in parentesis the concentration of the protein crude extract that was loaded on the column

GST_85 showed the highest specific activity among all the purified proteins with CDNB as assay substrate, while the one for the other substrates was very low. Due to the fact that two co-purified proteins were present in the assay, specific activity of the GSTs from strain M1 were calculated assuming that both GST_M1A and GST_M1B concurred to the total activity. None of the purified proteins showed a significant activity with DCNB as substrate and the specific activities obtained with the other substrates were in general low. Nevertheless, it is important to underlie the fact that the few available data on GST activities towards standard substrates do not allow a GST classification on this basis.

The different activity patterns shown by the different GSTs studied could reflect their different role in the cell metabolism of each strain.

3.4. **Physiological study of GST activity**

All purified proteins showed a well detectable activity with CDNB as substrate. This substrate was chosen to investigate if growth on increasing concentrations of phenol could affect GST activity with respect to the activity level determined with cells growing on a non-aromatic carbon source such as succinate. This study was performed with strains M1 and CF600, mainly because GST_M1A and GST_M1B do not match in sequence with other known GSTs, and GST_CF600 is very similar to that from Synecocystis sp. (Kaneko et al., 1996), which has not been characterized.

Fig. 2 shows the growth rates (Fig. 1A) and the activities of GST and of catechol 1,2-dioxygenase (Fig. 2B) or catechol 2,3-dioxygenase (Fig. 2C) in *P.* sp. M1 and *P.* sp. CF600, respectively.

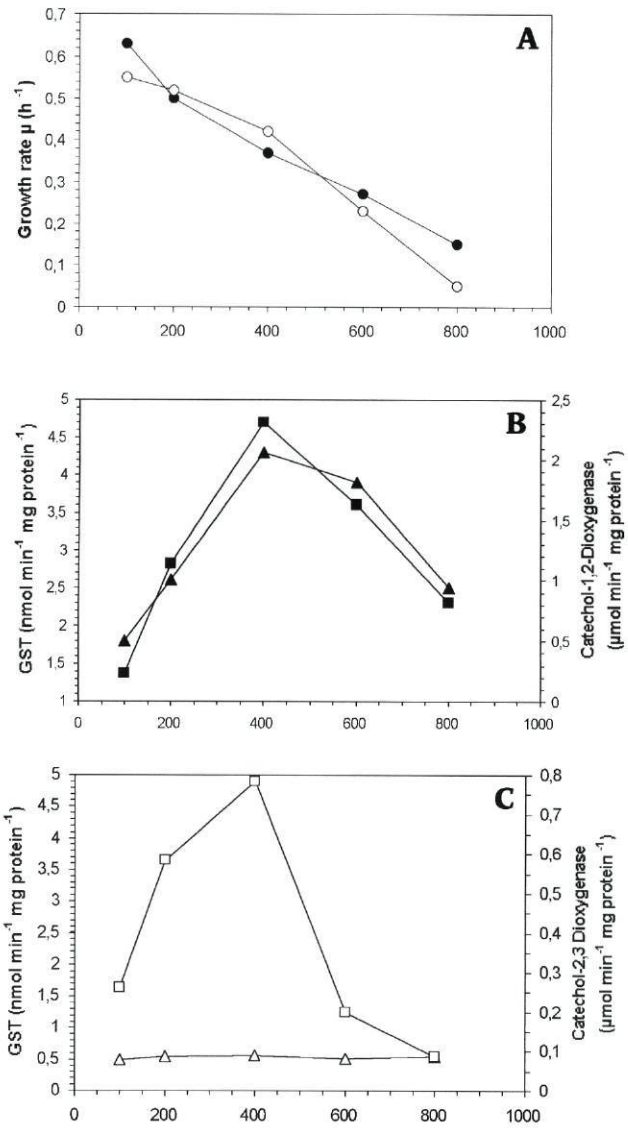


Fig. 2. Effect of phenol as sole carbon source. A. Growth rates. of *Pseudomonas* sp. M1 (solid circles) and *Pseudomonas* sp. CF600 (open circles). B. GST (solid triangles), and catechol 1,2-dioxygenase (solid squares) activities in *Pseudomonas* sp. M1. C. GST (open triangles) and catechol 2,3-dioxygenase (open squares) in *Pseudomonas* sp. CF600.

In both strains, a phenol concentration-dependent increase in the enzyme activity of the specific catechol-dioxygenase was observed. While no increase in GST activity was measured in *P.* sp. CF600 (Fig. 2C), a direct correlation between the GST activity and that of the catechol 1,2-dioxygenase was found in *P.* sp. M1 (Fig. 2B). Similar experiments showed a correspondence between dioxygenase and

GST activities also in *P. putida* PaW85(*pheBA*), while the behavior of *P. putida* F1 was very similar to that of CF600 (data not shown).

In cells grown on phenol as the only C-source, the maximum growth rate was achieved at concentrations of phenol lower than those which caused the maximum dioxygenase activity. This can be explained by the toxic effect of phenol. At higher concentrations of toxic compounds cell loose energy due to an increase in membrane permeability caused by the compound and need extra energy for the adaptive mechanisms to protect the cells (Heipieper and de Bont, 1994; Heipieper et al., 1994; Isken and de Bont, 1998). This energy is no longer available for growth (Isken et al., 1999).

In strain M1, the correspondence between the activity of catechol 1,2-dioxygenase and that of GST could indicate that the induction of GST activity is correlated to the activity of the catechol 1,2-dioxygenase and therefore with phenol catabolism.

