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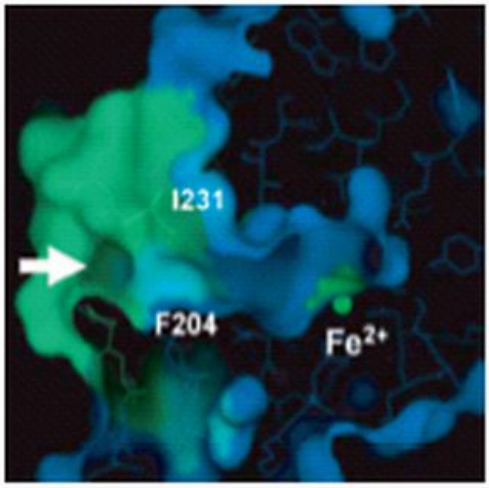
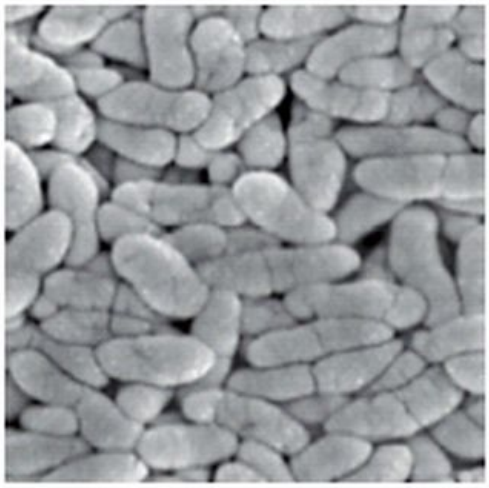
Pseudomonas

A Model System in Biology





Edited by Juan-Luis Ramos and Alain Filloux

Pseudomonas

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Edited by Juan-Luis Ramos, *CSIC, Granada, Spain*

Volume 1: Genomics, Life Style and Molecular Architecture
Volume 2: Virulence and Gene Regulation
Volume 3: Biosynthesis of Macromolecules and Molecular Metabolism
Volume 4: Molecular Biology of Emerging Issues
Volume 5: A Model System in Biology

Pseudomonas

Volume 5
A Model System in Biology

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PREFACE

It all began 20 years ago when Jack Sokatch first published his outstanding contribution entitled *The Biology of Pseudomonas* back in 1986. This incursion into the world of *Pseudomonas* was followed by two books published by the American Society of Microbiology containing the presentations of the *Pseudomonas* meetings held in Chicago in 1989 and Trieste in 1991. The earlier volume was edited by Simon Silver, Al Chakrabarty, Barbara Iglewski and Sam Kaplan and the latter by Enrica Galli, Simon Silver and Bernard Witholt.

Back in 2002 we believed that the time was ripe for a new series of books on *Pseudomonas* due to its current importance in human and plant pathogenesis, biolfims, soil and rhizosphere colonization, etc. After a meeting with Kluwer (now Springer) in August 2002 during the XI IUMS conference in Paris (France), it was decided to take on such an endeavour. In less than a year from that meeting, the first three volumes of the *Pseudomonas* series saw the light thanks to a group of outstanding scientists in the field, who after devoting much of their valuable time, managed to complete their chapters under the guidance of Juan L. Ramos, who acted as Editor.

To ensure the high standard of each chapter, renowned scientists participated in the reviewing process. The three books collected part of the *explosion* of new vital information on the genus *Pseudomonas* grouped under the generic titles of "Vol. I. *Pseudomonas*: Genomics, Life Style and Molecular Architecture", Vol. II. *Pseudomonas*: Virulence and gene regulation; Vol. III. *Pseudomonas*: Biosynthesis of Macromolecules and Molecular Metabolism.

A rapid search for articles containing the word *Pseudomonas* in the title in the last 10 years produced over 6000 articles! Consequently, not all possible topics relevant to this genus were covered in the three first volumes. A new volume was therefore due. *Pseudomonas* volume IV edited

by Roger Levesque and Juan L. Ramos came into being with the intention of collecting some of the most relevant emerging new issues that had not been dealt with in the three previous volumes. This volume was arranged after the *Pseudomonas* meeting organized by Roger Levesque in Quebec (Canada). It dealt with various topics grouped under a common heading: "*Pseudomona*s: Molecular Biology of Emerging Issues".

Yet the *Pseudomonas* story was far from being complete and a new volume edited by Juan L. Ramos and Alain Filloux was deemed to be necessary. This fifth volume has been conceived with the underlying intention of collecting new information on the genomics of saprophytic soil *Pseudomonas*, as well as the functions related to genomic islands.

Pseudomonas are ubiquitous inhabitants and this new volume explores some fascinating biodegradative properties of soil and water *Pseudomonas* and their life styles and sheds further light on the wide metabolic potential of this group of microbes. This volume also explores how *Pseudomonas* responds and reacts to environmental signals, including detection of cell density in one of the most sophisticated quorum-sensing systems. It also explores issues related to pathogenesis and gene regulation.

Chapters in *Pseudomonas* volume 5 have been grouped under the following topics: Genomics, Physiology and Metabolism, Databases, Gene Regulation, Pathogenesis, and Catabolism and Biotransformations. The chapters under the heading Genomics constitute an in-depth analysis of the genome of *Pseudomonas fluorescens* and the organization of glycosylation islands in *Pseudomonas aeruginosa*. The Physiology and Metabolism section collects five chapters that deal with the catabolic potential of *Pseudomonas* against certain xenobiotic compounds (styrene, xylenes, carbazole), a naturally abundant chemical, such as phenylacetic acid, and how *Pseudomonas* reacts to stress at the membrane level. The section on Databases collects the current information on the collection of mini-Tn*5* mutants of *Pseudomonas putida* kept in Granada (Spain). Under Gene Regulation we find several chapters dealing with quorum sensing, analysis of the family of two-component systems and their role in *Pseudomonas*. One of the chapters focuses on the biophysical approaches necessary to understand regulator/effector interactions. The section on Pathogenesis includes an exciting chapter dealing with the mechanisms of internalization of a pathogen such as *P. aeruginosa*, and finally under Catabolism and Biotransformations we have grouped the current existing knowledge on histidine catabolism and biosynthesis of polyhydroxyalkanoates.

It would not be fair not to acknowledge that this fifth volume would never have seen the light if it were not for a group of outstanding scientists in the field who have produced enlightening chapters to try to complete the story that began with the four previous volumes of the series. It has been an honour for us to work with them and we truly thank them.

The review process has also been of great importance to ensure the high standards of each chapter. Renowned scientists have participated in the review, correction and editing of the chapters. Their assistance is immensely appreciated. We would like to express our most sincere gratitude to:

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Juan-Luis Ramos and Alain Filloux

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GENOMICS OF *PSEUDOMONAS FLUORESCENS* PF-5

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1. INTRODUCTION

Pseudomonas spp. are ubiquitous inhabitants of soil, water, and plant surfaces, where they represent an important component of microbial assemblages.[7,138] Members of the species *P. fluorescens* typically exist as saprophytes or in a commensal relationship with plant or animal hosts. These bacteria are known for their capacity to utilize a striking variety of organic compounds as energy sources[126] and to produce a wide variety of metabolites.[64] Certain plant-associated strains produce phytohormones[24,72,92] or metabolites that alter plant hormone levels,[112] and these influence the growth and development of their plant associates.[2,3] Other strains produce proteins that alter the plant's capacity to avoid frost injury.[63,68] Still others can alter the availability of key nutrients[35,130] and enhance the nutritional status of their plant hosts.[65] Antibiotic production by rhizosphere-inhabiting

Juan-Luis Ramos and Alain Filloux (eds.), Pseudomonas, 3–30.

strains of *Pseudomonas* spp. can influence both the fitness of the producing strain[77] and the composition of microbial assemblages, including pathogens that would otherwise jeopardize plant health.[38] Commensal *Pseudomonas* spp. are intricately enmeshed in plant and soil biology through all of these diverse activities, and their functions as biological control agents have distinguished them as microorganisms with immense effects on agricultural productivity.

2. *PSEUDOMONAS* SPP. AS BIOLOGICAL CONTROL AGENTS

Throughout the history of agriculture, humans have struggled to reduce the adverse effects of plant disease on their crops. Early agriculturalists realized the benefits of cultural practices such as crop rotation and the use of organic soil amendments in promoting plant productivity. It is now well established that many of these effects are achieved by promoting the natural microbiological processes that keep plant disease in check. Cultural practices were mainstays of traditional agricultural systems and still provide the primary approaches for management of many soilborne diseases today. For example, disease suppressive soils, into which pathogen(s) can be introduced without causing the expected levels of disease severity, can result from alterations in cropping patterns or other cultural practices. *Pseudomonas* spp. that produce specific antibiotics have been associated convincingly with soil suppressiveness.[139] This compelling evidence for the role of *Pseudomonas* spp. in natural processes of biological control has built even greater enthusiasm for the potential of these bacteria as biological control agents.

The potential of specific strains of *Pseudomonas* spp. for suppression of plant disease in agriculture has been demonstrated in hundreds, if not thousands, of experiments worldwide. Typically, a collection of strains isolated at random from plant surfaces are inoculated individually onto seeds, roots, or aerial plant tissues and inoculated plants are compared to noninoculated plants in disease assays. *Pseudomonas* spp. have been identified repeatedly for their suppressive effects on plant pathogens in these studies. For example, in greenhouse studies in which a random collection of culturable bacteria were screened directly for suppression of *Pythium* damping-off, fluorescent pseudomonads comprised a large proportion (33–100%) of the effective biocontrol strains.[25,41,69,124,143] There is no question that strains representing diverse genera of Gram-negative and Gram-positive bacteria can suppress plant disease, but fluorescent pseudomonads are commonly among the most

effective antagonists selected for suppression of both soilborne and aerial diseases of plants.[68,88,127,138] Therefore, the genus has been the focus of ecological and mechanistic research evaluating biological control of plant disease.

2.1. *P. fluorescens* Strain Pf-5

Strain Pf-5 was isolated from the soil in College Station, Texas, USA in the late 1970s. It was first described for its capacity to suppress soilborne diseases of cotton caused by *Rhizoctonia solani*[45] and *Pythium ultimum*.[46] *R. solani* and *P. ultimum* are widespread pathogens with broad host ranges that constrain food and fiber production worldwide.[75] Since it was first described, Pf-5 has been shown to suppress these pathogens on plant hosts including cucumber,[60] pea, and maize (M. D. Henkels and J. Loper, unpublished). Pf-5 also suppresses a number of other soilborne or residue-borne fungal pathogens. When inoculated onto wheat straw residue, Pf-5 suppresses ascocarp formation by the tan spot pathogen of wheat, *Pyrenophora tritici-repentis*.[95] Pf-5 also suppresses dollar spot of turf grass caused by *Sclerotinia homoeocarpa* and leaf spot of turf grass caused by *Drechselera poae*. These are widespread, destructive diseases affecting golf courses, home lawns, and amenity turf areas.[107] In addition, Pf-5 suppresses Fusarium crown and root rot of tomato, caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*[113] and seed piece decay of potato caused by the bacterial pathogen *Erwinia carotovora*.[145]

2.1.1. Pf-5 Produces a Broad Spectrum of Antibiotics

Pf-5 produces a suite of antibiotics (Figure 1) including pyrrolnitrin,[45] pyoluteorin,[46,61,85,87] and 2,4-diacetylphloroglucinol (DAPG)[86]; it also produces hydrogen cyanide[60] and two siderophores: a pyoverdine of unknown structure and pyochelin (or a related compound). In addition, three gene clusters encoding for the production of unknown secondary metabolites were found in the recently completed genomic sequence of Pf-5. Each of the known secondary metabolites produced by Pf-5 has a different spectrum of activity against plant pathogens, and their roles in biological control have been established in various biological control organisms.[39,103] The spectrum of antibiotics produced by Pf-5 is remarkably similar to that produced by the well-characterized strain *P. fluorescens* CHA0, which was isolated from roots of tobacco grown in a soil near Payerne, Switzerland.[39,123] Many other biological control strains produce a subset of metabolites produced by Pf-5 and CHA0, whereas other strains

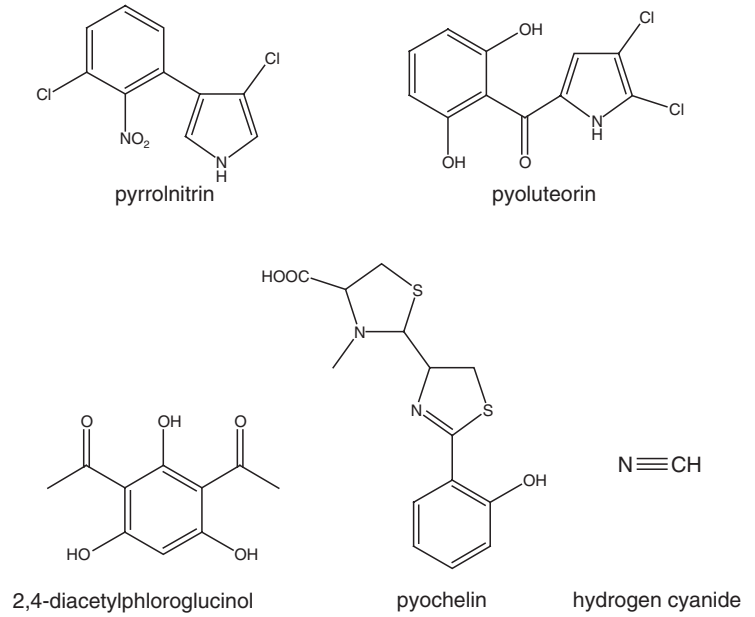

Figure 1. Secondary metabolites produced by *P. fluorescens* Pf-5.

produce different antibiotics (such as phenazines) that are not produced by Pf-5.

2.1.2. Regulation of Antibiotic Production in Pf-5

Antifungal metabolite production by biological control strains of *Pseudomonas* spp. is controlled by complex regulatory networks that respond to environmental and density-dependent signals and are coupled to the physiological status of the bacterium.[39,96,97] Loci known to regulate the production of antifungal metabolites in Pf-5 include a two-component regulatory system encoded by *gacS* and *gacA*[20,141]; the sigma factor σ^{S}[109,141]; the protease Lon[142]; *ptsP*, a paralog of sugar phosphotransferase enzyme I[140]; and regulators present in biosynthetic gene clusters.[11,85] Many of these loci control multiple phenotypes including stress response in *P. fluorescens*,[109,120,141,142] indicating that regulation of antibiotic production is intricately enmeshed in the physiology of the bacterial cell. A classic quorum-sensing system based upon *N*-acyl homoserine lactones has not been found in Pf-5, but pyoluteorin and DAPG serve as autoinducers of their own production.[10,11]

3. THE GENOMIC SEQUENCE OF *P. FLUORESCENS* PF-5

The genome of Pf-5 is composed of a single circular chromosome of 7.07 Mb (Figure 2).[94] To date, it is the largest of the sequenced genomes of *Pseudomonas* spp. Below, we present a brief overview of the general features of the genomic sequence in relation to the lifestyle and biological control properties of this bacterium. The Pf-5 genomic sequence data discussed below was published previously[94] and can be accessed readily on the internet (www.pseudomonas.com and http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

3.1. Environmental Fitness

As a rhizosphere inhabitant, Pf-5 must have access to the varied nutrients found in seed and root exudates, and survive stresses imposed by the environment and microbial competitors in the natural environment.

3.1.1. Nutrient Acquisition

The genome of Pf-5 has genes coding for the consumption of a broad spectrum of organic acids, sugars, and amino acids, including those typically found in seed or root exudates.[71,94] A complete metabolic pathway prediction generated by an automated analysis using Pathway Tools,[90] analogous to the EcoCyc database of *Escherichia coli* metabolism,[54] is available at www.pseudomonas.com.

For iron acquisition, the genome specifies the biosynthesis of two siderophores, pyoverdine and pyochelin. Pyoverdine biosynthesis and uptake genes are typically organized in two to three clusters in *Pseudomonas* spp.,[104] and Pf-5 fits this pattern, with three gene clusters devoted to these functions. Pyochelin biosynthesis and uptake genes are found in a single gene cluster in the Pf-5 genome, although the organization of the region differs from the well-characterized pyochelin gene cluster of *Pseudomonas aeruginosa*.[80]

Pseudomonas spp. are known to utilize siderophores produced by other microorganisms as sources of iron.[99] In natural habitats on root surfaces, these bacteria can acquire iron by uptake of exogenous siderophores via TonB-dependent, ferric-siderophore receptors, obviating the need to rely on siderophore production alone.[70,102] The Pf-5 genome has 45 genes predicted to encode TonB-dependent receptors, which exceed the 20–30 TonB-dependent receptors found in the genomes of other *Pseudomonas* spp. sequenced to date. Determining the roles of these outer membrane receptors in the ecology of Pf-5 in the soil will be an illuminating subject for future inquiry.

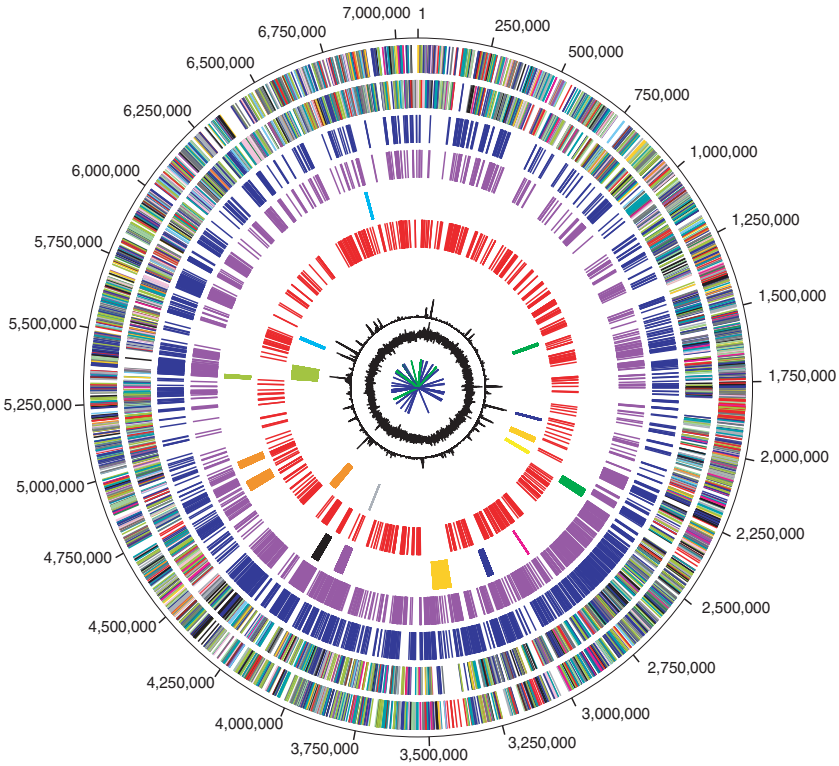

Figure 2. Circular representation of the genome of *P. fluorescens* Pf-5. The outer scale designates coordinates in base pairs (bps), with the origin of replication at 1 bp. The first circle (outermost circle) shows predicted coding regions on the plus strand color-coded by role categories: violet, amino acid biosynthesis; light blue, biosynthesis of cofactors, prosthetic groups and carriers; light green, cell envelope; red, cellular processes; brown, central intermediary metabolism; yellow, DNA metabolism; light gray, energy metabolism; magenta, fatty acid and phospholipid metabolism; pink, protein synthesis and fate; orange, purines, pyrimidines, nucleosides and nucleotides; olive, regulatory functions and signal transduction; dark green, transcription; teal, transport and binding proteins; gray, unknown function; salmon, other categories; blue, hypothetical proteins. The second circle shows predicted coding regions on the minus strand color-coded by role categories. The third circle shows the set of 1,489 *P. fluorescens* Pf-5 genes that are not found in the genomes of *P. aeruginosa* PAO1, *P. syringae* pv. tomato DC3000, and *P. putida* KT2440 (see Figure 4). The fourth circle shows the set of 1,472 genes that are not found in the genomes of *P. fluorescens* SBW25 or PfO-1 (see Figure 5). The fifth circle shows nine secondary metabolite gene clusters coded by color as follows (clockwise from the origin): green, cyclic lipopeptide; pink, hydrogen cyanide; blue, pyoluteorin; gold, the uncharacterized polyketide synthase; purple, pyochelin; black, pyrrolnitrin; orange, pyoverdine; olive, the uncharacterized nonribosomal peptide synthetase; cyan, DAPG. The sixth circle shows REP repeat elements. The seventh circle shows the PFGI-1 mobile island in olive, and putative phage regions as follows (clockwise from the origin): green, prophage 1; blue, prophage 2; gold, prophage 3; yellow, prophage 4; gray, prophage 5; orange, prophage 6; cyan, prophage 7. The eighth circle shows trinucleotide composition. The ninth circle shows percentage G + C in relation to the mean G + C in a 2,000-bp window. The tenth circle shows rRNA genes in green, tRNA genes in blue.

3.1.2. Self-Defense

Efflux of toxic compounds represents an important mechanism of self-protection for *Pseudomonas* spp, and the Pf-5 genome has a large collection of efflux systems predicted to function in resistance to toxic compounds. This collection includes characterized families of multidrug efflux pumps[93]: ATP-Binding cassette (ABC), drug/metabolite transporter (DMT), major facilitator (MFS), and resistance-nodulation-cell division (RND) families (Table 1). Within these families are genes with established or predicted roles in the efflux of secondary metabolites produced by Pf-5 (Table 2). Among the other roles of the many efflux systems in the Pf-5 genome is the export of exogenous toxic compounds, and Pf-5 is known to be resistant to a range of drugs including ampicillin, chloramphenicol, tetracycline, and spectinomycin, and streptomycin (J. Loper, unpublished). Other genes possibly involved in self-defense include homologs of genes conferring resistance to tabtoxin, a phytotoxin produced by *Pseudomonas syringae* pv. tabaci; fusaric acid, a toxin produced by the soilborne plant pathogen *F. oxysporum* that serves as a signal repressing the production of DAPG by *P. fluorescens* CHA0[84]; and copper,[71] a chemical used in agriculture to control plant diseases.

Table 1. Comparison of transporters in different species of *Pseudomonas*.

	P. aeruginosa PAO1	*P. fluorescens* Pf-5	*P. putida* KT2440	*P. syringae* pv. tomato DC3000
Total transporter proteins	423	475	386	322
Genome size (Mb)	6.26	7.06	6.18	6.54
No. of transporters per Mb genome	68	67	63	49
No. of transporters in various classes				
ATP-Dependent	116 (27%)	149 (31%)	124 (32%)	124 (39%)
ATP-binding cassette (ABC) family	104	140	117	119
Ion channels	20 (5%)	16 (3%)	18 (5%)	16 (5%)
Phosphotransferase system (PTS)	6 (1%)	10 (2%)	5 (1%)	4 (1%)
Secondary transporter	277 (65%)	296 (62%)	237 (61%)	174 (54%)
Drug/metabolite transporter (DMT) family	31	36	8	11
Major facilitator (MFS) family	89	95	83	71
Resistance to homoserine /threonine (RhtB) family	11	24	16	7
Resistance-nodulation-cell division (RND) family	17	14	18	12
Unclassified	4 (1%)	4 (1%)	3 (1%)	4 (1%)

[a]For a complete listing of transport systems in these species, see http://www.membranetransport.org

Table 2. Selected examples of transporters with putative roles in secondary metabolite efflux by *P. fluorescens* Pf-5.

Gene encoding efflux pump		Predicted substrate[a]	Homologs in *Pseudomonas* spp.		
			P. putida KT2440	*P. syringae* DC3000	*P. aeruginosa* PAO1
The ATP-binding cassette (ABC) superfamily					
Membrane	ATP-binding				
PFL_2149[b]		Cyclic lipopeptide	N/A[c]	PSPTO2832	N/A
PFL_2797 (*pltK*)		Pyoluteorin[11,47]	N/A	N/A	N/A
PFL_2798 (*pltN*)		Pyoluteorin[11,47]	N/A	N/A	N/A
	PFL_2796 (*pltJ*)	Pyoluteorin[11,47]	N/A	N/A	N/A
PFL_2980[b]		MCF toxin	PP4927	N/A	N/A
PFL_2982[b]		MCF toxin	N/A	N/A	N/A
PFL_3208[b]		Protease	PP2560	PSPTO3330	N/A
PFL_3494[b]		Pyochelin	N/A	N/A	N/A
PFL_3495[b]		Pyochelin	N/A	PSPTO2604	N/A
PFL_4082[b]		Pyoverdine	PP4210	PSPTO2159	PA2390
PFL_4091[b] (*pvdE*)		Pyoverdine[78]	PP4216	PSPTO2153	PA2397
PFL_4175		Pyoverdine	PP3803	PSPTO2139	PA2409
	PFL_4174	Pyoverdine	PP3802	PSPTO2140	PA2408
The drug/metabolite transporter (DMT) superfamily					
PFL_4655		Peptide synthesized from uncharacterized NRPS	N/A	N/A	N/A
The major facilitator superfamily (MFS)					
PFL_5958 (*phlE*)		DAPG [1,6]	N/A	N/A	N/A
The resistance to homoserine/threonine (RhtB) family					
PFL_2800 (*pltP*)		Pyoluteorin[11,47]	N/A	N/A	N/A

[a]Substrate predicted solely by proximity to biosynthetic genes unless a reference is provided.
[b]ABC family transporters with fused ATP-binding domain and permease domain.
[c]N/A indicates no clear homologs found (cut-off was at least 50% identity over half the length).

The genome of Pf-5 also has multiple copies of genes conferring tolerance to oxidative stress (i.e., ten peroxidases, six catalases, and two superoxide dismutases). The numerous copies of these genes support the proposed importance of oxidative stress tolerance in environmental fitness of *Pseudomonas* spp. in the rhizosphere.[57]

3.1.3. Secondary Metabolites and Other Secreted Products

Nearly 6% of the Pf-5 genome is devoted to the production of secondary metabolites, based upon the sizes of the nine biosynthetic gene clusters identified to date. The nine gene clusters specify the biosynthesis of pyoluteorin, DAPG, pyrrolnitrin, HCN, the siderophores pyoverdine and pyochelin and three unknown secondary metabolites. The three cryptic gene clusters were identified by the presence of characteristic sequences of polyketide synthases or nonribosomal peptide synthetases. The structure predicted from bioinformatic analysis of the nucleotide sequence of one of the nonribosomal peptide synthetases[94,101] has been confirmed by purifying the compound from cultures of Pf-5 and subjecting it to chemical analysis.[36a] The compound is a novel lipopeptide with surfactant properties, which can lyse zoospores of *Phytophthora ramorum*, an Oomycete plant pathogen.[36a] Biosurfactant production also has a role in swarming motility of Pf-5.[36a] The lipopeptide biosurfactant and the two remaining cryptic secondary metabolites have not yet been characterized with respect to their roles in biological control. Nevertheless, their discovery provides new directions for research evaluating mechanisms of biological control.

In addition to secondary metabolites, Pf-5 produces other exported products, including exoenzymes, bacteriocins, and an insect toxin that could contribute to biological control. The capacity to degrade chitin, an important fungal cell wall component, and proteins,[114] has been implicated in biological control. Pf-5 produces chitinase and the genome contains a homolog of *chiC*.[29] As typical for the genus, Pf-5 produces an extracellular alkaline protease(s) and has two homologs of *aprA*. The genome also has two homologs of *llpA*, which encode a bacteriocin related to LlpA.[91] Intriguingly, a homolog of *mcf* (for *m*akes *c*aterpillars *f* loppy) is present in the Pf-5 genome. Mcf is an insect toxin produced by the bacterium *Photorhabdus luminescens*, an inhabitant of the gut of entomopathogenic nematodes.[21] If injected into the hemocoel, Pf-5 can kill insects (J. Loper and D. Bruck, unpublished), but the role of Mcf in this toxicity has not been established to date.

3.1.4. Regulatory Circuitry

Pf-5 has an extensive collection of regulatory genes, as predicted for a bacterium with a large genome that lives in a rapidly changing environment. The Pf-5 genome has 68 predicted histidine kinases and 113 predicted response regulators, which exceeds the number predicted for the other sequenced genomes of *Pseudomonas* spp.[56] Pf-5 holds the record for the greatest number of sigma factors in the extracytoplasmic factor (ECF) class among the *Proteobacteria*,[55] with 28 genes encoding

ECF sigma factors in the genome. ECF sigma factors commonly coordinate transcriptional responses to extracellular signals, and have diverse functions in iron acquisition, stress response, metal resistance, cell development, virulence, and the production of extracellular products.[43] In the Pf-5 genome, 18 of the genes encoding ECF sigma factors are adjacent to genes predicted to encode a TonB-dependent outer-membrane protein and an anti-sigma factor. Because many TonB-dependent membrane proteins serve as receptors of ferric-siderophore complexes, the linked sigma factors could function in iron acquisition. These 18 sigma factors, along with two others present in the pyoverdine locus (PFL_4190, a *pvdS* homolog; and PFL_4080), form a cluster distinct from the other eight ECF sigma factors found in the Pf-5 genome in a phylogenetic tree (Figure 3). Three of the remaining ECF sigma factors have homologs that are well characterized in other *Pseudomonas* spp. *algU* (also called *algT*) encodes σ^E, which regulates alginate production and other phenotypes in *P. fluorescens* CHA0,[111] *P. aeruginosa*,[28] and *P. syringae*.[53] SigX controls expression of *ompF* and other genes in *P. aeruginosa*[9] and PrtI regulates extracellular protease production in response to growth temperature in another strain of *P. fluorescens*.[14] The functions of the remaining five ECF sigma factors in the Pf-5 genome cannot be deduced from the functions of surrounding genes or sequence similarities to characterized sigma factors. The numerous genes with putative roles in transcriptional regulation indicate that exceedingly complex regulatory networks exist in this environmental bacterium.

3.1.5. Lack of Key Pathogenicity Factors

P. fluorescens Pf-5 lacks a number of virulence factors found in pathogenic *Pseudomonas* spp. No evidence for a type III secretion system was found in the genomic sequence of Pf-5, although genes for these export systems have been found in many other strains of *P. fluorescens*.[76,100,105] Gene clusters for the biosynthesis of the phytotoxins tabtoxin, syringomycin, syringotoxin, or coronatine are not found in the Pf-5 genome. Pf-5 also lacks genes encoding for exoenzymes associated with degradation of plant cell walls and cell wall components.

4. COMPARATIVE GENOMICS

4.1. Genomic Comparisons Among *Pseudomonas* spp.

Pf-5 is one of six strains of *Pseudomonas* spp. whose genomic sequences have been published to date (September 2006). The sequenced genomes represent five species: *Pseudomonas putida*,[83] *P. aeruginosa*,[121] *P. fluorescens*,[94] *P. entomophila*,[136] and three pathovars of *P. syringae*:

Figure 3. Phylogenetic relationships among the sigma 70 factors in four species of *Pseudomonas*. Predicted peptide sequences of sigma factors in the sigma 70 family were compared among *P. fluorescens* Pf-5 (bold), *P. aeruginosa* PAO1, and *P. putida* KT2440, and *P. syringae* pv. tomato DC3000. Genes of *P. fluorescens* Pf-5 with linked genes encoding putative TonB-receptors and anti-sigma factors are denoted with a star. The maximum-likelihood phylogenetic tree was generated by using PHYML package.[37] Bootstrap support values are indicated next to the branch nodes.

tomato,[13] syringae,[27] and phaseolicola.[51] Members of the genus *Pseudomonas* are known to exhibit a high degree of ecological and metabolic diversity,[33] which is reflected in the genomic diversity displayed between species of *Pseudomonas*. Using a stringent approach to identify orthologs,[30] we determined that only 2,468 genes are conserved among strains representing four species (Figure 4). Therefore, the percentage of the proteome shared with all of the species varies from 40% for *P. fluorescens* Pf-5 (with 6,137 predicted protein-encoding genes) to 46% for *P. putida* KT2440 (with 5,350 predicted protein-encoding genes).

4.2. Genomic Sequencing of *P. fluorescens*

In addition to Pf-5, the genomes of two other strains of *P. fluorescens* have been sequenced to completion as of September 2006. *P. fluorescens* strain Pf0-1 was sequenced by the Joint Genome Institute of the US Department of Energy (GenBank accession number NC_007492), and *P. fluorescens* SBW25 was sequenced by the Sanger Centre (http://www.sanger.ac.uk/Projects/P_fluorescens).

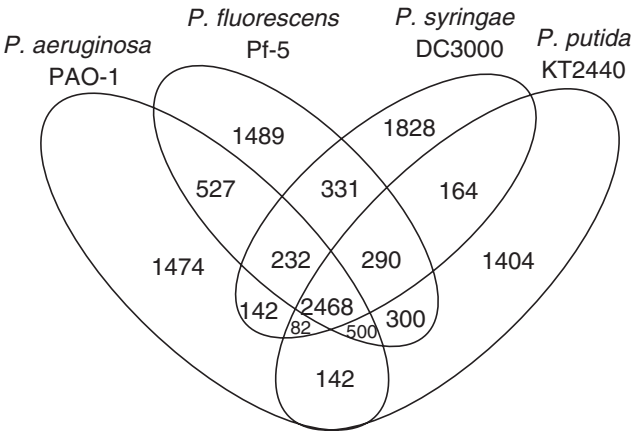

Figure 4. Venn diagram showing the number of proteins shared or unique for four *Pseudomonas* species. Protein sequences of four *Pseudomonas* genomes were compared, and bidirectional best matches that met the following criteria were scored as shared proteins: a p value less than or equal to 10^{-5}, identity of 35% or more, and match lengths of at least 50% of the length of both query and subject sequence.[30] By these criteria, 2,468 ORFs are conserved among the four species. This number is smaller than that reported earlier[94] because a more stringent standard was adopted in order to clearly identify shared orthologues rather than homologs.

4.2.1. Description of Strains of P. fluorescens whose Genomes have been Sequenced

P. fluorescens SBW25 was isolated from the phyllosphere of sugar beet in Oxfordshire, England[4] and has become a model strain for ecological and molecular studies of environmental bacteria. Although obtained from a leaf surface, the strain is also a rhizosphere colonist, and functions as a plant-growth promoting rhizobacterium and a biological control agent against the plant pathogen *P. ultimum*.[5,82] SBW25 has been the subject of extensive studies evaluating spatial distribution and aggregation of bacterial cells on plant surfaces[128,132]; metabolic activity of bacteria in soil[73,133] and on plant surfaces[132]; and factors that influence rhizosphere colonization, including motility,[131] root cap cells,[48] and the presence of other soil organisms such as nematodes.[59] SBW25 was the first free-living genetically modified bacterium to be released into the field in the UK, and it has been the subject of extensive research evaluating the biosafety of genetically modified bacteria[129] and the influence of genetic markers[22] and plasmids[66] on environmental fitness. SBW25 has also been the subject of landmark studies using in vivo expression technology (IVET) to identify genes expressed in the rhizosphere. Genes expressed by SBW25 in the rhizosphere have predicted roles in nutrient acquisition, stress response, and biosynthesis of phytohormones and antibiotics.[32,100] Subsequent analyses of rhizosphere-induced genes have resulted in the intricate characterization of the molecular basis of attachment and biofilm formation in this bacterium.[117,118] SBW25 was the first strain of *P. fluorescens* known to possess a gene cluster related to the type III secretion systems found in pathogenic *Pseudomonas* spp.[50,100] There are no published reports of antibiotic production by SBW25.

P. fluorescens Pf0-1 was isolated from an agricultural soil in the USA,[19] and has been the subject of studies evaluating the molecular basis of bacterial attachment to soil particles and seeds, environmental fitness, and bacterial gene expression in natural habitats. A transcriptional regulator (*adnA*), which influences flagellar synthesis, biofilm formation, and attachment was identified[16,23,106] and shown to provide a fitness advantage to Pf0-1, allowing it to spread and survive in soil under field conditions.[74] Genes expressed by Pf0-1 in the soil have been identified using IVET, and certain of these function in nutrient acquisition or in regulation. IVET analysis also identified cryptic promoters, which had escaped recognition by standard bioinformatic approaches used to identify ORFs in bacterial genomes.[115,116]

4.2.2. Comparative Genomics of P. fluorescens

Comparisons between the three sequenced *P. fluorescens* strains indicate that the high degree of diversity in this genus extends down to the species level. Between the three *P. fluorescens* strains, 3,688 genes are

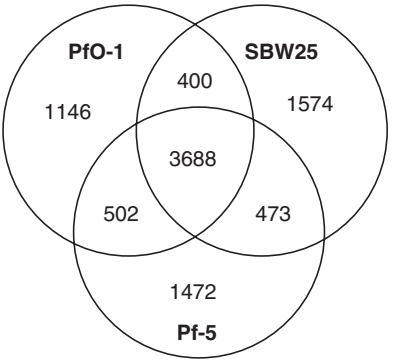

Figure 5. Venn diagram showing the number of proteins shared or unique for three strains of *P. fluorescens*. Sequences of predicted proteins from the genomes of Pf-5, Pf-01 (GenBank accession number NC_007492) and SBW25 (http://www.sanger.ac.uk/Projects/P_fluorescens) were compared. Bidirectional best matches that met the following criteria were scored as shared proteins: a *p*-value less than or equal to 10^{-5}, identity of 35% or more, and match lengths of at least 50% of the length of both query and subject sequence.[30] About 3,688 ORFs are conserved among the three strains of *P. fluorescens*, and each genome contains between 20 and 26% unique genes.

conserved as defined by the method of Fouts *et al.*,[30] representing 60–64% of the genome of each strain (Figure 5). Although the percentage of the proteome shared among strains of *P. fluorescens* is substantially greater than that shared among the *Pseudomonas* species (Figure 4), there is a large fraction of the proteome (1,146–1,574 genes) unique to each strain of *P. fluorescens* (Figure 5). The genomic diversity observed among the three strains of *P. fluorescens* is consistent with the tremendous phenotypic diversity, which is well recognized in this bacterial species.[33]

4.2.3. Unique Regions of the Pf-5 Genome

The majority of the genes unique to Pf-5 (Figures 4 and 5) are located in distinct clusters in the genome. The clustering of these unique genes can be visualized in Figure 2, which shows genes unique to Pf-5 compared with other species of *Pseudomonas* (circle 3) and other strains of *P. fluorescens* (circle 4). There is a high degree of overlap between these two sets of genes; and clusters of genes unique to Pf-5, whether defined at the strain or species level, also coincide on the genomic map (Figure 2). Therefore, the distribution of the 1,472 unique genes, defined at the strain level, provide another criterion that complements the four criteria already employed to identify genomic islands in the Pf-5 genome: (i) distribution of unique genes (defined at the species level), (ii) atypical trinucleotide composition, (iii) presence of putative integrated phages, and (iv) distribution of 1,052 copies of a 34-bp (base pair) REP element. The REP elements are clustered in the Pf-5 genome (circle 6, Figure 2) with distinct gaps that often correspond to regions of atypical nucleotide content (circle 8, Figure 2), the presence of prophages (circle 7, Figure 2), and genes unique to Pf-5 (circles 3 and 4, Figure 2).

Many of the genes unique to Pf-5 are located in clusters that contain genes for secondary metabolite biosynthesis (pyrrolnitrin, pyoluteorin,

DAPG, a novel polyketide, and a novel peptide), prophages, and the PFGI-1 genomic island.[94] A more detailed description of the latter two entities follows.

5. CHARACTERIZATION OF MOBILE GENETIC ELEMENTS IN PF-5

5.1. Genomic Island PFGI-1

As the number of sequenced bacterial genomes grows, so does the number of different types of mobile genetic elements (MGEs). Many of the newly described types are mosaic in nature and often combine key elements found in different "classical" MGEs such as plasmids, bacteriophages, and transposons.[89] Conjugative genomic islands (CGIs) represent one rapidly growing class of strain-specific mosaic MGEs that can have a profound impact on the adaptation and evolution of bacterial species.[40] CGIs vary in size from 10 to 500 kb, encode for mobility loci, and commonly exhibit anomalous G + C content and codon usage. Typical CGIs carry phage-like integrase genes that allow for site-specific integration, most often into tRNA genes. CGIs also encode plasmid-like replication and recombination functions, as well as conjugative machinery that contribute to horizontal transfer. Finally, they often carry gene clusters that encode functions that are not essential for the host but that provide an advantage under certain growth conditions. There is increasing evidence that plasmid-related CGIs are widely distributed among members of the genus *Pseudomonas*, where they encode host-specific pathogenicity traits, as well as traits essential for survival in natural environments.[31,42,58,62,98,144] For example, in the pathogens *P. aeruginosa* and *P. syringae*, CGIs encode pathogenicity factors that allow these organisms to successfully colonize a variety of hosts, as well as metabolic, regulatory, and transport genes that most probably enable the microorganisms to thrive in diverse environmental habitats.[27,42,58,62,98,110] An unusual self-transmissible CGI, the *clc* element from the soil bacterium *Pseudomonas* sp. B13, enables its host to metabolize chlorinated aromatic compounds.[31,134,135]

P. fluorescens Pf-5 harbors a 115-kbp mobile genomic island, PFGI-1 (Table 3), which resembles a large self-transmissible plasmid capable of site-specific integration into one of the two $tRNA^{Lys}$ genes. PFGI-1 exemplifies the first large MGE of this kind found in *P. fluorescens*. Of 96 putative PFGI-1 open reading frames, 50 were classified as hypothetical or conserved hypothetical genes, and 55 were unique to Pf-5 and absent from the genomes of the closely related strains *P. fluorescens* SBW25 and Pf-01.

Table 3. Genomic island and phage-related elements of *P. fluorescens* Pf-5 genome.