Another possible explanation is that in this strain GST takes part in the general mechanism of cell protection to the presence of a toxic compound and that GST induction shows a fortuitous parallelism with the catechol 1,2-dioxygenase activity. To test this possibility uncoupling of the catechol 1,2-dioxygenase activity from phenol toxicity was tried using other carbon sources in the presence of toxic concentrations of phenol. In fact, it is known that more favorable carbon sources often exert a catabolite repression effect on the induction of catabolic operons for aromatic compounds (Cases and de Lorenzo, 2001; Müller et al., 1996; Part A-II). Studies on aromatic hydrocarbon-degrading strains have shown that succinate, the best substrate for most *Pseudomonas* strains, exerts a strong repression on the induction of catabolic operons, while pyruvate can be considered as a non repressive carbon source (Collier et al., 1996; Müller et al., 1996). For other carbon sources, such as lactate, probably there is a threshold concentration which allows induction of the catabolic genes for aromatic degradation before the preferred carbon source is depleted (Müller et al., 1996; Part A-II). Therefore, succinate, lactate and pyruvate were tested as carbon sources, for strains M1 and CF600, to test the effect of phenol when it was added to exponentially growing cells. After an addition of phenol, cells continued to grow exponentially, but with reduction in the growth rates (with respect to the control) that directly depended on the phenol concentration added. Three hours after the addition of phenol, cells were collected and dioxygenase and GST activities determined. The results obtained in these experiments are presented in Table 4 and show that several aspects influence the levels of the enzymatic activities.

Table 4. Effect of the carbon and energy source on the activities of GST and the strain-specific dioxygenases of *Pseudomonas* sp. M1 and *Pseudomonas* sp. CF600 in the presence of phenol.

		Strains					
		Pseudomonas **sp. M1**			*Pseudomonas* **sp. CF600**		
C-source	**phenol (mg/l)**	$\mu(h^{-1})^a$	**GST**[b]	**C1,2O**[c]	$\mu(h^{-1})^a$	**GST**[b]	**C2,3O**[d]
	0	0.93	1.3	<0.01	0.97	0.49	<0.01
Succinate	400	0.55	1.4	0.05	0.69	0.54	0.20
	600	0.39	1.8	0.06	0.35	0.53	0.13
Lactate	0	0.89	1.6	<0.01	0.86	0.45	<0.01
	400	0.51	1.8	0.10	0.57	0.45	0.21
	600	0.32	2.4	0.09	0.27	0.52	0.14
	0	0.50	2.8	<0.01	0.47	0.42	<0.01
Pyruvate	400	0.38	3.8	0.46	0.41	0.45	0.26
	600	0.25	6.0	0.63	0.23	0.46	0.19

a growth rate

b Glutathione S-transferase specific activity given in nmol min^{-1} mg $protein^{-1}$

c Catechol 1,2-dioxygenase specific activity given in mol min^{-1} mg $protein^{-1}$

d Catechol 2,3-dioxygenase specific activity given in mol min^{-1} mg $protein^{-1}$

In strain M1, the expression of the catabolic operon for phenol degradation, measured as catechol 1,2-dioxygenase activity, was dependent on the carbon source present in the growth medium. Succinate and, to a lower extent lactate, caused a catabolite repression effect on the system, whereas the catechol 1,2-dioxygenase activity showed the highest values in the presence of pyruvate. In strain CF600, the expression of the phenol catabolic system, dmp operon, measured as catechol 2,3-dioxygenase activity, was nearly the same whatever the carbon source present in the growth medium. Nevertheless, the activity differences seem to correlate with the observed growth rates and this is in agreement with previous observations (Sze and Shingler, 1999; Sze et al., 1996).

Considering GST activity, in strain M1, when phenol was present in succinate and lactate-grown cultures, its levels were significantly lower than the ones found in cells grown on phenol as sole carbon source. This result indicates that a repression of phenol catabolism determines a concomitant repression of GST induction, suggesting that these two processes are strictly related. However, it was also found that, in the presence of phenol, GST basal activity showed a negative correlation with the growth rate specific for each substrate. GST levels increased upon addition of phenol and the extension of such an increase depended on phenol concentration and on the carbon source used. This was particularly evident in pyruvate plus phenol medium, where to a relatively low level of catechol 1,2-dioxygenase activity corresponded a high level of GST activity. In this case, phenol toxicity seems to play an important role in GST induction, even if phenol catabolism can also contribute to such an induction. In fact, the catechol 1,2-dioxygenase specific activity found in this growth condition was about ten times higher than the one found in succinate-grown cells.

As a whole the results obtained with strain M1 suggest that GST activity can be correlated with the general metabolic and energetic status of the cells, also in the absence of a toxic compound. A less favorable carbon source (pyruvate) determines higher levels of GST activity. The different levels of GST activity when the same phenol concentration was added to cells growing on different substrates might mean that GST activity induction is connected to a specific metabolic condition, which determines a (stress) signal for its induction. The occurrence of this specific condition could depend both on the presence of toxic phenol concentrations and on the capability of the cell to prevent the stressing signal by acquiring energy through the preferred carbon source metabolism. On the other hand, there are strong indications that the stressing signal is connected to the activation of the phenol catabolic pathway. Actually, an increase in GST activity was mainly observed when phenol catabolism took place, as measured by the activity of catechol 1,2-dioxygenase, whereas not degradable aromatic compounds (e.g. salicilate, styrene) did not cause an induction of GST even at toxic concentrations in any of the studied strains (Appendix 6).

In strain CF600, although the cells showed a strong increase in the catechol 2,3-dioxygenase activity upon phenol addition no increase in GST activity was observed at any of the investigated carbon sources and phenol concentrations. Probably, in this strain GST enzyme has a different function, as also suggested by its N-terminal sequence compared with that of strain M1.

3.5. Dimerization of GST_M1AB and GST_CF600

GSTs with known structures are homodimeric enzymes. Each monomer folds into two domain conformations: the N-terminal domain includes most of the glutathione-binding site, and the C-terminal domain is involved in the binding of hydrophobic substrates (Vuilleumier, 1997). GST enzymes function as dimers, therefore their activity depends on the dimerization degree of the monomeric forms present inside the cells.

Hence, GST_M1AB and GST_CF600 obtained from cells grown on pyruvate in the presence and in the absence of phenol, and on phenol as the sole carbon source were analyzed in native conditions

(Fig. 3). Considering the M1 protein patterns, it is evident that the lower B band, which likely corresponds to the monomer, is present only in the samples from cells grown on pyruvate in the absence of phenol (lane 2). No monomeric forms were obtained from extracts of cells grown on pyruvate plus 400 mg/l phenol or on phenol (lanes 1 and 3, respectively). A different situation was obtained with GST_CF600 where the monomeric form is present in all the analyzed conditions. These data are qualitatively in a good agreement with GST activities found in the different growth conditions and suggest that the GST dimerization level can be a controlled step in the regulation of GST activity. However, quantitative considerations are difficult to do because larger volumes culture would be necessary for reliable protein purification, and the growth phase and cell metabolic status could be different from the ones that occurred during GST and catechol dioxygenase activities determination.

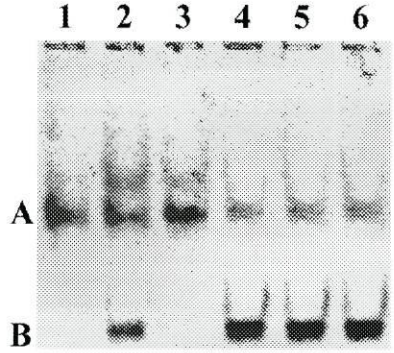


Fig. 3. Native-PAGE of GST_M1AB and GST_CF600 purified proteins obtained from cells grown on different conditions. *Pseudomonas* sp. M1 (lane 1,2, and 3) and *Pseudomonas* sp. CF600 (lane 4, 5, and 6) were grown on pyruvate in the presence (lane 1 and 6) of 400 mg/l phenol or in its absence (lane 2, 5), or in 400 mg/l of phenol (lane 3 and 4) as sole carbon source.

4. Discussion

In this work we report the purification, N-terminal sequencing and activity studies on GSTs extracted from four different *Pseudomonas* sp. strains which have in common the ability to grow on phenol plus from *P. fluorescens* ST. In these strains GST-coding genes, to present time knowledge, are not located within the different phenolic operons present in these strains. The purification procedure by affinity chromatography, their activity towards standard GST substrates and the characteristic amino acidic residues found in their N-terminal sequences concur to confirm these proteins as real GSTs. From their N-terminal sequences all the purified GSTs, except GST_CF600 and GST_F1, resulted to be different proteins. However, native gel electrophoresis proved that also these two proteins are actually different GSTs. Many bacterial GSTs belong to the theta class of GSTs, which includes representatives from plants, insects, mammals and fungi. Members of this class are characterized by a considerable sequence variability and appear to use a serine residue, instead of a tyrosine residue, in the activation of the sulfhydryl group of bound GSH. This has also been proven by site direct mutagenesis performed on theta class GSTs (Board et al., 1995). In the purified proteins presented here a serine residue is located in a restrict range of positions, from position 7 to 12, which are coincident with that of other bacterial GSTs. Moreover, other characteristics of these GSTs are in common with other GSTs, such as a low activity towards CDNB and a low concentration inside the cell.

It was shown that in strain M1, when grown on phenol, GST activity levels correlate positively to phenol concentration and to catechol 1,2-dioxygenase activity. This result could indicate a direct correlation between GST activity and phenol catabolism. The implication of GSTs in aromatic compounds degradation has been receiving greater attention since several GST coding genes have been found within catabolic operons or gene clusters involved in the metabolism of these compounds (Taira et al., 1992;. Bartels et al., 1999; Zhou et al., 2001; Hofer et al., 1994; Lloyd-Jones and Lau, 1997; Daubaras et al., 1996). However, their role in the catabolic process is not evident since, apparently, they are not required for the utilization of the corresponding aromatic compounds (Bartels et al., 1999; Hofer et al., 1994). The widespread presence of GST encoding genes in aromatic compounds-degrading bacteria does not seem to be fortuitous and their implication in improving cell fitness has been proposed (Bartels et al., 1999). It was demonstrated that, in strain M1, not only phenol toxicity and/or catabolism increases GST activity, but that different organic acids also affect GST levels. In particular a negative correlation between the specific growth rate determined for each substrate and GST activity levels has been observed. Moreover also the levels of GST induction upon addition of phenol depended on the organic acid present in the growth medium. These results suggested that GST induction can represent the response to a specific metabolic stressing condition determined by the presence of phenol and by the metabolic status of the cell. Indeed, a significant GST induction was not found when cells were grown on phenol at non-toxic concentrations, nor

in agreement with the fact that in the laboratory standard conditions, GST activity does not seem to be necessary for cell growth on aromatic compounds. However, in the natural environment, bacteria are subjected to stressing conditions and the presence of GST-encoding genes linked to or located within catabolic operons for aromatic compounds degradation could represent an evolutionary advantage.