Feature	5′ end	3′ end	Size (bp)	G + C %	Presence of integrase gene	Type of feature
Prophage01	1,386,082	1,402,957	16,875	62.6	No	SfV-like prophage
Prophage02	2,042,157	2,050,549	8,392	46.8	Yes[a]	Defective prophage in $tRNA^{Ser}$
Prophage03	2,207,060	2,240,619	33,559	61.2	Yes	P2-like prophage
Prophage04	2,338,296	2,351,794	13,498	56.3	Yes	Defective prophage in $tRNA^{Pro}$
Prophage05	3,979,487	3,982,086	2,599	55.3	Yes[a]	Defective prophage in $tRNA^{Cys}$
Prophage06	4,338,335	4,395,005	56,670	57.3	Yes	Lambdoid prophage in $tRNA^{Ser}$
Island PFGI-1	5,378,468	5,493,586	115,118	56.4	Yes	Putative mobile island in $tRNA^{Lys}$
Prophage07	5,728,474	5,745,256	16,782	51.5	Yes	Defective prophage in $tRNA^{Leu}$

[a]The predicted integrase gene contains a frameshift mutation(s).

A unique ~35-kb DNA segment of PFGI-1 is absent from closely related CGIs and contains genes that are not immediately related to integration, plasmid maintenance, or conjugative transfer. These genes encode proteins with predicted regulatory functions, as well as putative short-chain alcohol dehydrogenases, hydrolases, proteases, a cardiolipin synthase, a GGDEF domain protein, a nonheme catalase, and components of cytochrome *o* ubiquinol oxidase complex. How could these genes contribute to the survival of *P. fluorescens* Pf-5 in the rhizosphere? At least some of aforementioned enzymes may facilitate protection from environmental stresses. For example, nonheme catalases are bacterial antioxidant enzymes that contain a dimanganese cluster, which catalyzes disproportionation of toxic hydrogen peroxide into water and oxygen.[17] In *P. putida*, the cardiolipin synthase was implicated in adaptation to membrane-disturbing conditions such as exposure to organic solvents,[137] whereas the cytochrome *o* oxidase complex was shown to be highly expressed under low-nutrient conditions such as those found in the rhizosphere, and to play a crucial role in a proton-dependent efflux system involved in toluene tolerance.[44,125] The GGDEF domain proteins represent an emerging class of bacterial regulators involved in the synthesis of bis-(3′–5′)-cyclic dimeric GMP, which acts as a global signaling messenger,[108] and two-component signal-transduction systems are widely employed by Gram-negative bacteria to sense changes in the environment and appropriately modulate the expression of certain genes.[119]

5.2. Characterization of Phage-Related Elements in Pf-5

Temperate bacteriophages represent an important part of the bacterial flexible gene pool and actively participate in horizontal gene transfer.[15,34] Recent analyses of sequenced bacterial genomes have revealed that most of them contain prophages, which form when temperate bacteriophages integrate into the host genome.[15] In addition to genes encoding phage-related functions, many prophages carry nonessential genes that sometimes can dramatically modify the phenotype of the host and allow it to colonize a new ecological niche.[15,79] *P. fluorescens* Pf-5 harbors seven prophage regions that vary in G + C content from 62.6 to 46.8% (Table 3). Four of these regions, namely prophages 02, 04, 05, and 07, represent prophage remnants with reduced size and/or complexity that carry several mutated phage-related genes. The remaining three prophages, 01, 03, and 06, each exceed 15 kb in length and contain genes for transcriptional regulators, DNA metabolism enzymes, structural bacteriophage proteins, and lytic enzymes.

Prophage 01 spans 16,875 bp and carries genes encoding a myovirus-like tail, a holin-lysozyme lytic cassette, a putative chitinase gene, and a repressor protein. The absence of integrase, head morphogenesis genes and simple overall organization suggests that this prophage encodes a phage tail-like bacteriocin similar to R-type pyocins of *P. aeruginosa*.[81]

The second large *P. fluorescens* Pf-5 prophage, prophage 03, spans 33.5 kb (Table 3) and represents a unique region with no homologs present in *P. fluorescens* Pf-01 or *P. fluorescens* SBW25. Prophage 03 is a chimeric genetic element that contains a siphovirus head morphogenesis region and a myovirus-like tail assembly region. The prophage carries a putative integrase gene, regulatory genes, genes encoding the lytic enzymes holin and lysozyme, and a P2-like tail assembly region closely resembling the R2-specific part of the R2/F2 pyocin locus of *P. aeruginosa* PAO1.[81] However, the presence of genes involved in DNA modification and head morphogenesis suggests that prophage 03 represents the genome of a temperate bacteriophage rather than an R-type pyocin region.

The 56-kb prophage 06 encodes a temperate lambdoid phage integrated into the $tRNA^{Ser}$ gene and represents the largest prophage region of *P. fluorescens* Pf-5 (Table 3). It is mosaic in nature with no homologs present in *P. fluorescens* Pf-01 or *P. fluorescens* SBW25. Prophage 06 contains a lambda-type phage integrase gene and modules that are involved in head and tail morphogenesis and DNA recombination. Prophage 06 also contains a regulatory circuit, a gene for a putative cytosine C5-specific methylase, and host lysis genes encoding holin and phage lysozyme.

Although our knowledge of the ecological role of *Pseudomonas* prophages is rather limited, data from other bacterial species suggests a number of ways in which prophage regions may affect the survival of

P. fluorescens Pf-5 in natural habitats. It is not known whether the antagonistic activity associated with prophages and/or bacteriocins contributes to success in the competition between Pf-5 and closely related strains of *Pseudomonas* spp. in the plant rhizosphere. However, prophages 01 and 04 are associated with *llpA1* and *llpA2* genes, which encode related plant lectin-like low-molecular weight bacteriocins that are operational in Pf-5 under in vitro conditions and kill closely related sensitive strains of *Pseudomonas* spp. via yet unidentified mechanisms.[91] The fact that both *llpA* copies are located in the vicinity of prophage repressor genes, as well as involvement of a *recA*-dependent SOS response in LlpA production in a different *Pseudomonas* strain,[26] suggests that the association of *llpA* genes with prophages is not accidental and that prophages may be involved in the regulation of bacteriocin production in *P. fluorescens* Pf-5.

Temperate bacteriophages, similar to those encoded by prophages 03 and 06, are capable of development through both lysogenic and lytic pathways. The lytic pathway ultimately results in phage-induced host cell lysis, and it has been reported that the presence of virulent bacteriophages may have a deleterious impact on rhizosphere-inhabiting *P. fluorescens* strains.[12,52,67] Similarly, bacteriophage tail-like bacteriocins, such as the one encoded by prophage 01, also are capable of killing both closely and more distantly related strains of bacteria, presumably through destabilization of the cell membrane.[8,18,36,49,122] Interestingly, a genetic locus closely related to prophage 01 is expressed in another strain, *P. fluorescen*s Q8r1-96, under in vitro conditions, and encodes a fully functional lytic cassette capable of lysing *E. coli* cells (D. Mavrodi, unpublished). The presence of certain prophages also may protect the host, making it immune to superinfection by closely related bacteriophages.[15]

6. CONCLUSIONS

The genomic sequence of the rhizosphere bacterium *P. fluorescens* Pf-5 provides a variety of insights into this organism's lifestyle in association with plants in the natural environment. It reveals pathways for utilization of an extensive array of carbon substrates found in plant root and seed exudates as well as siderophores produced by soil inhabiting microorganisms. The antibiotic- and oxidative-stress-resistance capacities found in the genome are likely to provide a foundation for the fitness of *P. fluorescens* Pf-5 in the rhizosphere. The genome of *P. fluorescens* Pf-5 is larger than those of other *Pseudomonas* spp. sequenced to date, a difference that can be attributed to the presence of genomic islands that contain prophages, or gene clusters for secondary metabolite or polysaccharide biosynthesis or transport and catabolism of alternative carbon sources derived from

plants. The genomic sequence also highlighted gene clusters for three secondary metabolites with unknown structures and biological activities. The structure of one of these cryptic metabolites could be predicted bioinformatically, and has since been confirmed as a novel lipodecapeptide that functions as a biosurfactant. Its role and the roles of the other two cryptic metabolites in biological control are intriguing areas for future study. Finally, comparisons among the genomic sequences of six strains of *Pseudomonas* spp. reveal a high level of diversity at both the strain and species level, which is consistent with the tremendous phenotypic diversity characterizing this group of bacteria.

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2

GLYCOSYLATION ISLANDS OF *PSEUDOMONAS* SPECIES

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1. INTRODUCTION

Protein glycosylation, originally thought to be restricted to eukaryotes has now been recognized as an important posttranslational modification process in prokaryotic organisms, particularly of cell surface-associated or secreted molecules.[10,11,22,24,25,46,48,58,59,79,80,95,99] Many bacterial proteins, including potential virulence factors of pathogenic bacteria, such as pili of *Neisseria gonorrhoeae*,[79] *Neisseria meningitidis*,[62,65] *Escherichia coli*,[89] *Moraxella bovis*,[47] *Dichelobacter nodosus*,[41] *Eikenella corrodens*,[35] *Vibrio cholerae*,[87] *Ralstonia solanacearum*,[50] *Xanthomonas* species,[55] and *Pseudomonas aeruginosa*,[13] an adhesin of *Chlamydia*,[43] a surface-exposed immunodominant protein of two *Ehrlichia* species,[56] and the TiBA adhesin of ETEC[48] are now known to undergo glycosylation. Moreover, the subunits of the flagellar filaments in a variety of bacterial species are similarly modified, including those of *P. aeruginosa*,[11] *Pseudomonas syringae* pv. *tabaci* 6605,[83,84] pv. *glycinea*[86] and pv. *tomato*,[82,86] *Campylobacter coli* and *Campylobacter jejuni*,[23] *Treponema pallidum*,[97] *Borrelia burgdorferi*,[26] *Helicobacter felis*,[37] *Caulobacter crescentus*,[46] *Agrobacterium tumefaciens*,[20]

Juan-Luis Ramos and Alain Filloux (eds.), Pseudomonas, 31–56.

and *Listeria monocytogenes*.[73] However, glycosylation is not restricted to proteins found on the bacterial surface. A broad range of glycoproteins has been identified in *C. jejuni*[81] and in *Mycobacterium tuberculosis*,[69] indicating that glycosylation may be more common than currently appreciated.

In general, glycosylation of bacterial proteins represents a specific modification process, which is carried out by a dedicated set of genes. This modification is not the result of a secondary activity of enzymes involved in the biosynthesis of polysaccharides or glycolipids, although it is apparent that substrates may be utilized from other synthetic pathways. For example, glycosylation of meningococcal pilin requires the product of the *pglA* gene that although related to a number of glycosyltransferases, is not involved in the biosynthesis of lipopolysaccharide (LPS). Similarly, the proteins of the general glycosylation system in *C. jejuni*[81] encoded by *pglA-G* genes are involved in the modification of a number of proteins, including flagellin, and do not participate in the synthesis of LPS. Moreover, bacterial glycosylation appears to resemble the analogous process in eukaryotic cells with asparagines, serine, and threonine residues serving as the acceptors for the first sugar on the protein.[12,67]

In many of the glycosylation systems reviewed, the genes required for glycosylation are closely linked to their targets,[64] e.g., the genes involved in glycosylation of *Pseudomonas* flagellar proteins have been found to be present in a cluster called a "glycosylation island" (GI), which is located among the flagellar genes. As mentioned above a number of reports have demonstrated the presence of flagellar glycosylation genes in several bacterial species. However, far too few strains have been examined in most species to provide an idea of the extent and variability of flagellar glycosylation process among strains of a given bacterial species. Here we mainly review this process in *P. aeruginosa* and related species where the glycosylation has been examined to a greater degree.

2. *P. AERUGINOSA* FLAGELLINS

P. aeruginosa flagellins are classified into two groups (a- and b-types) based on their molecular weights and reactivity with specific anti-sera.[3,45] The discrepancy between the predicted molecular mass by sequence of the a-type flagellin (45 kDa) and the observed molecular mass on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (40 kDa) was attributed to a posttranslational modification,[90] which was later shown to be an addition of glycan chains.[11,72] No such discrepancy was noted for b-type flagellin bearing strains. The a-type flagellins exhibit variability in their molecular masses, in the range of 45–52 kDa, and differences in amino acid sequences. These features further classified these proteins into

A1 and A2 subtypes.[8] The b-type flagellins have more conserved sequences and show invariant molecular masses of about 53 kDa.[11]

3. GENETIC ORGANIZATION OF THE GLYCOSYLATION REGION LINKED TO THE FLAGELLAR GENES OF *P. AERUGINOSA*

3.1. A-type *P. aeruginosa* Strains

An approximately 16 Kb genomic island localized between *flgL* and *fliC* was discovered in *P. aeruginosa* strain PAK, which is known to produce a-type flagellin.[6] This island was likely acquired through horizontal gene exchange, although the sequences that reflect sites of transposition or recombination have not been detected. This unique genomic island consisted of 14 putative genes, *orfA* to *orfN* and was shown to be essential for glycosylation of the type a flagellin[6] (Figure 1). It has been further demonstrated that the inactivation of either *orfA* or *orfN* (now annotated as *fgtA* for flagellar glycosyl transferase)[92] flanking the ends of the GI abolished flagellin glycosylation demonstrating the role of these genes in this process.[6] The annotation of these 14 *orfs* is summarized in Table 1.[6] Two of the *orfs* (*orfI* and *orfK*) encode products of unknown function, and these are designated as hypothetical unknown genes. The *orfH* and *orfL* genes had low levels of homology with the nodulation protein NoeI of

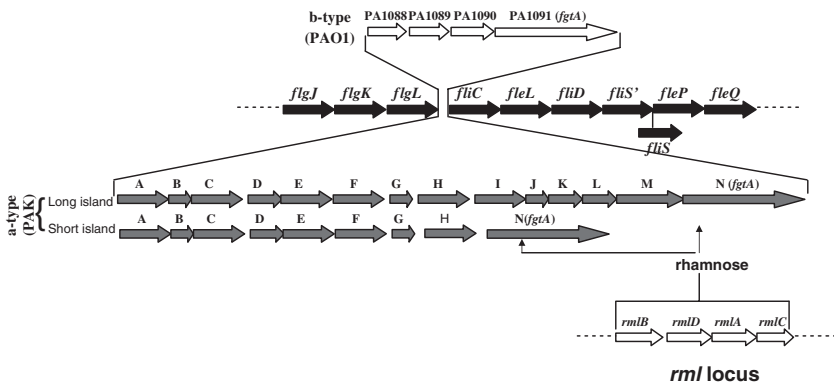


Figure 1. Schematic diagram showing the structure of GIs of a- and b-type *P. aeruginosa* strains. The diagram shows the region of the *P. aeruginosa* chromosome where the GIs are located. The location of the GI insertion is shown to be in the middle of flagellar genes *flgL* on the 5′ end and *fliC* at the 3′ end. The PAK a-type, long and short island *orfs* are shown by black filled arrows. The PAO1, GI with 4 *orfs* is shown by arrows with no fill. The *rml* locus located somewhere on the *P. aeruginosa* chromosome provides rhamnose that is utilized by *fgtA*.

Table 1. Similarities of *P. aeruginosa* PAK ORFs to proteins in the databases.

ORF	Size, aa	Homologue/organism	% identity, aa	Function of homologues	Accession number of homologue
A	467	VioA/*E. coli*	58	Synthesis of dTDP-4-amino-4,6 dideoxyglucose (dTDP-viosamine) (nucleotide sugar transaminase)	AF125322
B	75	Putative acyl carrier protein/*C. jejuni*	33	Carrier of the growing fatty acid chain in fatty acid biosynthesis	AL139078
C	334	FABH, 3-oxoacyl-[acyl-carrier-protein] -synthase/*Streptomyces coelicolor*	36	Catalyzes the condensation reaction of fatty acid synthesis by the addition of an acyl acceptor of two carbons from malonyl-ACP	T34914
D	220	FABG, 3-oxoacyl-[acyl-carrier-protein] -reductase/*Chlamydia pneumonia*	36	First reduction step in the fatty acid biosynthesis pathway	Q9Z8P2
E	211	Serine *O*-acetyltransferase/*Bacillus subtilis*	33	Involved in methylation of Nod factors (NoeI)	E70037
F	376	Putative aromatic ring hydroxylating dioxygenase alpha subunit/ *Sphingomonas sp.*	24	Involved in napthalene catabolic pathway	U65001
G	207	Putative acetyl transferase/ *Legionella pneumophilia*	21	Involved in LPS biosynthesis	AJ007311
H	380	Nodulation protein NoeI/*S. fredii*	33	Involved in Nod factor biosynthesis	AF228683
I	426	No homologue			
J	256	2-deoxy-manno-octulosonate cytidyl transferase/*E. coli*	35	Activates KDO for incorporation into LPS in Gram-negative bacteria	P42216
K	190	No homologue			
L	368	3-demethylubiquinone-9, 3-methyltransferase/*N. meningitidis*	24	Involved in ubiquinone biosynthesis pathway	AL162753
M	539	*cmtG*, 4-hydroxy-2-oxovalerate aldolase/*P. putida*	27	Involved in *p*-isopropylbenzoate (*p*-cumate) catabolic pathway	Q51983
fgtA	1138	O-antigen biosynthesis protein RfbC/*Myxococcus xanthus*	29	O-antigen biosynthesis	Q50864

Modified from Arora et al., 2001[6].

Sinorhizobium fredii and methyltransferase of *N. meningitidis*, respectively.[63] A group of genes within the island is similar to genes encoding proteins that function in various polysaccharide biosynthetic pathways. These include *orfsB*, *C*, and *D*, which encode homologues of carriers or modifiers of acyl groups. Some of the genes mentioned earlier encode products with similarities to enzymes involved in the biosynthesis or modification of polysaccharides. These genes include the product of *orfA*, a homologue of a nucleotide-sugar aminotransferase; *orfE*, encoding an acetyltransferase (potentially involved in modification of sugars or serine); and *orfJ*, a sugar-cytidine transferase similar to the enzymes involved in the biosynthesis of the core portion of the Gram-negative LPS. Finally, *fgtA*, which is present in both a-type and b-type strains, specifies a glycosyltransferase, with significant similarity to the enzymes involved in the addition of sugars to the O-side chains of LPS[30] or formation of polysaccharides by *Enterococcus faecalis*.[98] The functions of most of these genes remain unknown due mainly to the technical difficulties of doing sugar analyses on mutants of the *orfs* of this island. The major problem is the lack of proper standards, to help identify the unknown sugar moieties that may be unique to *P. aeruginosa*. In addition, the annotations of the genes besides *orfA* and *fgtA* in the PAK glycosylation island are based on relatively ill-defined functions, which include fatty acid biosynthesis, the naphthalene catabolic pathway, and nodulation factor biosynthesis, and as such provide few clues to the likely structures that make up this unique flagellar glycan component. Other aspects of the functioning of the genes including their regulation are also unknown, but unpublished observations from our laboratory indicate that the promoters of the genes of this island are very weak. It is however likely that their activities need to be coordinated with flagellar assembly and they are thus conceivably under the control of σ^{28} or σ,[54] the transcription factors involved in flagellar biogenesis.[18]

3.2. Polymorphisms of the Glycosylation Island of a-type Flagellin

The GI was assumed to be present in all the strains expressing a-type flagellin, however differences in the observed molecular masses of flagellin from several a-type strains (Figure 2) suggested that there may be differences in the levels or nature of glycosylation of the flagellin molecule and hence in the genes encoded by the islands in different strains.[6] Therefore, the glycosylation islands of a number of *P. aeruginosa* strains of diverse origins was analyzed by DNA microarray and sequencing. For microarray analysis chromosomal DNA hybridizations were performed[8,96] using the *P. aeruginosa* genome microarray (Affymetrix). The microarray used in