In these experiments a maximum of three times induction of GST activity was observed. This is an indication that *gst* genes are not co-transcribed with the catabolic genes. However, a study on the activities of GST enzymes from several biphenyl degrading strains has shown a variation of induction factors between 2 and 30 when cells were grown on this substrate, even though most of the *gst* genes analyzed were located inside the corresponding catabolic gene cluster and apparently in the same position (Bartels et al., 1999). It is possible to hypothesize that some *gst* genes are under the control of an independent promoter or that the GST activity depends on post-translational regulation. In *Ochrobactrum anthropi* the transcription of a GST-encoding gene is induced by atrazine and other aromatic compounds, and also the GST protein amounts show corresponding increases. However, no increase in GST activity could be observed in induced conditions (Favaloro et al., 2000).

A correlation between GST activity and protein dimerization was found. In fact the monomeric form of GST extracted from strain M1 grown on phenol or on pyruvate plus phenol was completely absent, while the two forms were present when no induction, or a low induction, of GST activity was found. In CF600_GST, whose basal levels did not change in any of the investigated growth conditions, the monomeric form was always prevalent. These data suggest that the dimerization process may constitute an important aspect in the modulation of GST activity which would represent a quick response to environmental changes for cells.

In strain M1 at least two GSTs are present. At the moment, it is not possible to assign the monitored activity to either protein and further experiments will be necessary to understand the function of the two GSTs in this strain.

Concluding Remarks

...We are continually faced with a series of great oportunities disguised as insoluble problems ..

John W. Gardner

Thesis Achievements and future perspectives

The experimental work reported in this thesis was focused on some important aspects of aromatic compounds degradation in *Pseudomonas* spp. mainly concerning the relationship between bacteria and environment in the biodegradation process. Bacteria that colonize polluted sites are subjected to extremely tough competition with other microbial residents of the same niche. Therefore, the successful assembly of a degradation pattern does not guarantee per se the survival of a particular strain. Transcriptional regulation of biodegradative genes and operons became a critical aspect, in that it must ensure both responsiveness to the polluting substrate and a suitable connection with the physiological state of the bacteria.

In the latest years, after a huge amount of data had been accumulated on catabolic genes sequence, structure and function as well as on their regulation, the integration of the metabolism of a specific compound within the general cell metabolism has attracted the attention of many researchers.

The results herein reported focus on the styrene degradation in *Pseudomonas fluorescens* ST as model system. The regulation of this pathway depends on a specific two-component regulatory system. This kind of regulation associated to aromatic compounds degradation has been only found in few microorganisms and has been poorly investigated.

The first approach was a physiological characterization of the expression of styrene catabolism genes in the presence of alternative carbon sources. We found that this catabolic pathway was subjected to catabolite repression, whereas the regulatory operon was constitutively expressed. This finding suggested that the main regulatory element was the active/inactive form of the regulator, that is its phosphorylated state. For this reason we started to purify the StyR regulator. Unfortunately most of the over-expressed protein was only found in inclusion bodies which were purified to obtain anti-StyR serum. Studies on StyR led to findings that somehow changed our previous conclusions. In fact, we found that StyR amounts were modulated in the different growth conditions and even growth phases. This means that StyR is subjected to a post-translational regulation and that not only its phosphorylation state, but also its availability are responsible for the catabolic operon gene expression. The presence of styrene in the growth medium increased StyR stability, while in its absence StyR concentration decreased to became almost absent in the stationary phase.

In order to achieve new insights with respect to the regulation of *sty* catabolic genes we performed a detailed analysis of the *PstyA* (promoter of the *sty* catabolic genes) sequence. Several putative *cis*-acting elements were found and their importance was checked in bandshift experiments and promoter expression experiments. Activities of *PstyA* fusions with a reporter system, ranging from 0 to 150 induction fold, were obtained and allowed the assignment of the critical regions involved in this

promoter's tight expression. Furthermore, for the first time, the involvement of the integration host factor (IHF) in the regulation of a promoter controlled by a two-component regulatory system was clearly demonstrated. This factor is responsible for 75 % of the induction level of the system most probably by bringing closer two distal regions where StyR can bind. Still, also for the first time, the binding of StyR with the *PstyA* was demonstrated in an innovative type of experimental procedure (South-Western) taking the advantage of the anti-StyR serum produced. Based on the results obtained, a possible model of *PstyA* regulation in induced and uninduced conditions has been proposed.

Another important factor which can affect not only biodegradation efficiency, but also cell survival is the toxicity of aromatic compounds. Hence, a strategy was developed in order to identify genes codifying enzymes that function as protective agents or are part of protective mechanisms against styrene toxicity. This kind of genes were not obtained, probably due to the screening strategy. However, the enzymatic results demonstrated that styrene triggers a sort of "regulative noise". These data suggested that this compound may activate an unknown regulator that is able to recognize other catabolic operons as a fortuitous inducer. This induction could lead to the synthesis of enzymes with low substrate specificity which are able to biotransform styrene into a less toxic compound. This mechanism can function as a detoxification process since it would leads to a decrease of styrene inside the cells.

Finally, the occurrence and possible role of a known protective enzyme, glutathione S-transferase (GST), in *Pseudomonas* phenol degradative strains was investigated. This enzyme was found in all the tested strains, and data obtained suggest that they play different roles within the cells. In particular, in one *Pseudomonas* strain, named M1, it was observed that GST activity was induced in the presence of phenol compounds and in less favorable metabolic growth conditions. Moreover, the GST induction level also depended on phenol concentration indicating that in this strain, GST functions as a protective agent against phenol toxicity. The observation that, in the presence of phenol, almost all the protein was in dimeric form, suggests that, induction of GST activity may be the result of the change from monomeric to dimeric form of this protein.

The results obtained can constitute the basis for the forthcoming research approaches on these subjects. In particular, strategies should involve the study of the StyR protein once a successful purification strategy has been found. This point is probably the borderline between the understanding of the role of phosphorylation on the control of *sty* gene expression and the actual knowledge.

Another interesting point to be deeply investigated is the hypothesis of the existence of degradative operons that can be activated to decrease the toxicity effects of the aromatic compounds within the cells. According to my knowledge this is the first time that such a mechanism is proposed.

The involvement of the GST protein in phenol catabolism is also demonstrated for the first time in this thesis. This is another interesting field of research which should be further investigated. Here, only a first approach was done. However, it seems most probable that some other GST proteins play a role as defense agents against aromatic compounds exposure. Together with the previous approach, the emerging data might contribute to elucidate some of the defense mechanisms against aromatic compounds stress.

Finally, the exponential development of new molecular and biochemical tools, like *Pseudomonas* bio-chips, and the elaboration of a functional database of the almost conclude *Pseudomonas putida* and *fluorescens* genome projects will be very useful for the understanding of the several molecular

mechanisms triggered during aromatic degradation.
The several approaches of this thesis resulted in:

a. the production of the following publications:

Santos, P. M., J. M. Blatny, I. Di Bartolo, S. Valla, and E. Zennaro. 2000. Physiological analysis of the expression of the styrene degradation gene cluster in *Pseudomonas fluorescens* ST. Appl. Envir. Microbiol. 66:1305-1310.
Santos, P. M., I. Di Bartolo, J. M. Blatny, S. Valla, and E. Zennaro. 2001. New broad-host-range promoter probe vectors based on the plasmid RK2 replicon. FEMS Microbiology Letters 195:91-96.
Santos, P. M., G. Mignogna, H. J. Heipieper, and E. Zennaro. 2002. Occurrence and properties of glutathione S- transferases in phenol-degrading *Pseudomonas* strains. Res. Microbiol. 153:89-98-
Santos, P. M., L. Leoni, I. Di Bartolo, and E. Zennaro. 2002. Integration host factor is essential for the optimal expression of *styABCD* operon in *Pseudomonas fluorescens* ST (submitted).

b. the deposite in the GeneBank or Swiss-Prot data bases of the following sequences:

AF187995 - Promoter probe vector pJB785TT, complete sequence
AF187996 - Promoter probe vector pPR9TT, complete sequence
AF32051 - Promoter probe vector pJB785TTKm1, complete sequence
P82996 -Glutathione S-transferase from *Pseudomonas putida* (strain PaW85)
P82997 - Glutathione S-transferase from *Pseudomonas* sp. (strain CF600)
P82998 - Glutathione S-transferase from *Pseudomonas putida* (strain F1)
P82999 - Glutathione S-transferase from *Pseudomonas* sp. M1
P83000 - Glutathione S-transferase from *Pseudomonas* sp. M1
P83001 - Glutathione S-transferase from *Pseudomonas fluorescens* ST

c. comunications in the following congresses:

"*Pseudomonas'97*"- Madrid, Spain, 4-8/09/97: Effect of solvent adaptation on the resistance to antibiotics in the solvent-resistant *Pseudomonas putida* S12. Isken, S, **P. M. A. C. Santos**, and J. A. M. de Bont (poster).
"Convegno congiunto ATTI" – Montesilvano, Italy, 1-4/10/98: Studio dell'espressione dei geni catabolici per lo stirene in *Pseudomonas fluorescens* ST. **P.M.A.C. Santos,** M. C. Terron, S. Iurescia, M. Ruzzi, A. Ficca, E. Zennaro (poster).
"I FISV" – Riva del Garda, Italy, 2-6/10/99: Regolazione della degradazione dello stirene in *Pseudomonas fluorescens* ST. Di Bartolo, I., **P.M. Santos**, E. Zennaro (poster).
"Biotechnology 2000" – Berlin, Germany, 3-8/09/00: Effects of different growth conditions on styrene degradation in *Pseudomonas fluorescens* ST. **P. M. Santos**, I. Di Bartolo, E. Zennaro (poster).
"XII Congresso Nacional de Bioquímica"- Póvoa de Varzim, Portugal, 28-30/09/00: Regulation of a two-component regulatory system involved in styrene degradation in *Pseudomonas fluorescens* ST. **P. M. Santos**, I. Di Bartolo, E. Zennaro (oral presentation).
"II FISV" – Riva del Garda, Italy, 30/09-04/10/00: Characterization of the regulatory system involved in styrene degradation in *Pseudomonas fluorescens* ST: **P.M. Santos**, I. Di Bartolo, L. Leoni, E. Zennaro (poster).
"10th International Congress in Biotechnology", Madrid, Spain, 8-11/07/01: Regulation of styrene gene cluster in *Pseudomonas fluorescens* ST. **P.M. Santos,** I. Di Bartolo, L. Leoni, E. Zennaro (poster).

conditions in phenol-degradative *Pseudomonas* strains. **P. M. Santos**, G. Mignogna, E. Zennaro, and H. J. Heipieper (poster).
"*Pseudomonas* 2001" – Bruxells, Belgium, 17-24/09/01: Involvement of IHF in the regulation of the expression of the styrene catabolic operon. **P. M. Santos**, I Di Bartolo, S. Merlo, L. Leoni, and E. Zennaro (poster).
"*Pseudomonas* 2001" – Bruxells, Belgium, 17-24/09/01: *Cis* acting elements in the promoter of *styABCD* operon of *Pseudomonas fluorescens* ST: L. Leoni, V. di Stefano, I. Di Bartolo, S. Merlo, **P. M. Santos**, and E. Zennaro (poster).
"III FISV" – Riva del Garda, Italy, 28/09-03/10/01: Identification of regulatory DNA motifs involved in transcriptional control of the *styABCD* styrene catabolic operon. L. Leoni, V. di Stefano, I. Di Bartolo, S. Merlo, **P. M. Santos**, and E. Zennaro (poster).