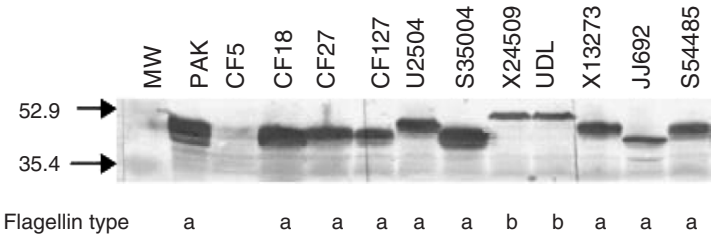


Figure 2. Glycan size heterogeneity of *P. aeruginosa* strains. Whole-cell lysates from different *P. aeruginosa* strains were separated by SDS-PAGE and were subjected to Western blot analysis with FliC-specific antibodies. The flagellin type for each strain is indicated at the bottom of the panel. The predicted masses of flagellins are almost identical thus, the different sized a-type flagellins suggest glycan heterogeneity. Data are from Arora *et al*., 2004.[8]

this study, included probes for the sequenced GI identified in strain PAK. The b-type strains, including PAO1, apparently lacked genes with identity to those present in the GI of the a-type strain, while other a-type strains had complete or partial islands when examined by microarray and or PCR and sequencing. Those with partial islands showed no hybridization with probes from *orfsH*, *I*, *J*, *K*, *L*, and *M*. In addition, *orfD* and *orfK* were designated uncertain in some strains based on their *P* values. Comparison of the b-type *P. aeruginosa* strain PAO1 genome sequence with the PAK GI sequence had previously shown that PAO1 had an open reading frame homologous to the *fgtA* of the PAK GI,[4] but the level of homology was too low for it to be detected by the DNA microarray hybridization. Thus the GI of a-type strains is polymorphic.[8] Microarray analysis showed that many of the genes found in strain PAK were absent from other strains or were probably not identical in sequence. A recently sequenced a-type strain CF27 whose genome is not annotated has 14 *orfs* found in PAK as also shown previously[8] (Figure 3a, Table 2). Another strain CF5 is also shown to have all the 14 ORFs in the microarray hybridization experiment. Analysis of strains lacking the complete GI, by microarray, PCR and sequencing, confirmed that many *P. aeruginosa* strains carried an abbreviated version of the GI (short island) in which *orfsD*, *E*, and *H* are polymorphic and *orfsI*, *J*, *K*, *L*, and *M* are absent[8] (Figure 3a, Table 2). For example, strains JJ692 and X13273 lack *orfsI*, *J*, *K*, *L*, and *M*. Strains CF127, CF18, S35004, S54485, and U2504 have a deletion of *orfsI*, *J*, *K*, *L*, and *M*. However, due to poor hybridization signal in microarray analysis, the absence of *orfH* in the strains CF127, CF18, S35004, S54485, and U2504 and of *orfD* in S54485 and *orfK* in U2504 could not be confirmed[8] until further experiments are performed (Figure 3a, Table 2). Thus, the strains possessing either short or long islands appear to have polymorphisms in their genes, indicating that the glycan chains may be quite varied among strains. Some of the other newly sequenced strains, mucoid a-type strain

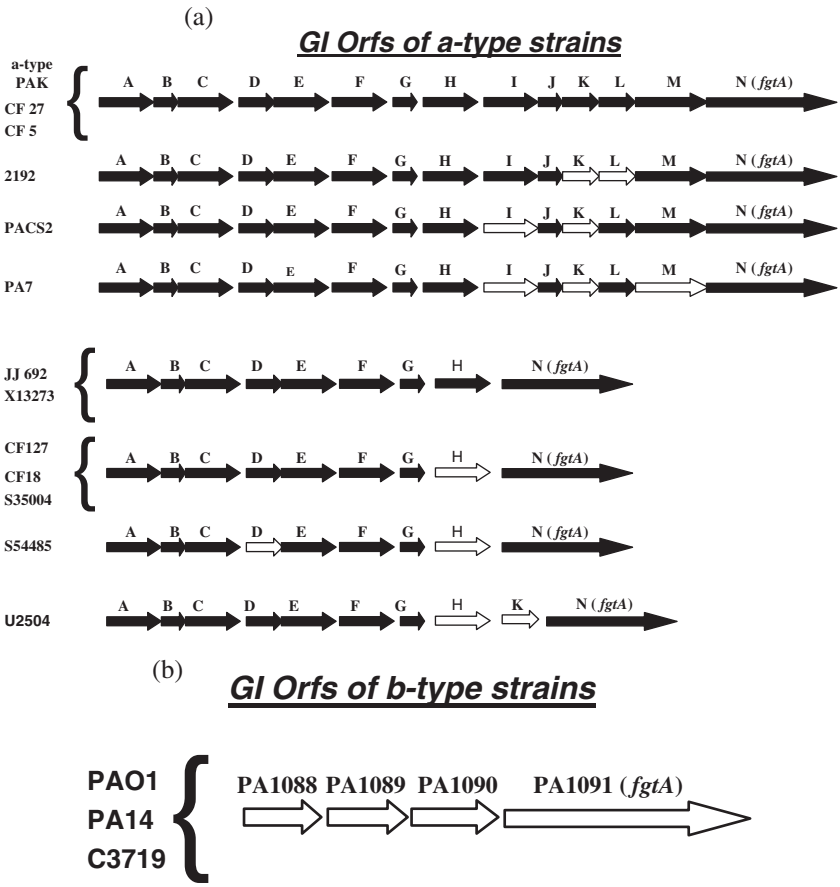


Figure 3. Schematic diagram showing the structure of GIs of a- and b-type *P. aeruginosa* strains. (a). GIs of a-type strains. A-type strains CF27 and CF5 have the 14 *orfs* found in strain PAK. The presence of *orfsK* and *L* is uncertain in strain 2192. Similarly, *orfsI* and *K* in PACS2 and *orfsI*, *K*, and *M* in PA7 are uncertain. A-type strains JJ692 and X13273 have short islands with a deletion of *orfsI*, *J*, *K*, *L*, and *M*. Short island a-type strains CF127, CF18, and S35004 have been shown to have a deletion of *orfsI*, *J*, *K*, *L*, and *M*. The presence of H is uncertain owing to the poor hybridization of DNA probes in microarray analysis. This gene is however likely to be present but polymorphic, since it was found in JJ692 and X13273. Strain S54485 and U2504 have also been shown to have short island, however the presence of *orfsD* and *H* in S55585 and *orfsH* and *K* in U2504 is uncertain due to the lack of hybridization signal in the microarray analysis. (b). GI of b-type strains PA14 and C3719 are almost identical to that of PAO1 strain.

2192, PACS2, and PA7 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome) also have GI's (Figure 3a, Table 2). However, *orfsK* and *L* in strain 2192, *orfsI* and *K* in PACS2, and *orfsI*, *K*, and *M* in PA7 are either absent or are polymorphic.

Table 2. Comparative analysis of *P. aeruginosa* PAK glycosylation island genes with the other type-a strains.

PA ORF identity	% similarity to ORFs of other type-a strains	Function (COG/PFAM prediction)	Accession number of homologues
orfA	99.1 (2192)	Predicted pyridoxal phosphate-dependent enzyme apparently involved in	ZP_00976554
	100 (JJ692)	regulation of cell wall biogenesis [amino acid transport and metabolism]	AAP35713.1
	100 (PACS2)		ZP_01364451
	97 (PA7)		ZP_01295778
orfB	100 (2192)	Acyl carrier protein [lipid metabolism/secondary metabolites biosynthesis,	ZP_00976555
	100 (JJ692)	transport, and catabolism]	AAP35714.1
	100 (PACS2)		ZP_01364452
	100 (PA7)		ZP_01295779
orfC	100 (2192)	3-oxoacyl-[acyl-carrier-protein] synthase 111	ZP_00976556
	100 (JJ692)		AAP35715.1
	100 (PACS2)		ZP_01364453
	99 (PA7)		ZP_01295780
orfD	99 (2192)	Dehydrogenases with different specificities (related to	ZP_00976557
	84.1 (JJ692)	short-chain alcohol dehydrogenases)	AAP35716.1
	91 (PACS2)		ZP_01364454
	68 (PA7)		ZP_01295781
orfE	100 (2192)	Acetyltransferase (isoleucine patch superfamily)	ZP_00976558
	-(JJ692)[a]		–
	99 (PACS2)		ZP_01364455
	56 (PA7)		ZP_01295782
orfF	99 (2192)	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases,	ZP_00976559
	98.8 (JJ692)	large terminal subunit [inorganic ion transport and metabolism/general	AAP35717.1
	99 (PACS2)	prediction only]	ZP_01364456
orfG	99 (2192)	Acetyltransferase (isoleucine patch superfamily)	ZP_00976560
	97.5 (JJ692)		AAP35718.1
	98 (PACS2)		ZP_01364457
	46 (PA7)		ZP_01295785

orfH	99 (2192)	SAM-dependent methyltransferases	ZP_00976561
	77.6 (JJ692)		AAP35719.1
	90 (PACS2)		ZP_01364458
	56 (PA7)		ZP_01295783
	99.7 (CF5)		AAP35721
orfI	100 (2192)	Hypothetical protein	ZP_00976562
	99.5 (CF5)		AAP35722.1
orfJ	100 (2192)	CMP-2-keto-3-deoxyoctulosonic acid synthetase [cell wall biogenesis,	ZP_00976563
	52 (PACS2)	outer membrane]	ZP_01366446
	51 (PA7)		ZP_01296582
	100 (CF5)		AAP35723.1
orfK	98.9 (CF5)	Unknown	AAP35724.1
orfL	12 (PACS2)	Similar to 3-demethylubiquinone-9, 3-methyl transferase of *N. meningitidis*	ZP_01366619
	12 (PA7)	[coenzyme metabolism]	ZP_01293437
	100 (CF5)		AAP35725.1
orfM	99 (2192)	Isopropylmalate/homocitrate/citramalate synthases	ZP_00976565
	44 (PACS2)		ZP_01364584
	99.6 (CF5)		AAP35726.1
fgtA	98 (2192)	Glycosyltransferases involved in cell wall biogenesis	ZP_00976566
	81.6 (JJ692)[b]		AAP35720.1
	97 (PACS2)		ZP_01364459
	93 (PA7)		ZP_01295786

[a]The nucleotide deletion caused a frameshift so that *orfE* in JJ692 was prematurely terminated and consisted of only 30 amino acids instead of the 211 amino acids in PAK and CF5 (Arora *et al*., 2004).

[b]A nucleotide insertion caused a frameshift at the 3′ end of *fgtA* of *P. aeruginosa* strain JJ692, leading to a drop in homology after amino acid 867 of the PAK *fgtA* (Arora *et al*., 2004).

In order to ascertain whether there was co-inheritance of the short island and specific flagellin sequence variants, the complete or partial nucleotide sequences of flagellin genes from 24 a-type *P. aeruginosa* strains were determined.[8] Two distinct flagellin subtypes were noticed, designated A1 and A2, with A2 flagellins having a short deletion in the central region. Long GI were found only associated with A1- type strains, whereas strains carrying the short island were associated with both A1- and A2-type flagellins suggesting that there may be structural constraints on glycosylation imposed by having the A1-type flagellin. These polymorphisms indicate that *P. aeruginosa* probably has the capacity to further diversify the antigenicity of this surface protein by the use of its GIs. The origin of these short islands is also unknown and evidence for transposition is also lacking.

3.3. B-type Flagellin Glycosylation in *P. aeruginosa* Strains

The b-type flagellin of *P. aeruginosa* was thought to be nonglycosylated since the apparent mass on SDS–PAGE fit the predicted mass. In addition, the glycosylation machinery of the a-type flagellin was not able to modify b-type flagellin.[6] Nevertheless, the identification of a possible small GI near the flagellin gene on the PAO1 genome suggested that the PAO1 flagellin might also be glycosylated, and this was recently demonstrated to occur.[92] However, in comparison to strain PAK that has an extensive flagellar glycosylation island, the GI in PAO1 comprises of only four genes, PA1088, PA1089, PA1090, and PA1091, in the same chromosomal location as in a-type strains (Figures 1 and 3b). PA1091 now annotated as *fgtA*, is homologous but not identical to the corresponding gene in a-type strains, and most likely serves the same purpose as the glycosyltransferase that attaches a deoxyhexose to the protein. One of the other genes, PA1090 also appears to play a role in glycosylation, encoding a nucleotidyltransferase, another gene, PA1089 maybe involved in attaching a phosphate group to the glycan chain. To date the genomic sequences of three strains with b-type flagellins have been completed; PAO1 (www.pseudomonas.com), PA14 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = Genome), and CF3719 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = Genome). Each has the same four genes, PA1088-PA1091 with almost complete sequence identity (Figure 3b, Table 3). PCR analysis of 12 other b-type strains yielded identical sized glycosylation islands suggesting that all b-type strains possess this simple island.[92]

3.4. Sites of Glycosylation

Schirm and colleagues determined the structural nature of flagellar glycosylation in type a *P. aeruginosa* strains.[72] For the identification of type and location of glycosylation sites, flagellins from strains PAK and

Table 3. Comparative analysis of *P. aeruginosa* PAO1 ORFs with the other type-b strains.

PA ORF identity	% similarity to ORFs of other type-a strains	Function (COG /PFAM prediction)	Accession number of homologues
PA 1088	98 (PA14) *100 (C3719)*	Putative methyl Transferase, UbiE/COQ5 methyltransferase family	ZP_00138676, *ZP_00970425*
PA 1089	100 (PA14) *100 (C3719)*	Phosphoserine phosphatase (amino acid transport & metabolism)	ZP_00138677 *ZP_00970426*
PA 1090	100 (PA14) *100 (C3719)*	Predicted sugar nucleotidyl-transferases (cell envelope biogenesis, outer membrane)	ZP_00138678 *ZP_00970427*
PA 1091 (*fgtA*)	99 (PA14) *99 (C3719)*	Putative glycosyl/glycerophos-phatase transferases involved in teichoic acid biosynthesis TagF/TagB/EpsJ/RodC	ZP_00138679 *ZP_00970428*

The PA orfs are defined in the *Pseudomonas* genome database version 2 as homologues of previously reported genes of unknown function or with no similarity to any previously reported sequences.

JJ692 were digested with trypsin and subjected to Liquid chromatography (LC)–nanospray-MS/MS analysis. Flagellin from the a-type strain PAK was shown to be modified at Thr 189 and Ser 260 with a heterogeneous glycan comprising of up to 11 monosaccharide units that were O-linked through a rhamnose residue to the flagellin backbone.[72] In addition, *orfA* and *fgtA* were shown to be required for attachment of the heterogeneous glycan and the proximal rhamnose residue, respectively.[72] By contrast, the chymotryptic or tryptic digest of b-type flagellin protein of strain PAO1 when subjected to liquid chromatography–nanoelectrospray-MS/MS analysis revealed an unknown deoxyhexose to which is attached unknown modifications that have a linked phosphate group. Additionally the sites of glycosylation of the PAO1 flagellin are adjacent to each other, Ser 191 and Ser 195.[92] Thus, there may be structural constraints on glycan attachment, which dictate which of the many serines and threonines in flagellin are glycosylated, or the specificity of the glycosyltransferase (*fgtA*) for these substrates may be strictly controlled. The detailed glycan structures of both a-type and b-type flagellin glycan remain to be elucidated owing to the lack of proper standards, to help identify the unknown sugar moieties.

3.5. Glycosylation Island Substrates

The location of the glycosylation island within the flagellar locus suggests that flagellin is the primary substrate for this modification process, however, the possibility that other proteins in *P. aeruginosa* may

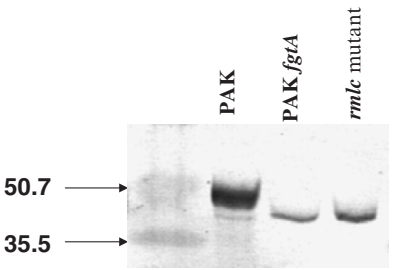


Figure 4. Flagellins of PAK, PAK *fgtA*, and *rmlC* mutant. Whole-cell lysates from PAK, PAK *fgtA* mutant and *rmlC* mutant in a PAK background were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were subjected to Western blot analysis with FliC-specific antibodies. The flagellin from *rmlC* mutant migrates at the same position as that of PAK *fgtA*, indicating that it is not glycosylated.

also be glycosylated and that other accessory genes provide substrates for this pathway cannot be excluded. For example, the *rml* locus composed of four genes designated as *rmlB*, *rmlD*, *rmlA*, and *rmlC*[31,66,68,70] in *P. aeruginosa*, provides rhamnose for synthesis of the core oligosaccharide and O polysaccharide of LPS. In our previous studies[6,72] we had not examined whether this may also be the case for the rhamnose found on the a-type flagellin. This possibility was examined by Western immunoblotting of purified flagella isolated from the *rmlC* mutant of strain PAK provided by J. S. Lam.[66] The flagellin from this strain migrated in the same position as flagellin from the nonglycosylated *fgtA* mutant, suggesting that the *rml* locus provided the first sugar that is attached to the protein (Figure 4). L-Rha is synthesized as an activated nucleotide derivative (i.e., dTDP- L-Rha) by four enzymes designated RmlA, RmlB, RmlC, and RmlD.[42] The proposed mechanism of L-Rha utilization by flagellar pathway is depicted in Figure 1. It is therefore likely that other unknown sugars are provided to the glycosylation island enzymes by accessory pathways.

4. OTHER PSEUDOMONADS REPORTED TO HAVE FLAGELLAR GLYCOSYLATION MACHINERY

4.1. Pseudomonas syringae

Other *Pseudomonas* species have also been shown to possess glycosylation islands as part of their flagellar regulons.[82,83,84,86] For example, the flagellar regulon of *P. syringae* carries a small island comprising of three ORFs, *orf1*, *orf2*, and *orf3* in a similar location between the *flgL* and *fliC* genes (Figure 5) and was also shown to possess glycosylated flagellin.[82,83,84,86] Similarities of *P. syringae* pv. *glycinea* ORFs to proteins in databases has been shown in Table 4. Glycosylation of flagellin in this plant pathogen appears to be involved in specific host cell recognition of distinct pathovars.[86] The putative proteins encoded by *orf1* and *orf2* exhibited 31.7 and 37.8% similarity, respectively, to *fgtA* of the GI in *P. aeruginosa* strain PAK.[6] The proteins encoded by *orf1* and *orf2* exhibited 27.9% identity to each other. Deletion of *orf1* and *orf2* resulted

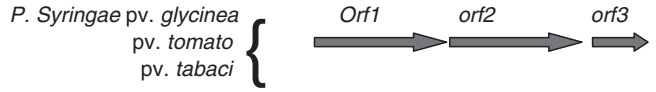


Figure 5. Glycosylation island of *P. syringae* pv. *glycinea*, pv. *tomato*, and pv. *tabaci*. The island is composed of three *orfs*, which are indicated by black filled arrows. The nucleotide sequences are almost identical, with few differences in their amino acids.

Table 4. Similarities of *P. syringae* pv. *glycinea* ORFs to proteins in databases.

Gene	Size (aa)	Protein and function/organism/homology	% aa homology
orf 1	1,191	Putative glycosyl transferase (*P. syringae* pv. *tomato*)	92.9
		Putative glycosyl transferase (*P. syringae* pv. *tabaci*)	99.6
		fgtA, putative glycosyltransferase (*P. aeruginosa*)	31.7
		Rfbc, O-antigen biosynthesis (*M. xanthus*)	30.1
		Y4gl, biosynthetic protein for rhamnose-rich lipopolysaccharide (*Rhizobium* sp. Strain NGR234)	30.3
orf 2	968	Putative glycosyltransferase (*P. syringae* pv. *tomato*)	91.6
		Putative glycosyltransferase (*P. syringae* pv. *tabaci*)	99.3
		fgtA, putative glycosyltransferase (*P. aeruginosa*)	37.8
		Rfbc, O-antigen biosynthesis (*M. xanthus*)	29.9
		Y4gl, biosynthetic protein for rhamnose-rich lipopolysaccharide (*Rhizobium* sp. Strain NGR234)	28.3
orf 3	308	Putative 3-oxoacyl-(acyl-carrier-protein) synthase III (partial sequence) (*P. syringae* pv *tabaci*)	100
		Putative 3-oxoacyl-(acyl-carrier-protein) synthase III (*P. syringae* pv *tabaci*)	98.7
		OrfC, unknown (*P. aeruginosa*)	29.2

Modified from Takeuchi et al., 2003.[86]

in nonglycosylated flagellin and partially glycosylated flagellins, respectively, indicating that both gene products are necessary for flagellin glycosylation.[86] In this system, two glycosyltransferases are thought to function in a certain order. It has been indicated that the *orf1* product initially transfers some saccharide(s) to flagellin proteins, followed by modification by *orf2*.[86]

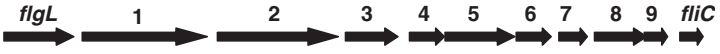
The *orf1, orf2*, and *orf3* of *P. syringae* pv. *tabaci* 6605 exhibited 99.6%, 99.3%, and 99.7% homology at the amino acid level to the corresponding ORFs in *P. syringae* pv. *glycinea*.[86] It has been previously demonstrated that posttranslational modification of flagellin correlates with the ability to induce hypersensitive reaction (HR) cell death.[82,83] Based on this observation, it is speculated that the slight differences at the amino acid level among the pathovars *tabaci* and *glycinea* may determine substrate specificity or sugar binding preferences, both of which play crucial roles in the specificity of pathogen–plant interactions.[84] The glycosylated amino

acid residues on the flagellin molecule of *P. syringae* pv. *tabaci* have been identified at positions 143, 164, 176, 183, 193, and 201 by generation of site-directed Ser/Ala-substituted mutants.[84] Further Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) analysis revealed that each glycan was about 540 Da. Based on the predicted tertiary structure of flagellin, S176 and S183 are expected to be located on most external surface of the flagellum.[84]

Recently, Taguchi and colleagues investigated the role of *orf3* gene in the glycosylation island of flagellin gene cluster of *P. syringae* pv. *tabaci* 6605.[85] The *orf3* gene has significant homology to the 3-oxoacyl-(acyl-carrier-protein (ACP)) synthases III of *E. coli*, *Salmonella enterica* serovar *typhimurium*, and *Pseudomonas putida* strain KT2440.[60,85] The Δ*orf3* mutant had a significantly reduced ability to form acyl-homoserine lactones (AHLs), quorum-sensing molecules, suggesting that Orf3 is required for AHL synthesis. Moreover, the phenotypes of the Δ*orf3* mutant and AHL synthesis mutant (Δ*psyI*) were found to be similar. Recent papers have suggested that each process of biofilm formation might be regulated by expression of quorum-sensing genes.[2,19] It is speculated that the *orf3* gene in relation to flagella expression[85] might regulate the dynamic alterations in gene expression for biofilm formation.