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Appendix

Appendix I

Growth of *Pseudomonas fluorescens* ST in styrene

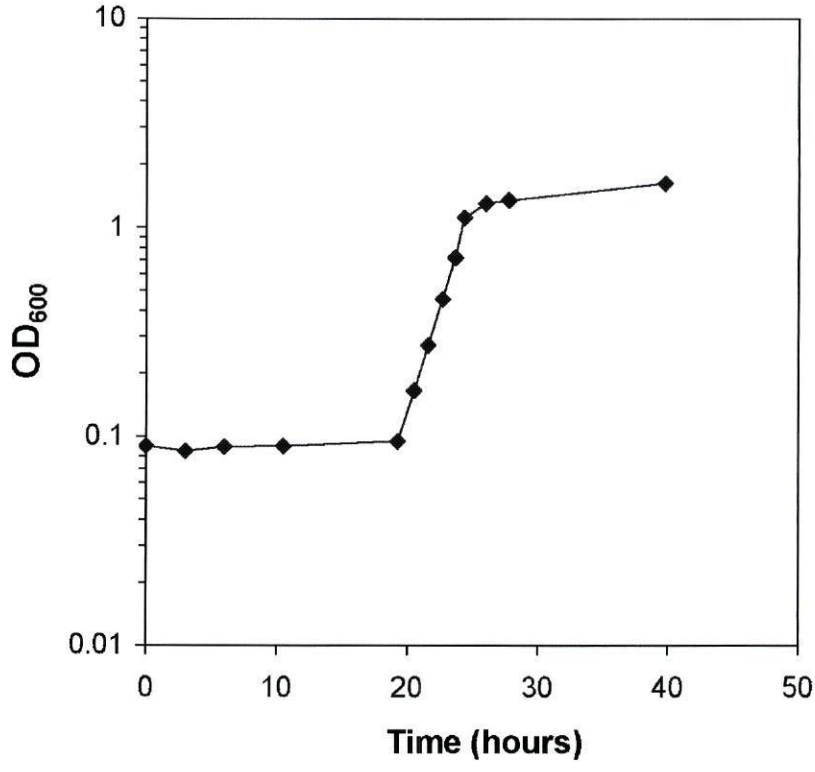


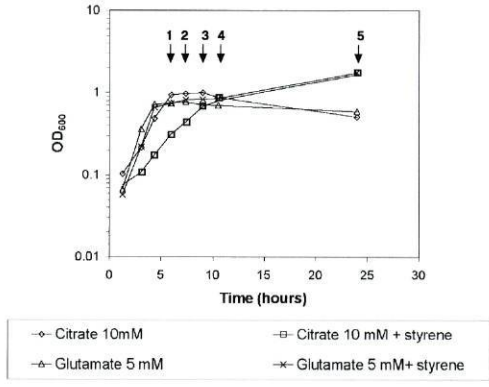
Figure. Growth of *Pseudomonas fluorescens* ST in mineral medium (Hartmans et al., 1989) containing styrene as sole carbon source

Appendix II

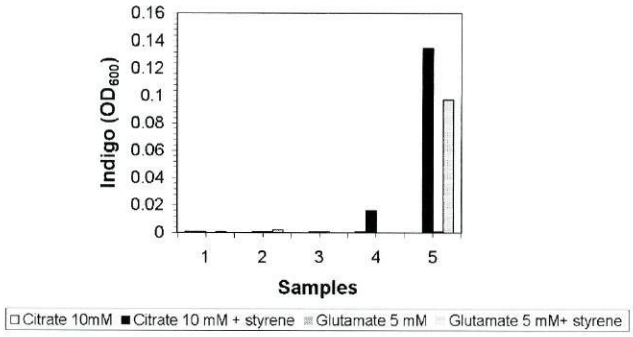
Expression of the *styABCD* catabolic operon in different carbon sources

1. Repressing carbon sources (citrate and glutamate)

A



B



C

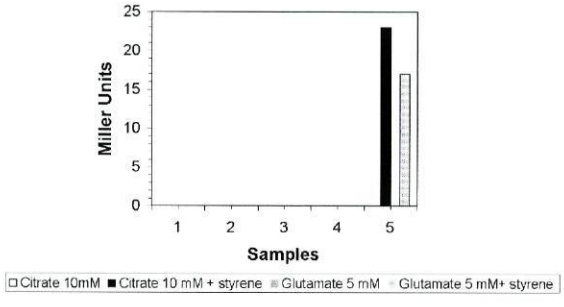


Figure. A. Growth of *Pseudomonas fluorescens* ST harboring pPR9TTPa in 10 mM citrate or in 5 mM glutamate in the presence or in the absence of styrene. In **B** the activity of SMO and in **C** the activity of *PstyA::lacZ* are shown, respectively. Each growth condition is referred on the respective legend. Samples (1 to 5) were collected for activity assays along the growth at the times indicated by the arrows.