4.2. Pseudomonas fluorescens

P. fluorescens strain pf5 an environmental pseudomonad whose genome was recently sequenced (http://www.ncbi.nlm.nih.gov/entrez/querygenome) but not annotated, has a glycosylation island that lies between the *flgL* and *fliC* genes. The genetic arrangement of this locus is depicted in Figure 6. The first two genes of this island show high homology to putative glycosyl transferase of *P. syringae*. The rest of the genes likely have a role in flagellin modification. The last two genes are conserved hypothetical genes. The details about the function of these genes and their homologues are listed in Table 5.



The Genetic arrangement of glycosylation locus in *P. fluorescens* Pf5 in between *flgL* and *fliC* Genes.

Figure 6. Glycosylation island of *P. fluorescens* PF5. The genes present in between *flgL* and *fliC* have been annotated as follows: (1) Glycosyl transferase, group 2 family (1190 aa), (2) Glycosyl transferase, group 2 family (978 aa), (3) Flagellin modification protein FlmB (389 aa), (4) Acylneuraminate cytidyltransferase (232 aa), (5) FlaR protein (501 aa), (6) NeuB family protein (350 aa), (7) 3-oxoacyl-(acyl-carrier-protein) (308 aa), (8) Conserved hypothetical protein (429 aa), and (9) Conserved hypothetical protein (89 aa).

Table 5. Homologues of the proteins encoded by genes numbered 1 to 9 (Figure 6) of *P. fluorescens* PF5 in the microbial database.

Protein ID accession number	Function	% similarity to homologue/ homologue function	Accession number of homologue
AAY90917	Glycosyl transferase, group 2 family protein	73, Putative glycosyl transferase (*P. syringae* pv. *tabaci*)	BAC75964.2
		72, Putative glycosyl transferase (*P. syringae* pv. *tomato*)	NP_791769
		50, flagellar glycosyl transferase (*P. aeruginosa*)	AAK15339.1
AAY90918	Glycosyl transferase, group 2 family protein	70, glycosyl transferase, family 2 [*P. fluorescens* PfO-1]	ABA73260.1
		67, putative glycosyltransferase [*P. syringae* pv. *tabaci*]	BAC75965.2
		66, glycosyl transferase, group 2 family protein [*P. syringae* pv. *tomato*]	NP 791770.1
		56, hypothetical protein PaerPA_01001566 [*P. aeruginosa* PACS2]	ZP01364459.1
AAY90919	Flagellin modification protein FlmB	86, DegT/DnrJ/EryC1/StrS aminotransferase [*P. fluorescens* PfO-1]	ABA73262.1
		75, ORF 7; similar to DegT/DnrJ/EryC1/StrS family [*P. aeruginosa*]	AAM27873.1
AAY90920	Acylneuraminate cytidylyltransferase	83, acylneuraminate cytidylyltransferase [*P. fluorescens* PfO-1]	ABA73264.1
		66, ORF 10; similar to cytidylyltransferase [*P. aeruginosa*]	AAM27856.1
		65, Cjejd_01001548 [*C. jejuni* subsp. *doylei* 269.97]	ZP01377713.1
AAY90921	FlaR protein (flaR)	76, GCN5-related *N*-acetyltransferase [*P. fluorescens* PfO-1]	ABA73265.1
		50, ORF 9 predicted glycosyltransferase [*P. aeruginosa*]	AAM27875.1
AAY90922	NeuB family protein	93, NeuB [*P. fluorescens* PfO-1]	ABA73266.1
		70, ORF 11; similar to NeuB family [*P. aeruginosa*]	AAM27877.1
		85, Sialic acid synthase [*Nostoc punctiforme* PCC 73102]	ZP00109033.1
AAY90923	3-oxoacyl-(acyl-carrier-protein) synthase III	96, 3-oxoacyl-(acyl-carrier-protein) synthase III, putative [*P. fluorescens* PfO-1]	ABA73267.1
		94, 3-oxoacyl-[acyl-carrier protein] synthase [*P. syringae* pv. *syringae* B728a]	AAY38499.1
		94, putative 3-oxoacyl-[acyl-carrier-protein] synthase III [*P. syringae* pv. *tabaci*]	BAD24669.1
		93, 3-oxoacyl-(acyl-carrier-protein) synthase III, putative [*P. syringae* pv. *tomato* str. DC3000]	AAO55466.1
		91, 3-oxoacyl-(acyl-carrier-protein) synthase III [*P. putida* KT2440]	AAN69957.1
AAY90924	Conserved hypothetical protein	78, conserved hypothetical protein [*P. fluorescens* PfO-1]	ABA73268.1
AAY90925	Conserved hypothetical protein	65, hypothetical protein Pfl_1526 [*P. fluorescens* PfO-1]	ABA73269.1

Figure 7. Glycosylation island of *P. entomophila* L48. The four genes present in between *flgL* and *fliC* are annotated as follows: (1) glycosyltransferase, (2) glycosyltransferase, (3) glycosyltransferase, and (4) 3-oxo-(acyl-carrier-protein) synthase III.

4.3. *Pseudomonas entomophila* L48

P. entomophila L48 is an insect pathogen with multiple habitats. Its genome was recently sequenced (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi? db=Genome). The flagellar glycosylation island of this organism lying between *flgL* and *fliC* genes is depicted in Figure 7. The first three genes of this island are glycosyl transferase and show variable homologies to the glycosyl transferase of other organisms (Table 6). The last gene in this island is 89% homologous to 3-oxoacyl-(acyl-carrier-protein) synthase III of *P. putida*.

5. PILIN GLYCOSYLATION

The pili of *P. aeruginosa* are assembled from a monomeric subunit, pilin, that has a molecular weight in the range of 15 to 17,000 Da and has the characteristics associated with the type IV pili (TFP).[16,61] Many bacteria express the TFP. However, the most extensively characterized TFP are those of *N. gonorrhoeae*, *P. aeruginosa* and *V. cholerae*, for which crystal structures have been determined.[17,33,62] Pilins of *P. aeruginosa* could be divided into five distinct phylogenetic groups (designated I-V), based on amino acid sequence and the presence of unique accessory genes immediately downstream of *pilA*[44] Figure 8. Only pilins belonging to groups 1 and IV are known to be glycosylated.

P. aeruginosa strain 1244 pilins fall in group I. The pilin glycan of this strain is product of the O-antigen biosynthetic pathway.[13,14,21] The gene immediately downstream of *pilA* encodes the PilO/TfpO glycosyltransferse involved in transfer of the O-antigen unit to the C-terminal Ser residue of PilA. The *P. aeruginosa* PAO1 genome sequence[78] contains a completely unrelated gene also named "*pilO*" (ORF *PA5042*), which is not involved in pilin glycosylation. Therefore, to reduce future confusion Kus and colleagues have renamed the glycosylation gene *pilO* (adjacent to *pilA*) as *tfpO*.[44] The pilin of *P. aeruginosa* strain 1244 (O7 immunotype) is glycosylated with a trisaccharide: containing pseudaminic acid, xylose, and

Table 6. Homologues of the proteins encoded by genes in the glycosylation island of *P. entomophila* L48 in the microbial database.

Protein ID accession number	Function	% similarity to homologue/homologue function	Accession number of Homologue
YP_609332	Glycosyltransferases, probably involved in cell wall biogenesis	67, glycosyl transferase, group 2 family protein [*P. putida* F1]	ZP_00900536.1
		50, glycosyl transferase, family 2 [*P. fluorescens* PfO-1]	YP_347240.1
		49, glycosyl transferase, family 2 [*P. syringae* pv. *syringae* B728a]	YP_236539.1
		48, putative glycosyltransferase [*Pseudomonas savastanoi* pv. *glycinea*]	BAD01152.1
		48, glycosyl transferase, group 2 family protein [*P. syringae* pv. *phaseolicola* 1448A]	AAZ34985.1
		48, putative glycosyltransferase [*P. syringae* pv. *tabaci*]	BAC75964.2
		48, glycosyl transferase, group 2 family protein [*P. syringae* pv. *tomato* str. DC3000]	AAO55464.1
		48, glycosyl transferase, group 2 family protein [*P. fluorescens* Pf-5]	YP_258746.1
YP_609331	Glycosyltransferase	63, glycosyl transferase, group 2 family protein [*P. putida* F1]	ZP_00900535.1
		35, glycosyl transferase, family 2 [*P. fluorescens* PfO-1]	YP_347249.1
		34, glycosyl transferase, group 2 family protein [*P. fluorescens* Pf-5]	AAY90918.1
		34, glycosyl transferase, family 2 [*P. syringae* pv. *syringae* B728a]	YP_236538.1
YP_609330	Glycosyltransferase	46, glycosyl transferase, family 2 [*Methylobacillus flagellatus* KT]	YP_546754.1
		49, hypothetical protein PaerP_01002283 [*P. aeruginosa PA7*]	ZP_01295786.1
		48, hypothetical protein PaerPA_01001566 [*P. aeruginosa* PACS2]	ZP_01364459.1
		48, *fgtA* a flagellar glycosyltransferase [*P. aeruginosa* 2192, PAK]	ZP_00976566.1
YP_609329	3-oxoacyl-(acyl-carrier-protein) synthase 111	89, 3-oxoacyl-(acyl-carrier-protein) synthase III [*P. putida* F1]	ZP_00900525.1
		78, 3-oxoacyl-(acyl-carrier-protein) synthase III [*P. putida* KT2440]	NP_746493.1
		78, 3-oxoacyl-[acyl-carrier protein] synthase [*P. syringae* pv. *syringae* B728a]	YP_236537.1
		77, 3-oxoacyl-(acyl-carrier-protein) synthase III, putative [*P. syringae* pv. *tomato* str. DC3000]	NP_791771.1
		77, putative 3-oxoacyl-[acyl-carrier-protein] synthase III [*P. syringae* pv. *tabaci*]	BAD24669.1

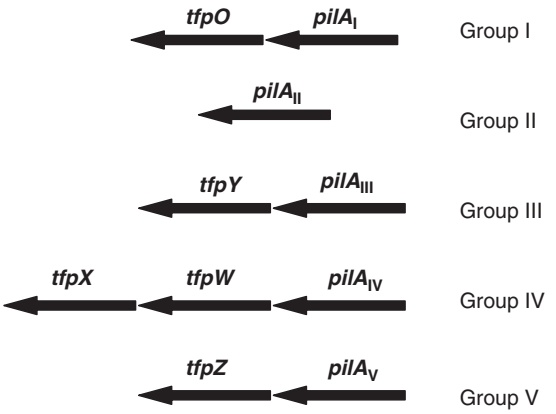


Figure 8. Pilin alleles and associated accessory gene(s) in *P. aeruginosa*. Phylogenetic grouping (designated I-V) of *P. aeruginosa* pilins based on amino acid sequence and the presence of unique accessory genes immediately downstream of *pilA*. The genes mentioned in all the groups are flanked by the conserved tRNA-Thr and *pilB*. Modified from Kus *et al*., 2004.[43]

N-acetylfucosamine [α-5NβOHC$_4$7NFmPse-(2→4)-β-Xyl-(1→3)-β-FucNAc].[13,15,54,55] This trisaccharide is the same as the O-antigen repeating unit found in the O7 immunotype LPS.

The common laboratory strains PAO1 and PAK (group II) have no accessory genes downstream of *pilA* and produce unmodified mature pilins as previously established.[15] All group II PilA sequences have a 12 aa C-terminal DSL (disulfide-bonded loop) region related to that of strains PAO1 and PAK.

Strains (Pa4494, Pa5024, Pa5112, Pa5122, Pa5223, and Pa141123 and PA14) have been assigned to group III. These strains have an *orf* named *tfpY* (*t*ype *f*our *p*ilin gene *Y*) that corresponds to a previously reported gene called *orf1*.[77] This *orf* lies immediately downstream of the pilin gene. The protein encoded by *tfpY* is identical in all strains carrying PilA$_{III}$ and is homologous to FimB, a pilin accessory protein of unknown function from *D. nodosus*.[41] These pilins do not appear to be glycosylated.

Group IV currently contains a single isolate, PA5196. There are two novel *orf*s immediately downstream of *pilA* in PA5196. The first, *tfpW*, encodes a large hydrophobic hypothetical transmembrane protein, while the second, *tfpX*, encodes a putative pilin accessory protein most similar to TfpZ and to PilB and FimB of *E. corrodens* and *D. nodosus*, respectively. Recently, it has been shown that the Group IV pilins continue to be modified in a LPS (*wbpM*) mutant of PA5196, showing that, unlike group I strains, the pilins of group IV are not modified with the O antigen unit

of the background strain.[94] The pilin glycan of Pa5196 was determined to be an unusual homo-oligomer of α 1, 5-linked D-arabinofuranose (D-Araf).[95] This sugar is uncommon in prokaryotes, occurring mainly in cell wall arabinogalactan.

Strains Pa271457, Pa081061, and Pa110594 have novel *pilA* genes and fall in group V. Adjacent to the *pilA* gene in each of these strains is a novel accessory gene *tfpZ*. This gene encodes a putative pilin accessory protein most homologous (31% identity, 53% similarity) to the PilB protein of *E. corrodens* strain VA1.[93]

At present, the proximity of *pilO* to the O-antigen gene cluster in strain 1244 is unknown because whole genome sequence information of serotype O7 is presently unavailable. Thus, existence of pilin glycosylation genes does not necessarily make the case for a glycosylation island but it extends the glycosylation repertoire of Pseudomonads. The importance of TFP in the biology and virulence of *P. aeruginosa* has made them attractive targets for vaccine development.[34,75] Saiman and colleagues raised cross-reactive monoclonal antibodies that recognized some pilin variants and reduced binding to eukaryotic cells.[71] Concerning the role of pilin glycosylation Smedely and colleagues showed in a mouse respiratory model that the presence of the pilin glycan allowed a significantly greater survival in the lung environment.[76] This suggests that the pilin glycan is a significant virulence factor and may aid in the establishment of infection.

6. ROLE OF FLAGELLAR GLYCOSYLATION

The hierarchy of flagellar gene regulation and assembly has been extensively studied for the peritrichously flagellated *E. coli* and *Salmonella typhimurium*[53] where the flagella are nonglycosylated. The genes and gene organization of polar motility systems of Gram-negative bacteria such as *Vibrio*, *Pseudomonas*, *Campylobacter*, *Aeromonas*, and *Caulobacter*, have received attention only recently.[6,28,29,46,51,52,57,88] A number of studies[29,38,46,49,91] however, have provided clues that glycosylation may be a unique feature of polar motility systems, where it could play a key role in the assembly process, adhesion, motility, inflammation, and virulence. Thus, the flagellar glycosylation may indeed contribute to the unique biological properties of these particular flagella.

The effect of glycosylation on flagellar assembly is poorly understood. In *P. aeruginosa* as well as in *P. syringae* the absence of glycosylation of the flagellin protein does not lead to a loss of flagellar filament assembly or a loss of motility. In contrast, two other polarly flagellated organisms, *Campylobacter* and *Helicobacter* become nonmotile and are unable to synthesize a flagellar filament when the glycosylation genes are

mutated.[27,74] In comparison to *P. aeruginosa*, the flagellar filaments of these two organisms are complex and are comprised of two flagellin monomeric proteins, FlaA, and FlaB, and the level of glycosylation per protein monomer is substantially higher. However, it remains to be determined whether either of these features is the reason for a more dramatic effect on flagellar assembly when glycosylation is inhibited.

Recently roles for protein-associated glycans has been suggested in bacterial pathogenesis as glycosylation defective mutants of several bacteria, have been shown to be attenuated in virulence attributes, such as adhesion, invasion[80], and colonization.[40] Flagellin glycosylation mutants, (*fgtA*) of strains PAK and PAO1 have been shown to be significantly attenuated in virulence in the burn wound infections caused by *P. aeruginosa*, suggesting a role for flagellin glycosylation in *P. aeruginosa* virulence.[7] Similar results have been reported in *P. syringae* where all glycosylation-defective mutants were impaired in their ability to cause disease in tobacco.[84]

The other possible functions of flagellin glycosylation are to provide antigenic variation of the flagellar surface as speculated for *Campylobacter*.[5,23] Glycosylation may also provide stability to proteins, e.g., it has been hypothesized that nonglycosylated FlaA (*C. jejuni*) is less stable than wild-type FlaA.[38,39] As has been described for pilins,[54] flagellin glycosylation might affect the interaction of the flagellin subunits among each other, or facilitate the dynamic interaction with their cognate chaperone FliS[9] during secretion. Thus, it might be involved in the process of correct secretion of late structural proteins via the flagellar type III secretion system, and even provide a means for flagellin stabilization coupled to secretion. Nevertheless, in *P. aeruginosa* neither flagellar assembly nor motility are affected by mutations in the GI, therefore the true function of flagellar glycosylation that has allowed its persistence in this primarily environmental organism that is an accidental pathogen, remains to be ascertained.

6.1. Flagellar Glycosylation and Inflammation

Many studies demonstrated that flagellin signals through TLR5 to induce the gene expression of host defense and proinflammatory molecules in various cell types.[1,4,32,91] The exact region of *P. aeruginosa* strain PAK flagellin molecule interacting with TLR5 has been determined.[36,91] It has been demonstrated that the IL-8 signaling from the flagella purified from the two glycosylation defective mutant strains is reduced up to 50% compared to the levels for their respective wild-type strains.[91] Further support for a role of *Pseudomonas* flagellar glycosylation in inflammation comes from the observation that the nonglycosylated whole flagella from the two said strains do not appear to differ in their stimulatory activities,

whereas the glycosylated forms differ significantly. Whether flagellin glycan moieties would aid in the binding of flagellin to TLR5 or glycans have some signaling activity of their own through another TLR, or other cellular receptor is not known.

Much needs to be learned about glycosylation islands in bacteria. Besides function, their evolutionary origins are obscure. Structural studies are also lacking, as a variety of unknown sugars exist on many of the glycans. It is anticipated that these missing pieces will contribute to understanding the reason for glycosylation, as some functions may be inferred when structural data becomes available.

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PHYSIOLOGY AND METABOLISM

3

STYRENE, AN UNPALATABLE SUBSTRATE WITH COMPLEX REGULATORY NETWORKS

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1. INTRODUCTION

Styrene, a volatile organic compound (VOC), is an important industrial material involved in the production of plastic, synthetic rubber and resin, insulation and other industrial materials containing molecules such as polystyrene, butadiene-styrene latex, styrene co-polymers and unsaturated polyester resins. Styrene exposure may cause contact-based skin inflammation, irritation of eyes, nose and respiratory tract. Neurological effects such as alterations in vision, hearing loss and longer reaction times, have been associated with styrene exposure in the workplace. In addition, styrene oxide may act as an established mutagen and carcinogen (www.epa.gov/ chemfact /styre-sd.pdf). It has been reported that, in 2002, 22,323 tons of styrene were released to the environment,[85] in spite of the US Clean Air Act mandate on reduction in the volume of allowable styrene emission (www.epa.gov/chemfact /styre-sd.pdf). Among a variety of emerging air pollution technologies, biofiltration is an attractive option for the treatment

Juan-Luis Ramos and Alain Filloux (eds.), Pseudomonas, 59–87.

of VOCs, because it is cost-effective and does not generate secondary contaminants.[47] Moreover, microbial biodegradation is the major route for the removal of non-aqueous compounds from soils. Styrene is also naturally present in non-polluted environments, since it derives from fungal decarboxylation of cinnamic acid.[93] Therefore it is not surprising that microorganisms of different families have been found to be able to degrade this compound.[33] The promising results obtained in the removal of styrene from contaminated waste-gases by biofiltration[5,41,107] have led to an increasing attention to the regulatory mechanisms underlying styrene degradation, with the aim to improve bioremediation processes. Despite the diffusion in nature of this degradative capability, only few strains, mainly belonging to the *Pseudomonas* genus, have been characterized.[69]

This chapter is focused on the up-to-now discovered regulatory mechanisms underlying the expression of the styrene-catabolism genes. Moreover, open questions on environmental and metabolic constrains that govern styrene degradation are discussed. Biotechnological relevance of styrene-degrading strains in fine chemicals production and bioremediation processes is not examined here. Main topics on these application fields have recently been reviewed by Dobson and co-workers.[69]

2. STYRENE-CATABOLISM SYSTEMS: A DESCRIPTION

Microbial metabolism of styrene has been extensively reviewed recently,[69] therefore only a brief summary on this subject will be presented here. Styrene degradation can occur both in anaerobic and aerobic conditions. Anaerobic degradation has been poorly investigated. In one study performed with microbial consortia styrene catabolism initiated through an oxidation–reduction reaction, most likely by the addition of water across the double bond in the alkenyl side-chain. The degradation proceeded through to carbon dioxide, as final product. Benzoic acid and phenol were transient compounds found in highest concentrations in the spent culture fluid and were suggested as the key intermediates of the transformation process, but the enzymatic activities and the genes involved in this transformation process have not been investigated.[31] Accordingly, benzoyl-coenzyme A (benzoyl-CoA) is considered a key common intermediate in the anaerobic degradation of monoaromatic benzene-toluene-ethylbenzene-xylenes (BTEX) hydrocarbons. This compound undergoes a ring reduction catalyzed by benzoyl-CoA reductase coupled with ATP hydrolysis, before ring cleavage and oxidation.[17]

In aerobic conditions, two principal pathways have been described for styrene degradation in bacteria, one in which the first step consists in the oxidation of the aromatic ring, and the other one in which the side-chain is first

oxidized. The best studied microorganism that degrades styrene through the ring attack is *Rhodococcus rodochrous* NCIMB 13259.[104] In this strain, styrene breakdown starts with the hydroxylation of the aromatic nucleus by a dioxygenase, leading to the formation of vinylcatechol. This compound can be substrate of both 1,2 and 2,3 dioxygenases, but only the *meta* pathway, in which the vinyl group is eliminated as acrylic acid, is productive.[104]

Styrene conversion to phenylacetic acid (PAA) via the vinyl chain oxidation is referred to as the upper pathway of the styrene degradation. The entire sequence of the catabolic *styABCDE* and regulatory *stySR* operons of this pathway has been obtained in *Pseudomonas fluorescens* ST[8,56] and in *Pseudomonas* spp. Y2,[101,103] while in *Pseudomonas putida* CA-3[65,68] and in *Pseudomonas* spp. VLB120[73] some sequence gaps are still present. In any case, the organization of the genes and their high sequence homology allow us to refer to the *sty* genes without making any difference among these four strains (Figure 1).

Styrene degradation starts with the oxidation of the vinyl double bond to styrene oxide by styrene monooxygenase (SMO), a two-component flavin-dependent oxygenase encoded by the *styA* and *styB* genes, whose reaction mechanism has recently been proposed.[71] *styC* codes for styrene oxide isomerase (SOI), which converts styrene oxide to phenylacetaldehyde that is oxidized to PAA by phenylacetaldehyde dehydrogenase (PADH), encoded by *styD*.[8,56,65,73,101,103] The *styE* gene has recently been identified in *P. putida* CA-3 by Dobson and co-workers as coding for a 431 amino acids protein, homologous to several membrane-associated ATPase-dependent transporters, involved in the active transport of aromatic compounds.[60] In the

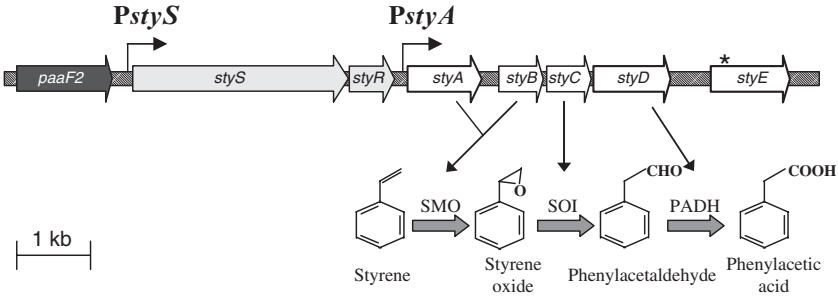

Figure 1. General organization of the *sty* genes and styrene upper pathway in *Pseudomonas* spp. *paaF2*, phenylacetyl-CoA-ligase (previously reported as *paak*); *stySR*, sensor histidine kinase; *styR*, response regulator; *styAB*, styrene monooxygenase (SMO); *styC*, styrene oxide isomerase (SOI); *styD*, phenylacetaldehyde dehydrogenase (PADH); *styE*, styrene transport protein. P*styS* and P*styA* are the promoters of the regulatory and catabolic operons, respectively. The asterisk indicates the position of the IS*1162* insertion in *P. fluorescens* ST (modified from ref [53]).

same paper the authors demonstrated that *styE* is co-transcribed with the *styABCD* genes and that it is essential for growth on styrene. Moreover, overexpression of *styE* led to an increase of both SMO activity and *styA* transcript amount. These data strongly support the involvement of StyE in styrene uptake and suggest that styrene passive diffusion is a limiting factor in the expression of the catabolic pathway.[60] StyE is also present in Y2[103] and ST strains,[8] while no data on the VLB120 strain are available. However, in ST the *styE* sequence is interrupted at nt 198 by the presence of IS*1162*[8,96] and this does not impair growth on styrene. It is not known whether the remaining part of this gene is expressed, if the truncated product (368 amino acids out of 434) is functional, or whether a full copy of this truncated gene is present elsewhere in the genome.

The *styS* and *styR* regulatory genes, encoding a two-component system (TCS), are located upstream of the catabolic operon. *styS* encodes the StyS sensor kinase and *styR* the cognate response regulator (RR), StyR, that was demonstrated to be essential for the activation of P*styA*, the promoter of the catabolic operon.[68,86,103]

PAA, the final product of the styrene upper pathway, is a common substrate for *Pseudomonas* spp., probably because degradation of many aromatic compounds (styrene, 2-phenylethylamine, *trans*-styrylacetic acid and phenylalkanoic acids with an even number of carbon atoms) converges to PAA formation.[22,40,55,70] In the PAA catabolic pathway, that represents the lower pathway for styrene degradation, PAA is activated to phenylacetyl-CoA (PA-CoA) and then degradated through a β-oxidation-like process.[27,28,70] Note that the genes coding for the PAA pathway are named *paa* or *phe* and that *paa*/*phe* genes coding for the same enzymatic function are differently named in different microorganisms.[1,40] Here we refer to the PAA-catabolism genes of the styrene-degrading *Pseudomonas* sp. Y2. Two *paa* gene clusters, named *paa1* and *paa2*, have been identified in this strain.[1,7] Although the individual genes of each set are highly homologous, the gene organization and the genetic context where they are localized are different. The *paa1* gene cluster is located upstream of the *sty* genes.[1] The gene organization of this cluster is different from that of *paa2* and from the one found in *P. putida* KT2440,[40] and seems to have been subjected to extensive rearrangements, as also demonstrated by the duplication of genes coding for PA-CoA ligase (named *paaF* and *paaF2*) and of genes coding for a ring opening enzyme (named *paaN* and *paaN2*). In the Y2 strain, one *paa* gene cluster can functionally substitute for the other one, as demonstrated by the fact that only when both the two set of *paa* genes were inactivated, Y2 cells were unable to grow on PAA and on styrene.[7] The existence of a pathway homologous to *paa1* also in the other *Pseudomonas* styrene-degrading strains is strengthen by the presence of the *paaF2* gene, coding for the PA-CoA ligase enzyme, just upstream of

the *sty* genes.[8,103] The linked localization of the upper and lower styrene pathways in all the styrene-degrading strains suggests that the entire *paa-sty* region has been inherited by horizontal gene transfer and that the two clusters could derive from a common evolutionary history.[1,103] However, the G + C content analysis, performed in *Pseudomonas* spp. Y2, clearly indicates that the two clusters are evolutionary unrelated. More interestingly, an analysis of single gene codon usage revealed that not only *sty* and *paa* genes are unrelated, but also that *sty* catabolic genes and *sty* regulatory genes have probably evolved separately.[1] We found that, in *P. fluorescens* ST, inactivation of *paaA* and *paaC* genes, coding for enoyl-CoA hydratase/isomerase and hydroxyacyl-CoA dehydrogenase, respectively,[1] did not affect growth on PAA or on styrene (unpublished results), indicating that also in this strain an additional *paa* gene cluster could be present. Alternatively, these mutations could be complemented by corresponding enzymatic activities of other β-oxidation pathways.

The regulation of PAA catabolism has been poorly investigated. The analysis of the intergenic regions of the *paa1* cluster in Y2 identified five putative σ^{70}-dependent promoters. Two possible regulators, PaaX and PaaY, have been identified in most of the PAA catabolic pathways sequenced.[40] While the function of PaaY is still unknown, the role of PaaX as a transcriptional regulator of *paa* gene expression in *Escherichia coli* K12 has been established.[26] PaaX is a repressor protein of the GntR family that binds the specific operator site of the target promoter. The interaction of PaaX with PA-CoA, the first intermediate of PAA catabolism, leads to its release from the promoter, so allowing transcription.[26]

3. STYRENE AS A TOXIC AND A NUTRIENT SIGNAL

Styrene and the other solvents, such as toluene, benzene and xylenes, with a partition coefficient in an octanol–water mixture (log $P_{o/w}$ value) between 1.5 and 4 are extremely toxic to cells because they accumulate in the cytoplasmic membrane, disorganizing its structure and impairing vital functions.[20,95] As experienced by researchers who work with microorganisms that grow on hydrophobic compounds, a pre-adaptation at a low concentration of the organic solvent is necessary in order to obtain a good growth at higher concentrations. If this pre-culture is not done, only a fraction of the cells (about 0.001%) survives to a sudden exposure to high concentrations of the toxic compound.[81]

Several possible mechanisms concurring to solvent tolerance have been described.[78] In addition to the adaptative response at the cytoplasmic membrane level, such as *cis*/*trans* isomerization of unsaturated fatty acids and changes in the head group formation of the membrane phospholipids,[37,42,78]

tolerance to these chemicals is mainly achieved by a number of energy-dependent active efflux pumps belonging to the RND (acronym for Resistance-Nodulation-cell Division) family.[45,46,62,79,83] Studies on hydrocarbon tolerance in *Pseudomonas* spp. have been mainly performed in three different *P. putida* strains: the styrene-degrading strain S12,[34] and the two toluene-degrading strains DOT-T1E[80] and F1.[30] Interestingly, in DOT-T1E and F1 strains toluene catabolic gene expression is under the control of the two-component regulatory system TodS/TodT, very similar to the StyS/StyR one.[50,63] Moreover, these two systems respond to the presence of both styrene and toluene.[2,16,49,63] To our knowledge, the only styrene-degrading strain studied for styrene tolerance is *P. putida* S12,[105] in which the styrene-catabolism system is probably similar to that of the other above described styrene-degrading strains, since it contains a nearly identical sequence of the *styAB* genes.[43] In this strain, an active efflux pump, named SrpABC, has been identified.[39,45] This energy-dependent export system is involved in the resistance to a wide variety of organic solvents, including aromatic and aliphatic compounds and alcohols, that induce transcription of the *srpABC* genes. On the other hand, the SrpABC efflux system does not appear to be involved in excreting hydrophobic antibiotics, or to respond to general stress conditions, such as temperature, pH, heavy metals and osmotic stress.[45] The presence of active efflux pumps specifically dedicated to solvents extrusion has been reported in other *P. putida* strains. In the toluene-degrading strain *P. putida* DOT-T1E, the TtgDEF efflux pump, one of the three efflux systems found in this strain,[83] is induced by toluene and styrene and is mainly involved in removing these hydrocarbons.[62,63] The other two efflux pumps TtgABC and TtgGHI exhibit broader effector specificity, pumping out, besides solvents, also antibiotics such as chloramphenicol and tetracycline.[25,83] In *P. putida* F1, *sepABC* genes code for a solvent-specific efflux pump and their expression is induced by aromatic compounds but not by aliphatic solvents or alcohols.[77] Interestingly, the *ttgDEF* and *sepABC* genes are linked to the *tod* genes for toluene metabolism in DOT-T1E and F1 strains, respectively.[62,77]

Solvent tolerance also involves a complex and probably multi-step reprogramming of sugar and energy metabolism, as highlighted by proteomic analyses of *P. putida* KT2440[24] and DOT-T1E[90] cells, after short-term and long-term exposure to toluene, respectively. Comparing the different patterns of proteins expressed in these two conditions, it is tempting to speculate that the success of the quick response, supposed to be aimed to redirect the available transcriptional resources to counteract cell damage and assure survival,[24] might constitute the input signal to start a new metabolic reprogramming directed to gain solvent tolerance.

Among the solvent-sensitive transposon mutants isolated both in the styrene-degrading S12 and in the toluene-degrading DOT-T1E strains, mutations in the flagella biosynthetic pathway have been found. In S12,

mutations in flagella structural proteins, in the transcriptional activator FleQ and in the flagellum-specific RNA polymerase sigma factor FliA led to a decrease of *srpABC* gene expression, resulting in a reduced tolerance to organic solvents.[44] The relationship between motility and toluene tolerance has been investigated in DOT-T1E and the results obtained indicated that an intact flagellar export apparatus plays a direct or indirect role in toluene tolerance.[89,91] There is therefore a structural or regulatory connection between chemotactic apparatus and solvent tolerance.

In contaminated sites, bacterial chemotaxis is an important feature that allows microbial degraders to sense and gain access to poorly soluble hydrophobic compounds that can represent important carbon sources.[51] Bacterial chemotaxis towards environmental pollutants has been investigated only in few microorganisms, compared with the huge number of strains known for their capability to degrade these compounds.[72,74] Non-essential transporters of *p*-hydroxybenzoate[35] and 2,4-dichlorophenoxyacetate,[36] encoded by the *pcaK* and *tfdK* genes, respectively, have been found to be essential for chemotactic response towards these compounds, but it is not yet known if these proteins are chemoreceptors or play an indirect role in chemotaxis. As a general rule, chemotaxis seems to respond to the presence of attractants that can be degraded and/or recognized by the enzymes of the corresponding catabolic pathway. However, the degradation process *per se* is not always required for the chemotactic response.[72,74] In *P. putida* F1, mutants defective in toluene dioxygenase, which cannot grow on toluene, or in the TodX toluene permease, which coding gene *todX* is part of the *tod* operon, are chemotactic towards this compound. On the contrary, mutants defective in the TodS/TodT TCS, essential for the expression of the *tod* operon, do not exhibit chemotaxis towards toluene.[75] It has been suggested that TodS itself could function as a sensor, mediating a regulatory coordination between the expression of genes required for the chemotactic response and those for the degradation and uptake of the corresponding substrate.[75] Due to the similarity between the TodS/TodT and StyS/StyR regulatory systems and their capability to sense both toluene and styrene, it would be very interesting to investigate if a similar regulatory relationship determines chemotactic response to styrene.

As a whole, the few examples above reported strongly point out the existence of complex interactions between solvent tolerance, primary metabolism and the flagellar system. Probably microorganisms have developed sophisticated regulatory networks in order to survive in polluted sites where toxic compounds, either metabolizable or not, are simultaneously present. The specificity of the chemotactic response and the parallel broad range of compounds that are recognized by the efflux pumps constitute a good compromise between the need to counteract solvent toxicity and the necessity of scavenging possible carbon and energy sources.

4. PHYSIOLOGY OF STYRENE DEGRADATION

Aromatic hydrocarbons, though toxic to the cell, represent an important carbon source for microorganisms that are able to degrade them, especially in polluted sites. However, for the utilization of these compounds, cell must cope not only with their toxicity, but also with the demand of extra energy necessary to synthesize all the enzymes required to transform them in energy-yielding intermediates. This is probably the reason why all the known hydrocarbon catabolic systems, independently of the single evolutionary histories, are subjected to a repression when nutrients endowed with a more rapid assimilation are simultaneously present.

Physiological studies on the effects of different carbon sources on the expression of the styrene-catabolism genes in *P. fluorescens* ST, have shown that the expression of the *styABCDE* catabolic operon is induced by styrene and that such an induction is repressed to different extents by organic acids or carbohydrates. In particular, lactate and succinate exert a moderate repression, allowing the expression of the catabolic operon during the mid-exponential growth phase, when these carbon sources have not been completely depleted. In contrast, in cells grown on styrene plus glucose, acetate or glutamate, styrene utilization is induced only when the concentration of the preferred carbon source is not sufficient to further sustain cell growth.[86] In these experiments the enzymatic activities of both SMO, encoded by the *styA* and *styB* genes, and β-galactosidase, encoded by the *lacZ*-reporter gene under the P*styA* promoter control, were determined. The parallel induction pattern displayed by the two activities demonstrated that the modulation of the catabolic gene expression occurs at the transcriptional level.[86]

Similar studies performed in *P. putida* CA-3 on one hand confirmed the repressing effects of organic acids on the expression of the styrene catabolic operon, on the other hand showed differences in the pattern of the repressing carbon sources with respect to the strain ST.[65,66] The main difference concerns the effect of these compounds on the expression of the *stySR* operon. In fact, while we found that the *stySR* operon was expressed, even if at a lower level, also in the absence of styrene, this does not occur in CA-3 strain.[68] However, CA-3 growth in a continuous culture without styrene and under PAA limiting concentration led to the induction of both styrene catabolic and regulatory genes, besides the *paa* lower pathway.[67,69] Thus, the substrate of the lower pathway, PAA, can act as a strong repressor in batch cultures where its concentration is not limiting and as an inducer in growth limiting concentrations. Under inorganic (nitrate, sulphate or phosphate) nutrient-limiting conditions with saturating PAA concentrations, detectable levels of the *stySR* transcript, were observed.[67] These data show that signals other than styrene can induce *stySR* expression.

The different conditions that allow the expression of the regulatory genes in CA-3 and ST strains could reflect differences in their nutritional demands.

When non-induced cells are grown on both styrene and a preferred carbon source, the energetic-metabolic signals that dictate the induction of the styrene catabolic genes seem to be different from the ones operating when a repressive carbon source is added to styrene induced cells. In fact we found that the addiction of glucose to styrene growing cells exerted a less stringent repression on styrene catabolic gene expression with respect to what observed in induction experiments (see Section 5.3).[86,53] Probably, more complex metabolic requirements and, consequently, more regulatory checking points are implicated in the induction of a peripheral pathway, distant from the central metabolism, with respect to those required to repress an already induced peripheral catabolic system.

The possible regulatory mechanisms involved in the carbon catabolite repression of styrene metabolism are discussed in Section 5.

5. REGULATORY MECHANISMS IN THE EXPRESSION OF STYRENE-CATABOLISM SYSTEM

5.1. The Styrene Regulatory Genes

The *styS* and *styR* genes are located upstream of the *styABCDE* operon and are required for the styrene-dependent expression of the *styABCDE* operon in the heterologous host *E. coli*.[103] The *styS* and *styR* genes are co-transcribed and translationally coupled, and encode two proteins showing a high similarity with members of the superfamily of TCSs for signal transduction.[68,86,73,103] Although TCSs are the major signalling pathways in eubacteria,[97] they have been found to be involved in the regulation of genes for aromatic compound degradation only in styrene and toluene degradation in *Pseudomonas* spp. and in *Thauera* spp., and in biphenyls degradation in *Rhodococcus* sp. M5 and RHA1 strains.[19,48,50,82,100] The constituent proteins of TCSs are a sensor histidine kinase (HK; of widely different sizes, ranging from about 40 to 110 kDa) and a response regulator (RR; often around 25 kDa). In the presence of a proper chemical signal, the HK component catalyzes the ATP-dependent autophosphorylation at a specific histidine residue, subsequently transferring the phosphoryl group to a conserved aspartate residue at the N-terminal regulatory domain of the RR component. Such a phosphorylation triggers the activation of the RR C-terminal effector domain, which binds specific DNA sequences in most TCSs, regulating transcriptional initiation.[97]

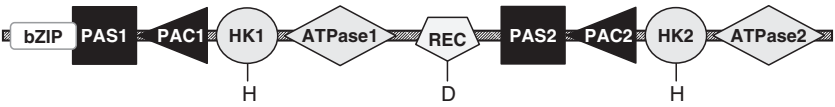

Figure 2. Domain organization of the StyS hybrid kinase, as inferred from the SMART program (http://smart.embl-heidelberg.de). bZIP, leucine zipper domain; PAS1/PAC1 and PAS2/PAC2 are the sensory sub-domains of the input domains 1 and 2, respectively; HK/ATPase, histidine kinase and ATP-binding domains; REC, receiver domain.