Appendix II

Expression of the *styABCD* catabolic operon in different carbon sources

2. Non-repressing carbon sources (Arginine and Glycerol)

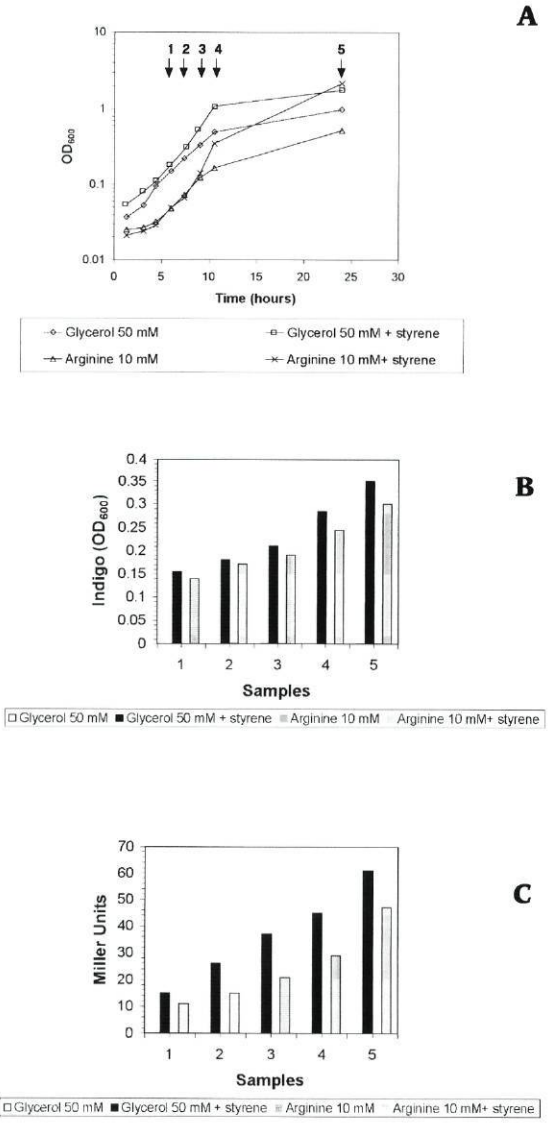


Figure. A. Growth of *Pseudomonas fluorescens* ST harboring pPR9TTPa in 50 mM glycerol or in 5 mM arginine in the presence or in the absence of styrene. In **B** the activity of SMO and in **C** the activity of *PstyA*::*lacZ* are shown, respectively. Each growth condition is referred on the respective legend. Samples (1 to 5) were collected for activity assays along the growth at the times indicated by the arrows.

Appendix III

Expression of the *styABCD* catabolic operon in 0.2 % succinate at pH 6.0 and 7.0

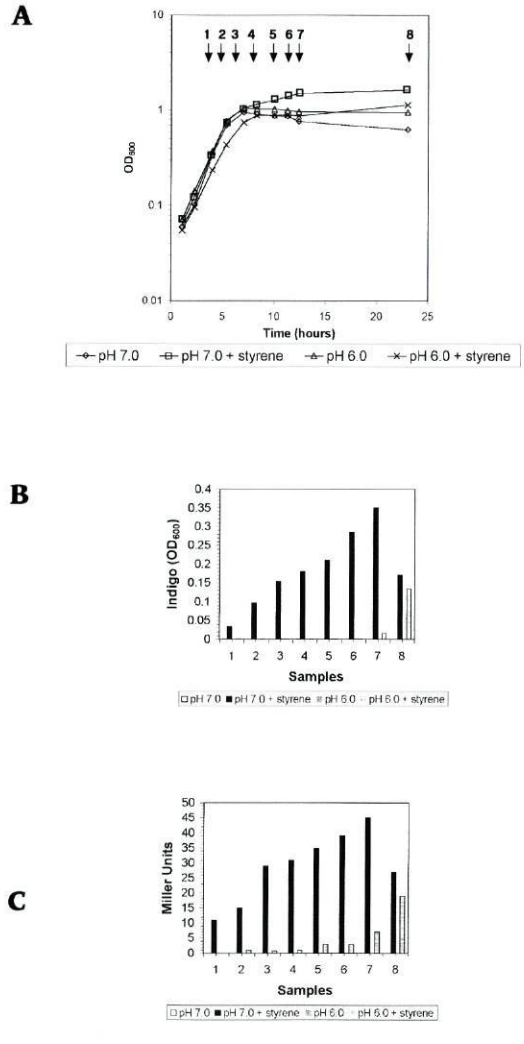


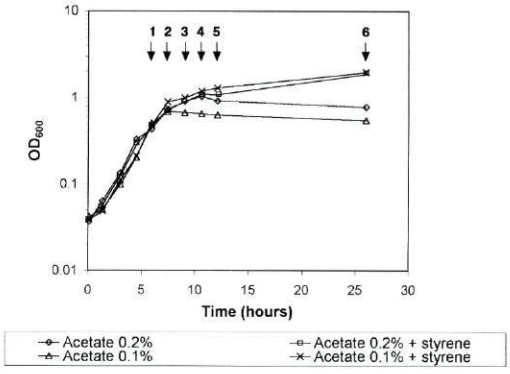
Figure. A. Growth of *Pseudomonas fluorescens* ST harboring pPR9TTPa in mineral medium (initial pH 6.0 or pH 7.0) containing 10 mM succinate in the presence or in the absence of styrene. In **B** the activity of SMO and in **C** the activity of *PstyA::lacZ* are shown, respectively. Each growth condition is referred on the respective legend. Samples (1 to 8) were collected for activity assays along the growth at the times indicated by the arrows.