The product of the *styS* gene, StyS, is a protein of about 109 kDa, predicted to be a hybrid HK with several distinct functional domains (Figure 2). Interestingly, the only functionally characterized sensor kinases showing an overall high level of homology and the same domain organization as StyS are the strictly related TodS of *P. putida* F1 and DOT-T1E strains, TmoS of *Pseudomonas mendocina* KR1 and TutC of *Thauera aromatica* T1, all involved in the regulation of toluene degradation.[19,50,63,82] A distinctive feature of these proteins with respect to other hybrid HKs is the presence of two distinct kinase cores (HK/ATPase domains in Figure 2). Moreover they contain an internal receiver domain and two putative input domains, consisting of the PAS and PAC sensory sub-domains. PAS is an acronym for *Drosophila P*eriod clock protein, vertebrate *A*ryl-hydrocarbon receptor nuclear translocator, and *Drosophila S*ingle-minded protein. In a current definition, the PAS domain is a highly conserved block (S1 box) of approximately 40 amino acid residues, that can be extended in the carboxyl direction by a second block, the S2 box or PAC motif. Complete PAS domains (including PAS/PAC motifs) were identified in hundreds of proteins from eukaryotes and prokaryotes.[99]

In general, PAS domains are very versatile, monitoring changes in light, redox potential, oxygen, small ligands and overall energy level of a cell. A recent work from the group of J. L. Ramos showed that the purified TodS protein is able to autophosphorylate in vitro and that toluene increases the phosphorylation rate. TodS is also able to transfer the phosphate to its cognate RR TodT in vitro. The analysis of the truncated variants of TodS revealed that toluene binds to the N-terminal input domain, but not to the C-terminal one.[49] However, amino acid substitutions in the receiver domain and in the second PAS domain of TodS were previously found to play a role in the substrate specificity of this sensor kinase.[18] Therefore, what is the stimulus recognized by the second PAS domain of TodS remains still an open question. Several proteins containing PAS domains are known to detect their signal by way of an associated co-factor. For instance, heme, flavin and a 4-hydroxycinnamyl chromophore can be used in different proteins.[99] Since the second PAS/PAC domains of StyS and TodT share some sequence homology with the heme-bound

oxygen-sensing PAS/PAC domain of the *Sinorhizobium meliloti* FixL protein, it has been postulated that this domain could bind heme and sense oxygen.[50,103] However, while heme-binding proteins produced in *E. coli* copurify with heme,[64] the purified TodS does not show absorption peaks in the wave-length range characteristic of heme-bound proteins.[49] Therefore, further structural and functional studies of the StyS and TodS proteins are still required in order to understand the stimuli to which these complex HKs respond, besides styrene and toluene, and their mechanism of action, especially considering their unique domain organization with respect to other hybrid HKs. In any case, the presence of two distinct input domains leads to speculate that StyS could respond to another stimulus besides styrene, probably related to the energetic status of the cell. Recent studies on the complex transcriptional regulation of the styrene upper pathway are in line with this hypothesis and will be discussed later (Section 5.3).

An analysis carried out using the most established topology prediction software available at ExPAsy (www.expasy.org/tools/) showed that neither StyS nor TodS contain predictable transmembrane helices, suggesting that these proteins are located in the cytoplasm (author's unpublished results; see ref [49]). Moreover, StyS and TodS contain at their N-terminus a leucine zipper (bZIP) dimerization and DNA-binding domain.[50,103] Indeed, a dimerized synthetic peptide corresponding to the TodS bZIP domain was shown to bind to a DNA region located upstream of the *todS* gene itself.[50] This preliminary result indicates that TodS could function as a dimer and control its own expression. Although this possibility needs to be corroborated by further investigations, StyS and TodS may be unique among known HKs in incorporating a bZIP motif.[50]

The *styR* gene encodes a protein, StyR, of about 23 kDa showing high level of homology with other RRs belonging to the FixJ family. Interestingly, the proteins sharing the highest levels of homology with StyR are the TodT, TmoT and TutR RRs, associated with TodS, TmoS and TutC HKs, respectively.[19,50,63,82]

The relationship between phosphorylation, oligomeric state and DNA-binding ability of StyR was investigated in *P. fluorescens* ST.[52] Like many RRs, StyR can be phosphorylated in vitro in the absence of the cognate sensor kinase, using acetylphosphate as a phosphate-donor. This made it possible to show that the phosphorylated form of StyR (StyR-P) is a dimer.[52] Both monomeric (not phosphorylated) and dimeric forms of StyR can specifically bind the P*styA* promoter, however the DNA-binding affinity of StyR-P is at least tenfold greater with respect to the unphosphorylated form.[52] This is in line with subsequent studies showing that the StyR-binding sites on the promoter of the *styABCDE* operon are palindromic (see below).

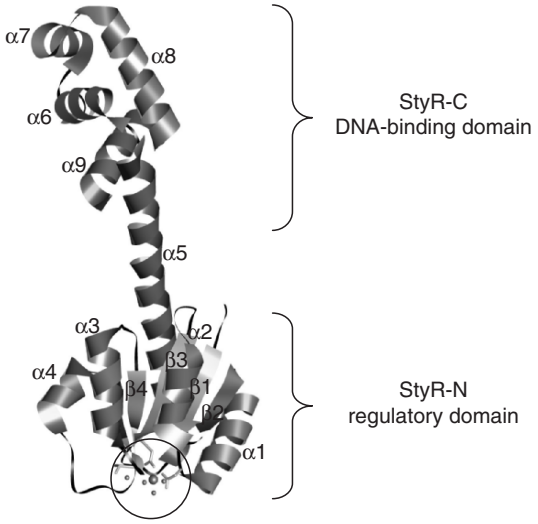

Figure 3. Overall fold of unphosphorylated StyR. The phosphorylation site is circled. More information regarding the structure is available at www.rcsb.org (PDB code: 1YIO) (modified from ref [59]).

The crystal structure of unphosphorylated, monomeric StyR has been resolved at a 2.2 Å resolution by Bolognesi's research group (Figure 3).[59] StyR discloses the typical domain organization of the TCSs RRs and is composed of a N-terminal regulatory domain (StyR-N) and a C-terminal DNA-binding domain (StyR-C). The StyR-N regulatory domain is structurally closely related to the regulatory domain of the FixJ protein of *S. meliloti* and displays the typical ($\beta\alpha$) fivefold observed in other RRs, that is built by five parallel β strands assembled in a central β sheet surrounded by five α helices (α1 to α5; Figure 3). The StyR-C DNA-binding domain is structurally homolog to the FixJ/ NarL RRs subfamily and is essentially composed of four helices (α6 to α9), where helix α8 plays the role of the DNA-recognition helix.[59]

In addition to StyR, only four three-dimensional structures of full-length RRs (NarL, CheB, DrrD and DrrB), all in their inactive unphosphorylated state, are present in the Protein Data Bank.[9] These four RRs structures are monomeric and display extensive contacts between their N- and C-terminal domains that are connected by a linker region substantially devoid of any secondary structure. Such packed inter-domain arrangement is in agreement with the current functional model of RR, postulating that phosphorylation would "open" the structure relieving the inhibition mediated by the N-terminal domain on the C-terminal domain effector activity.[97] Strikingly, the StyR structure displays a clear

separation of the N- and C-terminal domains by means of the extended α-helical arrangement of the linker region, which acts as an elongation of the StyR-N α5 helix (Figure 3). The resulting α-helix locates the StyR-N and StyR-C domains more than 16 Å apart. Therefore, the unphosphorylated StyR-C domain displays an orientation and accessibility that, in contrast with the other inactive RR effector domain structures mentioned above, can partially support DNA binding. Accordingly, unphosphorylated StyR binds DNA, albeit with lower affinity with respect to the phosphorylated species.[52] Thus, in contrast to other RRs, full activation of StyR upon phosphorylation may require mainly the achievement of the dimeric state, needed for palindromic DNA recognition, rather than the release of the inter-domain inhibition. The events that after phosphorylation shift StyR equilibrium from the monomeric to the dimeric "active state" may involve additional structural determinants, which dissecting will require a structural study of the StyR phosphorylated form.

5.2. The Promoter of the *styABCDE* Operon

The molecular mechanisms underlying the regulation of styrene degradation has been mainly performed in *P. fluorescens* ST. The sequence of the DNA region located between the *stySR* and *styABCD*E operons and encompassing the P*styA* promoter is shown in Figure 4. This uncoding region from *P. fluorescens* ST is highly conserved in the different *Pseudomonas* strains in which the *sty* genes have been characterized, suggesting that also the P*styA* regulation machinery could be very similar if not the same. As mentioned above, the StyS/StyR TCS is required for styrene-dependent P*styA* activity in the heterologous host *E. coli*, identifying the response regulator StyR as the major transcriptional regulator required for P*styA* activity. DNase I protection assays identified on P*styA* three distinct binding sites for the dimeric StyR-P protein, named STY1, STY2 and STY3 (Figure 4). Site STY2 contains the 5′-ATAAACCACGGTTTAT-3′ palindrome, formerly named sty-box.[73,103] Also sites STY1 and STY3 contain this palindromic sequence, although partially degenerated. The extension of the protected regions was similar in all the three sites, indicating that each site interacts with a distinct StyR-P dimer.[53]

StyR-P has different binding affinities for the three STY sites. The highest-affinity binding site is STY2, while StyR-P binding affinity for STY1 and STY3 is 4-fold and 20-fold lower, respectively, with respect to that for STY2. Interestingly, the binding of StyR-P to sites STY1 and STY2 is co-operative since, in the DNase I protection assay, it occurs on P*styA* at the same StyR-P concentration.[53] The possibility that StyR-P dimers located at the STY1 and STY2 sites can interact, forming tetramers, is supported by cross-linking experiments showing that StyR-P dimers

Figure 4. The P*styA* promoter of *P. fluorescens* ST. (a) Sequence of the promoter. The nucleotides are numbered with respect to the transcriptional start site.[103] The StyR-binding sites (STY1, STY2, STY3) are boxed. The IHF-binding site is indicated by grey-bar. The bracket indicates the URE region. The "extended" –10 consensus for σ^{70} is underlined. The TGA *styR* stop codon and the ATG *styA* start codon are in boldface. (b) Alignment of the STY sequences. The *arrows* indicate inverted repeats. Nucleotides identical to the STY2 palindrome are in uppercase and boldface (modified from ref [53]).

(about 50 kDa) can form higher-order multimers of about 100 kDa, a molecular weight compatible with the formation of tetramers, and that the formation of StyR-P tetramers is enhanced by the presence of DNA containing the binding sites STY1 and STY2 (Rampioni *et al.*, manuscript in preparation; see ref [59]). Taken together, the above results suggest that binding of a StyR-P dimer to the high-affinity site STY2 could stimulate the binding of another StyR-P dimer to the lower affinity site STY1, resulting in StyR-P tetramer formation and DNA looping.

In addition to StyR, also the Integration Host Factor (IHF) global regulator takes a relevant part in P*styA* regulation. In a previous study we found that IHF binds to P*styA*, and that SMO activity was reduced in an *ihf* deficient heterologous background, indicating a positive role for this regulator in controlling P*styA* activity.[87] Subsequently, DNase I protection experiments showed that the DNA region protected by IHF contains a consensus sequence for IHF (5′-WATCAANNNNTTR-3′), oriented in opposite direction with respect to the *styABCDE* operon (Figure 4).[53] Interestingly, the DNA regions protected by StyR-P on the STY1 site and by IHF overlap, suggesting that these proteins could compete for the binding to this DNA region, herein referred to as Upstream Regulatory Element (URE).[53] This has recently been confirmed by our group by DNase I

protection experiments showing that IHF is able to displace StyR-P from the URE, and vice versa (Rampioni *et al*., manuscript in preparation).

Recently, it has been demonstrated that, in *Pseudomonas* sp. Y2, PaaX, the regulator of the *paa* genes in *E. coli*[26] binds a DNA region downstream of the transcription start site and slows down transcription from P*styA* in the absence of PA-CoA.[21] The putative consensus sequence (GATACA-26 bp-TGTATC) for PaaX is also present in the same position in *P. fluorescens* ST. The possible role of PaaX in the modulation of *sty* catabolic gene expression will be discussed in Section 5.3.

As mentioned before, the TodS/TodT and TmoS/TmoT pairs are TCSs involved in toluene degradation in *P. putida* (strains F1 and DOT-T1E) and *P. mendocina*, respectively, and share a high similarity with the StyS/StyR pair. Thus, we wondered if also the TodS/TodT- and TmoS/TmoT-dependent promoters (named P*todX* and P*tmoX*), that are identical,[82] share common features with the P*styA* promoter.

The sequence of the P*todX* promoter shows an overall low degree of homology with the P*styA* promoter, however some critical features are conserved.[49] A first common feature is the presence of putative multiple binding sites for TodT on P*todX*. DNase I protection assays performed with unphosphorylated TodT showed that this protein bind a large region of P*todX* containing two palindromes (tod-box) highly similar to the STY2 palindrome of P*styA*.[49,53] Since electrophoretic mobility shift assays with unphosphorylated TodT revealed the presence of four retarded bands, it has been suggested that four TodT monomers could bind to the region protected in the footprinting. Another important common feature between P*todX* and P*styA* is the requirement of IHF binding to achieve full promoter activity.[49] The relative positions of the *cis*-acting elements of P*todX* are different with respect to P*styA*, that is, both the TodT-binding sites characterized up today are very close and do not overlap the downstream IHF-binding site. However, since phosphorylation increases the binding affinity of the RRs for their target sequences, additional low-affinity binding site/s for phosphorylated TodT could have escaped this analysis. Therefore it could be possible that the activity of P*todX* is regulated similarly to P*styA,* depending on the levels of phosphorylated TodT. However, this issue deserves further investigation.

5.3. Towards a Possible Model: Integrating Styrene-Specific and Global Regulation

The complex architecture of P*styA* is in line with the physiological studies showing that the activity of this promoter is finely modulated, being repressed to various extents by the presence of an alternative carbon source in addition to styrene.[86] In a first study, we showed that inactivation

of the high affinity StyR-binding site STY2 abolished promoter activity,[87] in line with the previous observations that StyR-P is required for P*styA* activity.[73,103] On the other hand, the StyR-P lowest-affinity binding site, STY3, is located downstream of the P*styA* transcription start point, a position consistent with the binding of a repressor.[53] A further level of complexity is given by the competition between IHF and StyR-P on the URE region (Rampioni *et al.*, manuscript in preparation; see ref [53]). We speculated that the activity of P*styA* could be finely modulated depending on the differential binding of StyR-P to the different *cis*-acting elements, in response to different metabolic conditions.

To address the functional role of STY3 and of the URE region, we constructed variants of P*styA* carrying either mutations in STY3 or a deletion of the URE region or both, fused to the *lacZ*-reporter gene, to allow the comparison of the promoter activity of these constructs with the wild-type promoter, under different growth conditions. Promoter activities were measured during the whole growth curve under conditions of full induction (growth on styrene as sole carbon source), full repression (growth on glucose as sole carbon source) or partial glucose-mediated repression (growth on styrene plus glucose).[53] The results of these experiments are shown in Figure 5, and more details on the experimental design are given in the corresponding legend.

As a whole, this study shows that StyR-P acts as an activator when is bound to STY2, since this site is necessary for promoter activity.[86] Conversely, StyR-P acts as a repressor when is bound to the negative regulatory site STY3, since inactivation of this site relieves the glucose-mediated repression. STY3 is the lowest-affinity binding site for StyR-P and must be occupied subsequently to sites STY1 and STY2, upon an increase of the cellular levels of StyR-P. Thus, it is likely that an increase of phosphorylated StyR leads to P*styA* repression through occupancy of all the three STY sites.

The contact between two dimers of StyR-P located on sites STY1 and STY2 probably leads to DNA looping and partial repression of P*styA*, since URE deletion causes relieve from glucose repression. This hypothesis is also consistent with the fact that when the URE region (containing STY1) and STY3 are simultaneously inactivated (pPR9*Pa4*; Figure 5), P*styA* is completely de-repressed in carbon catabolite repression conditions.[53]

In vivo, the co-operative binding of StyR-P to STY1 and STY2 is probably counteracted by IHF which, competing with StyR-P for binding to the URE, would exert a positive modulatory role on promoter activity, consistently with previous data showing that P*styA* activity is reduced in an *ihf* deficient heterologous background.[87] In this view, the fine modulation of P*styA* would depend on the relative cellular levels of StyR-P and IHF, in the different growth conditions. On the other hand, IHF cannot

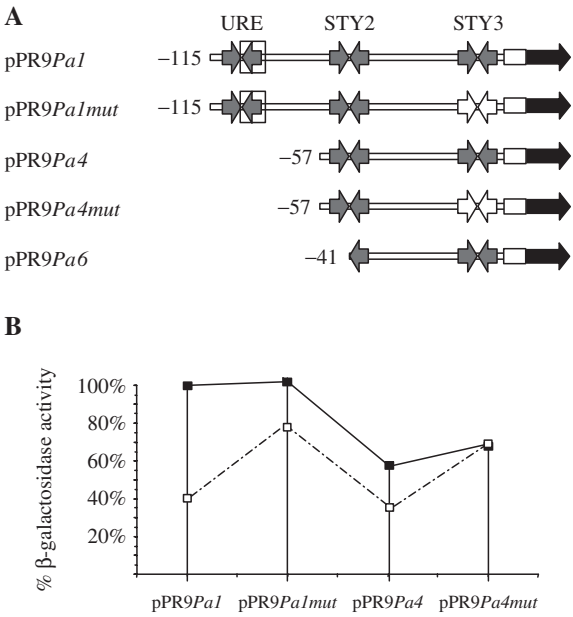

Figure 5. Functional analysis of the P*styA* promoter. (a) Schematic representation of the P*styA* deleted and/or mutated derivative fragments cloned in the promoter probe vector pPR9TT. The *grey inverted arrows* indicate the StyR-binding sites. The *white inverted arrows* indicate the mutated STY3 site. The IHF-binding site is boxed. The white rectangle indicates the *styA* open reading frame. The *black arrows* indicate the *lacZ* gene fused to *styA*. Nucleotide numbering refers to the P*styA* transcription start site.[103] (b) Graph representing the percentage (%) of β-galactosidase activity of *P. fluorescens* ST carrying the plasmids shown in (a) (pPR9*Pa6* is not reported in the graph since the partial deletion of STY2 abrogated β-galactosidase activity). Early exponential phase cultures growing on styrene as the sole carbon source were divided into three flasks, and either styrene or glucose (0.4%) or both were added. β-Galactosidase activities, measured after two exponential cell divisions, are reported as % of the pPR9*Pa1* activity in styrene, considered as 100%. The black squares and the black line represent the relative % of activity of the different constructs in styrene. The white squares and the dotted line represent the relative % of activity of the different constructs in styrene plus glucose. The levels of activity are normalized with respect to the basal β-galactosidase activity in glucose (modified from ref [53]).

only have the role of displacing StyR-P from the STY1 repressive site, since in this case the wild-type promoter and the URE-deleted derivative (pPR9Pa1 and pPR9Pa4, respectively; Figure 5) should have had the same activity in fully inducing conditions (growth on styrene as sole carbon source). Thus, IHF must exert a positive role *per se*. IHF levels do not change significantly during exponential growth both on styrene and on styrene plus glucose, suggesting that the amount of the activated form of StyR is the main element determining the activity of the P*styA* promoter

in different growth conditions (Rampioni *et al.*, manuscript in preparation). Additional studies will be required to better understand the interplay of StyR-P and IHF on the URE region.

The above results suggest that P*styA* promoter activity is determined by the different 3-D structures that this promoter can assume in the different growth conditions. We propose a working model for P*styA* regulation, shown in Figure 6, implying that in the full-repressed conformation, the levels of StyR-P are such that this protein binds to sites STY1, STY2 and STY3 (sites STY1 and STY3 are negative *cis*-acting elements). In the full-activated conformation the ratio StyR-P/IHF decreases, so that IHF binds to the URE while StyR-P binds only to site STY2 (both IHF-site and site STY2 are necessary for full promoter activity). Among these two extremes, P*styA* can probably assume intermediate conformations, corresponding to intermediate promoter activity.

Activation of StyR depends on StyS sensor kinase activity that is in turn determined by the sensed stimuli. Since StyS sensor contains two input domains, characterized by the presence of PAS domains, it is possible that the response to the main signals, that is, the presence of styrene and the energetic level of the cell, is integrated by the hybrid sensor kinase, leading to a fine modulation of the amount of RR active form and ultimately of the activity of the P*styA* promoter. This could constitute an efficient way to directly connect the expression of a peripheral catabolic pathway with the energetic status of the cell.

An open question concerns the role of PaaX in the modulation of the expression of the styrene-catabolism genes. As already mentioned, in strain Y2, this repressor binds a DNA region downstream of the transcription start site and slows down transcription from P*styA* in the absence of PA-CoA, the central intermediate of the styrene lower pathway (*paa* operon). Such a repression is released in the presence of PA-CoA.[21] In *P. fluorescens*

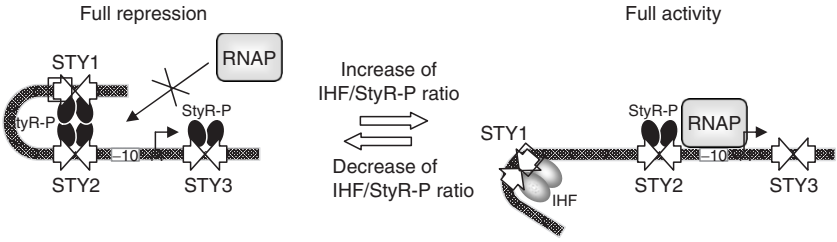

Figure 6. Proposed model for the regulation of P*styA*. In the full-repressed conformation, the levels of StyR-P are such that this protein binds to sites STY1, STY2 and STY3, impairing the binding of the RNAP to the promoter. In the full-activated conformation the levels of StyR-P are lower, so that this protein binds only to site STY2, while IHF binds to the URE, allowing the binding of the RNAP to P*styA* and promoting transcription.