Appendix IV

Expression of the *styABCD* catabolic operon in different concentrations of acetate and glucose

1. In different concentrations of acetate in the presence or in the absence of styrene

A



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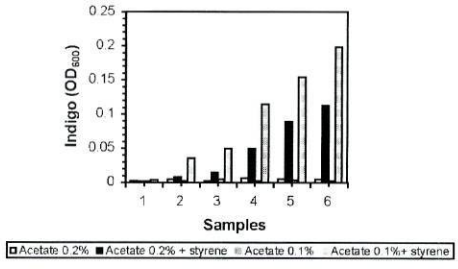


C

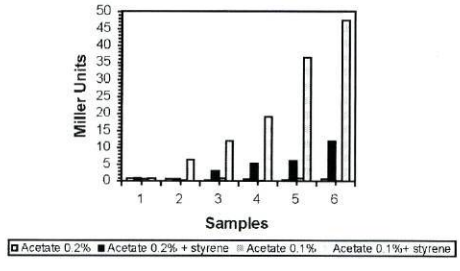


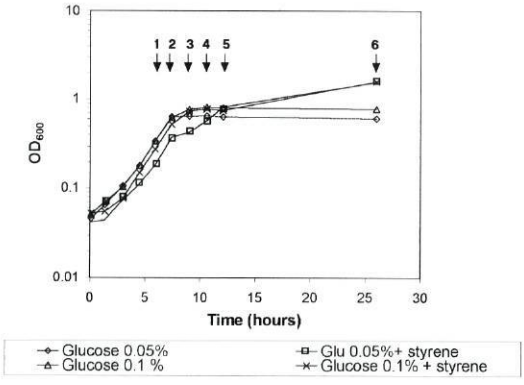
Figure. A. Growth of *Pseudomonas fluorescens* ST harboring pPR9TTPa in 0.1 or 0.2% acetate in the presence or in the absence of styrene. In **B** the activity of SMO and in **C** the activity of *PstyA::lacZ* are shown, respectively. Each growth condition is referred on the respective legend. Samples (1 to 6) were collected for activity assays along the growth at the times indicated by the arrows.

Appendixm IV

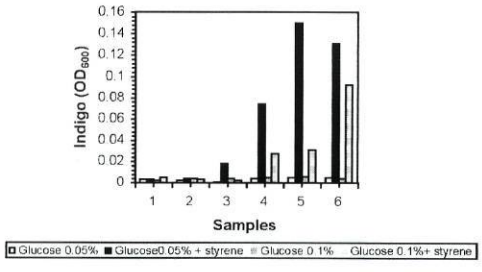
Expression of the *styABCD* catabolic operon in different concentrations of acetate and glucose

2. In different concentrations of glucose in the presence or in the absence of styrene

A



B



C

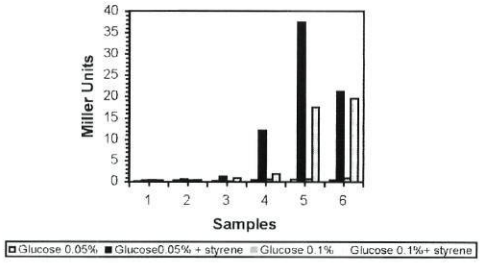


Figure. A. Growth of *Pseudomonas fluorescens* ST harboring pPR9TTPa in 0.05 and 01 % glucose in the presence or in the absence of styrene. In **B** the activity of SMO and in **C** the activity of *PstyA::lacZ* are shown, respectively. Each growth condition is referred on the respective legend. Samples (1 to 6) were collected for activity assays along the growth at the times indicated by the arrows.

Appendix V

Native gel of the purified GSTs from different *Pseudomonas* strains

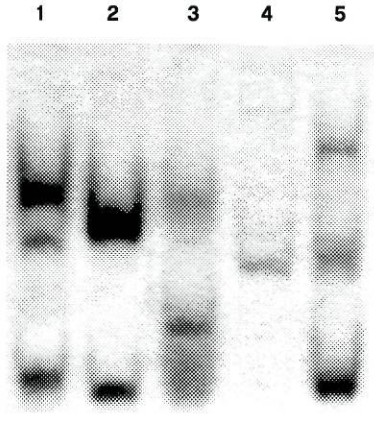


Figure. Native-PAGE of the affinity chromatography purified GSTs. 1-5, protein products upon purification of cell crude extracts from *Pseudomonas* sp. M1 (1), *Pseudomonas* sp. CF600 (2), *Pseudomonas putida* F1 (3), *Pseudomonas putida* PaW85(*pheBA*) (4) and *Pseudomonas fluorescens* ST (5).

Appendix VI

GST activity in *Pseudomonas* sp. M1 and *Pseudomonas* sp. CF600 cells toxified with non-degradable aromatic compounds

Table 4. Effect of increasing concentrations of salicylate and styrene on the activities of GST in *Pseudomonas* sp. M1 and *Pseudomonas* sp. CF600 grown in 10 mM succinate[a]

		Strains			
		Pseudomonas **sp. M1**		*Pseudomonas* **sp. CF600**	
Toxin	**Conc (mg/l)**	$\mu(h^{-1})$[b]	**GST**[c]	$\mu(h^{-1})$[b]	**GST**[c]
	0	0.93	1.3	0.97	0.49
Salicylate	400	0.85	1.4	0.89	0.54
	800	0.79	1.4	0.75	0.53
	1200	0.59	1.6	0.66	0.45
	1600	0.31	1.7	0.34	0.45
	0	0.93	1.4	0.97	0.42
Styrene	50	0.88	1.2	0.89	0.45
	100	0.75	1.3	0.71	0.46
	150	0.57	1.5	0.65	0.44
	200	0.24	1.5	0.21	0.42
	300	0.11	1.6	0.10	0.43

a Salicylate and styrene were added to the cultures after 3 h of exponential growth and growth was followed for further 3 h before sampling.
b growth rate
c Glutathione S-transferase specific activity given in nmol min^{-1} mg $protein^{-1}$