ST, the PaaX consensus overlaps the DNA region protected by StyR-P at the STY3 site. We have demonstrated that mutations in this site partially relieve P*styA* from glucose repression.[53] The STY3 mutated nucleotides, located in the spacer sequence between the two inverted repeats that constitute the putative consensus for PaaX binding, are not conserved in the *Pseudomonas* promoters in which this consensus has been found,[21] and it is therefore very unlikely that the introduced mutations can impair PaaX binding at this site, interfering with the meaning of our results. As also suggested by the authors, PaaX could have an additional role in coordinating the expression of the upper and lower styrene catabolic pathways to avoid the accumulation of toxic catabolic intermediates such as styrene oxide and phenylacetaldehyde.

6. CONCLUSIONS

In *Pseudomonas* the majority of the catabolic routes for toxic compounds are regulated by a specific mechanism responding to the actual substrate and by several mechanisms aimed to couple the promoter of the catabolic operon to cell physiology and stress response.[12] The response to metabolic signals is dictated by different global regulatory factors (e.g., sigma factors, IHF, Crc, CyoB, RelA, PtsN) whose activity or availability is determined by the metabolic status of the cell (reviewed by Cases and de Lorenzo[13] and Shingler [94]). Increasing evidence has led to the notion that this global regulation in *Pseudomonas* is multifactorial and that different regulators could have different impacts on the distinct catabolic routes. These regulators could constitute networks that in the different strains can vary in the type and hierarchy of the different elements involved, in a strain-specific manner (reviewed by Cases and de Lorenzo [13] and Shingler [94]).

Several genes have been described as involved in the catabolite repression of different pathways in *Pseudomonas*. The *crc* gene encodes for a regulatory protein (Crc) that probably acts at the post-transcriptional level on the substrate-specific regulator with a still unknown mechanism.[3,38,61,84] The *ptsN* and *ptsO* genes, encode components for an alternative phosphoenolpyruvate:sugar phosphotransferase system (PTS).[11,14,15] It has been speculated that this PTS may somehow be linked to some glucose/energy-sensing system.[12,94] A clear link between energy sensing and catabolite repression is given by independent studies showing that the genes of the *cyoABCDE* cluster are involved in catabolite repression of alkanes and phenol degradative pathways.[23,76] The *cyo* genes encode cytocrome *o*-ubiquinol oxidase, the main terminal oxidase of the *o*-type branch of electron transport chain under high-energy conditions.[23,76] It has been

suggested that the ATP yield generated by the intact *o*-type branch of electron transport chain may be an early step in the signal transduction causing catabolite repression.[76] Interestingly, a study considering the effect of the *crc-ptsN* double mutation on catabolite repression suggests that these genes could be elements of a common regulatory pathway.[3]

The styrene-catabolism regulation system is peculiar in several aspects with respect to the regulation of other catabolic pathways. Indeed TCSs have been rarely associated with aromatic compound catabolism.[19,48,50,82] The StyS sensor kinase, together with the homologous TodS sensor, is unique among the plethora of HKs known in prokaryotes, since it contains two HK core domains and a N-terminal bZIP domain. It is worthwhile to underlie that the two HK domains belong to the different HK families 1a and 4, respectively,[32] indicating that they are not the result of a gene duplication, but probably derive from the fusion of two distinct sensor HK-encoding genes.

Studies on the styrene-catabolism regulation under glucose repression conditions suggest that StyS can sense both styrene and the redox status of the cell, via the two PAS domains, possibly integrating the specific and the global response. This hypothesis is appealing, but needs to be validated. Unfortunately, it is not yet possible to predict the stimulus perceived by a PAS domain on the basis of its sequence. The N-terminal input domain of the TodS protein, containing the first PAS domain, binds toluene, while the effector of the second C-terminal PAS domain remains to be elucidated.[49] Due to the high homology between TodS and StyS it is likely that in StyS the N-terminal PAS domain could sense styrene, while, according to our regulatory model, the C-terminal PAS domain could be involved in redox-sensing. It has been pointed out that the quinones could be the best candidates as redox indicators, since they are the only components of the respiratory chain endowed of apparent free movement within the membrane.[29] In *E. coli*, the kinase activity of the sensor protein ArcB is switched off by the oxidized form of quinones.[29] This modular protein is constituted of a membrane-anchoring domain, and of cytoplasmic PAS, transmitter, intrinsic receiver and histidine-containing phosphotransfer domains.[88] It was shown that the ArcB soluble form, lacking the transmembrane domains, is responsive to oxidized / reduced ubiquinone in vitro.[29] Similar results have been obtained with the ArcB-related HKs BvgS and EvgS.[10] These findings demonstrate that the interaction of these proteins with the quinone must involve the cytoplasmic domains and that, in general, an intra-molecular signal transduction from transmembrane or periplasmic to cytoplasmic modules is not mandatory. Some HKs, such as CheA,[54,106] are soluble cytosolic proteins that interact with specific transmembrane receptors. It has been suggested that other predicted soluble HKs could have membrane-linked receptors that so far

have not been identified.[106] StyS appears to be a soluble protein, but it could be associated to a membrane protein. A possible candidate could be StyE, the putative styrene transporter protein.[60] To address this point, and to identify the real signals to which StyS responds, a structural and functional characterization of this protein is in progress in our laboratory.

Studies on the *cis*- and *trans*-acting elements of the P*styA* promoter led to the proposal of a model, according to which the response to the catabolic operon-specific substrate and to a preferred carbon source are integrated by the same regulatory device (the StyS/StyR TCS). This would be achieved by the StyS-dependent variation of the StyR-P levels, and consequently by the relative ratio of StyR-P and IHF, which in turn would determine variations of the promoter architecture and modulation of its activity. Even if sensor-depending changes in the level of activated response regulators for the regulation of the associated promoters is a paradigm in TCSs, styrene regulation is the first example of the involvement of such a system in the carbon catabolite repression of an aromatic compound catabolic pathway. Another example of a direct link between catabolic pathway expression and the metabolic status of the cells concerns the regulatory system for the *ortho*-cleavage pathway of 3-chlorocatechol degradation.[57] The specific inducer of the system (the metabolic intermediate 2-chloro-*cis*,*cis*-muconate) and the TCA-cycle intermediate fumarate, acting as key signalling molecule of the metabolic status of the cell, compete for direct binding to the LysR-type regulator ClcR and modulate positively and negatively its activity, respectively.[57] Thus, completely different regulators (LysR-type and TCS-type) can directly integrate, by mechanistically different means, the response to the specific stimulus with the cellular metabolism.

The molecular mechanism and the physiological role underlying the IHF-mediated activation of P*styA* remains another open question. In many promoters this protein binds to a site between the promoter and an upstream enhancer element, inducing DNA looping. This facilitates the interaction between an upstream activator and/or upstream regulatory elements and RNAP.[58] Our data rule out this mode of action for IHF since the minimum P*styA* fragment endowed with full styrene-dependent activity starts just upstream of the URE (Figure 5).[87] It is most likely that IHF acts as an activator by a different mechanism. In the *ilv*P_G promoter, IHF facilitates duplex destabilization in the −10 region and activates transcription without any direct interaction with the σ^{70} RNAP.[92] A similar mechanism of action for IHF has been hypothesized in the σ^{54}-dependent P_0 promoter for phenols degradation of *Pseudomonas* CF600.[98] Consistently, P*styA* shares common features with *ilv*P_G and P_0, such as an A + T rich region flanking the URE.

Concerning the physiological role of IHF, in *E. coli* this protein regulates more than 100 genes[4] and in *Pseudomonas* it is involved in the regulation of several processes, especially in toxic compound degradation.[94]

In *E. coli* the IHF levels are regulated by the ppGpp alarmone, the production of which is triggered by various nutritional and environmental stresses.[6] Moreover IHF levels increase upon entry into the stationary phase, both in *E. coli* and in *P. putida*.[6,102] However, although likely, a clear link between IHF and stress response is far to be proven in *Pseudomonas*.

In *P. putida* F1, toluene pathway is regulated by the StyS/StyR-homologous TodS/TodT system. In other strains the same pathway is under the control of a σ^{54}-dependent promoter.[12,94] Activation of the σ^{54}-dependent promoters directly depends on the availability of global regulators (σ^{54}, IHF, RelA) whose concentration is determined by the metabolic status of the cell. This assures a first intrinsic integration of a specific pathway in the global regulatory network and accounts for the success of these promoters in the catabolism of toxic aromatic compounds and in cellular processes that respond to variation in nutrient availability.[13] A further and more refined regulation requires the participation of other global regulators (Crc, PTS, CyoB, RpoS, σ^{32}) to fulfil the numbers of different metabolic conditions that microorganisms experience in the natural environments.[94] It is likely that also the regulation of styrene catabolism requires additional regulatory factors besides StyS/StyR and IHF. Indeed, the suggested coordination between the lower and upper styrene pathways, through the action of the PA-CoA-responsive PaaX regulator, would integrate styrene catabolism in the network of the degradation routes for aromatic compounds that converge to the central intermediate PA-CoA.[21] In any case, it seems that pathways for toxic compound degradation recruit regulatory expression systems that are directly integrated in a large regulatory network. This could be the unique way for a newly acquired pathway to be maintained and to evolve. Indeed, it has been speculated that the redundancy of global regulatory networks in the physiological control of a promoter could confer an advantage in horizontal gene transfer. Such a redundancy would make more probable to a newly acquired catabolic pathway to find a pre-existing compatible network for its integration in the host strain metabolism.[13] With this respect, the finding that TodS and probably StyS are somehow connected with chemotactic response to toluene and styrene and that in turn chemotaxis is associated to solvent tolerance could indicate that these unique HKs take part to a regulatory network that control the coordinate response to compounds that are both toxic and nutrient to the cell.

Note added in proof: After the submission of this manuscript a paper concerning the degradation of PAA in *P. fluorescens* ST was published: P. Di Gennaro et al., Arch. Microbiol. Mar 22; (Epub ahead of print)

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4

DEGRADATION OF *o*-XYLENE BY *PSEUDOMONAS STUTZERI* OX1 (*PSEUDOMONAS* SP. OX1)

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1. INTRODUCTION

Interest in the microbial degradation of methylbenzenes arose in the early 1970s, when both toxicological and ecological data started to alert people about their wide distribution in the environment and their effects on living organisms. Most of the early studies on bacterial methylbenzene catabolism focused on bacteria belonging to the genus *Pseudomonas*, which proved to be endowed with a formidable metabolic versatility.[20] Several *Pseudomonas* strains isolated then for their ability to degrade toluene, *m*- and *p*-xylene, and 1,2,4-trimethylbenzene, later become the archetypes for the studies on the biochemistry, genetics and regulation of methylbenzene degradation[28,44] (see also volume 2, Chapter 18 and volume 4, Chapter 7).

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Interestingly, none of these strains was reported to grow with the ortho isomer of xylene, which proved to be particularly recalcitrant to biodegradation. The first papers reporting on the isolation of bacteria able to utilize *o*-xylene as the only carbon and energy source appeared only in 1987, when two articles were published almost at the same time, dealing with a *Corynebacterium* and the *Pseudomonas* strain object of this chapter, respectively.[4,36] After then, aerobic *o*-xylene degradation was reported only as a side activity of toluene degrading bacteria. Only recently, some *Rhodococcus* strains have been characterized for their ability to grow on *o*-xylene.[17,25] Moreover, a number of bacterial strains proved to be capable of anaerobic *o*-xylene catabolism.[23]

Early studies showed the existence of two possible pathways for methylbenzenes aerobic degradation: one proceeding through the progressive oxidation of a methyl group[15,48] (see also volume 1, Chapter 6) and the other through the direct dioxygenation of the aromatic nucleus.[21,22] Later, a number of bacterial strains have been characterized for toluene degradation through monooxygenation of the aromatic nucleus (see volume 2, Chapter 18). None of these methylbenzenes' degradation pathways is able to catabolize *o*-xylene, except the one found in *Pseudomonas* sp. OX1.

2. GENETIC ORGANIZATION AND BIOCHEMICAL FEATURES OF THE PATHWAY

2.1. *o*-Xylene Degradation by *Pseudomonas* sp. OX1: A Peculiar Mix of Toluene and Phenol Catabolic Pathways

Pseudomonas sp. OX1 was isolated about 20 years ago from the sludge of a wastewater treatment plant for its ability to use *o*-xylene as sole carbon and energy source and tentatively classified as *Pseudomonas stutzeri* by conventional biochemical methods.[4] Recent genotypic characterization and phylogenetic analysis confirmed that this strain belongs to the *Pseudomonas* (*sensu stricto*) genus, but not to the *stutzeri* species.[32] On the contrary, the strain belongs to intrageneric cluster II and is related to the *Pseudomonas fluorescens–Pseudomonas syringae* complex. The catabolic versatility of *Pseudomonas* sp. OX1 became evident shortly after isolation. In fact, the strain could also grow on a broad range of aromatics, such as toluene, phenol, cresols and dimethylphenols. The strain was found to harbour a plasmid, named pPB, apparently not involved in the degradation of aromatic compounds and conferring mercury resistance by virtue of two *mer* operons.[5,33]

The first hypothesis about the nature of the *o*-xylene catabolic pathway in *Pseudomonas* sp. OX1 was based on analytical and physiological data suggesting the direct oxygenation of the aromatic nucleus followed by

extra-diol *meta*-cleavage of the aromatic ring.[4] Furthermore, cross-inducibility of toluene- and *o*-xylene-dependent O_2 uptake in cells grown on either toluene or *o*-xylene supported the notion that *o*-xylene shared with toluene the same catabolic pathway.

Later studies[2,7,8,12,13] confirmed these early hypotheses and described in detail the catabolic pathway, named in the meanwhile *tou*, standing for toluene and *o*-xylene utilization. As shown in Figure 1, in *Pseudomonas* sp. OX1 toluene and *o*-xylene are activated by sequential introduction of two adjacent hydroxyl groups to form first methylphenols and then methylcatechols, which are subsequently cleaved in extra-diol *meta* position into 2-hydroxymuconic semialdehyde derivatives and further processed into TCA intermediates. The early attack of toluene (Figure 2a)

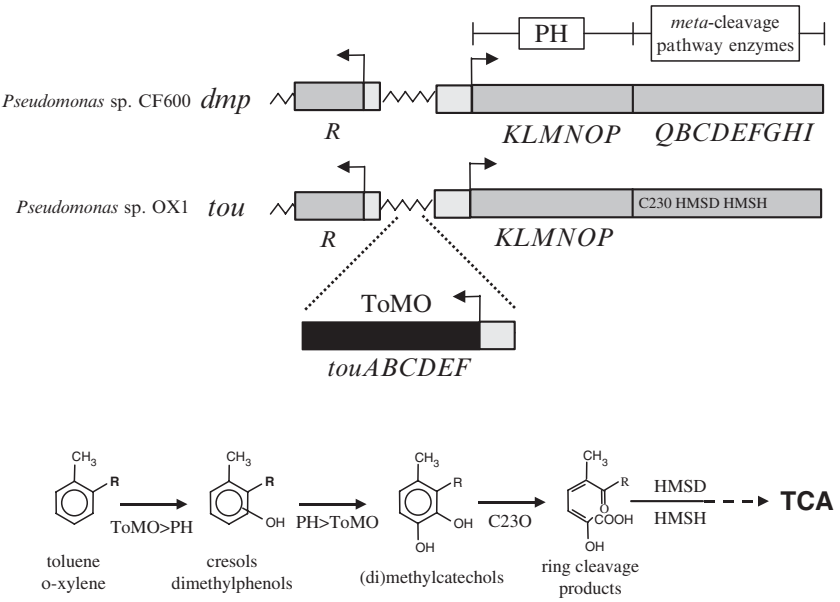

Figure 1. Genetic organization and biochemical features of the *tou* pathway. To emphasize the possible origin from a phenol-degrading system (see text), the *tou* divergent operons and the regulator gene *touR* are depicted below the gene arrangement of the *dmp*-system for phenol, cresols and 3,4-dimethylphenol degradation coded by pVI150 catabolic plasmid of *Pseudomonas* sp. CF600.[31] The concerted activity (see also Figure 2) of toluene *o*-xylene monooxygenase (ToMO) encoded by the left *tou* operon,[8] and phenol hydroxylase (PH) encoded by upstream genes of the right operon,[2,12] give rise to the production of (di)methylcathecols from both toluene and *o*-xylene through phenolic intermediates. Once methylcathecols are formed, they are processed by canonical *meta*-pathway enzymes, such as cathecol 2,3-dioxygenase (C23O) semialdehyde dehydrogenase (HMSD) and semialdehyde hydrolase (HMSH) to intermediates of TCA cycle. The location of the *tou* genes for C23O, HMSD, HMSH is inferred from gene hybridization and/or enzyme assay[2] and partial sequencing.[45] Differences in catalytic efficiency between ToMO and PH in the two steps of hydroxylation[13] are also indicated.

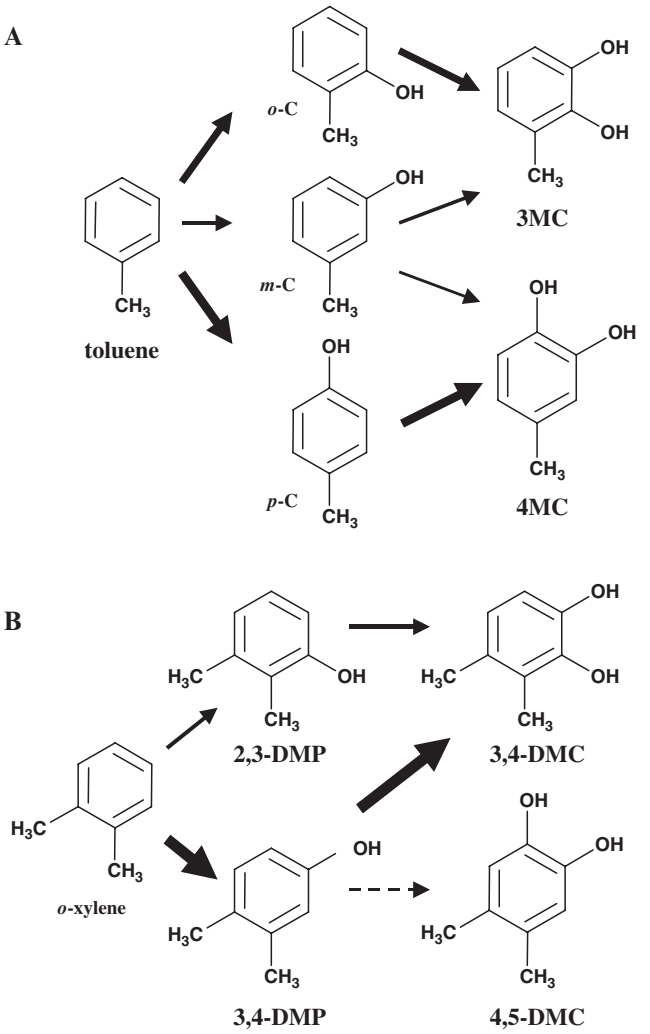

Figure 2. Biochemical steps for the conversion of toluene to methylcatechols (A) and for the conversion of *o*-xylene to dimethylphenols (B) catalyzed concertedly by ToMO and PH. The thickness of the arrows is roughly proportional to the relative abundance of each species produced. *o*-C, *o*-cresol; *m*-C, *m*-cresol; *p*-C, *p*-cresol; 3MC and 4MC, 3- and 4-methylcatechol, respectively; 2,3-DMP and 3,4-DMP, 2,3- and 3,4-dimethylphenol, respectively; 3,4-DMC and 4,5-DMC, 3,4-dimethylcatechol and 4,5-dimethylcatechol, respectively. The regioselectivities of ToMO and PH are different.[13] Cresols are produced by ToMO with a relaxed regioselectivity, preferentially yielding the *ortho* and *para* isomers. PH yields the three cresol isomers. However, it shows preference for the *ortho* isomer, which accounts for 70% of the product. Thus, PH is more restricted in its regioselectivity than ToMO. PH and ToMO still have opposite regioselectivities when they act on a hydroxylated substrate. For example, 4-MC is the major product of ToMO reactivity with *m*-cresol, whereas 3-MC is

(*Continued*)

produces a mixture of the three (mono)methylphenol isomers *o*-cresol, *m*-cresol, and *p*-cresol, which are hydroxylated to both 3- and 4-methylcatechol. Similarly, initial hydroxylation of *o*-xylene (Figure 2b) produces both 2,3- and 3,4-dimethylphenol, which are converted mainly into 3,4-dimethylcatechol. These initial hydroxylation steps are carried out by two evolutionarily distinct bacterial multicomponent monooxygenases[29] (BMMs), toluene *o*-xylene monooxygenase (ToMO), belonging to the family consisting of four-component aromatic/alkene monooxygenases (group 2 BMMs)[8,14,29] (see also volume 4, Chapter 8), and phenol hydroxylase (PH), belonging to the group consisting of toluene 2-monooxygenases (T2MO)/phenol hydroxylases (group 1 BMMs).[2,12] Once methylcathecols are formed, they are processed by canonical *meta*-pathway enzymes such as cathecol 2,3-dioxygenase, semialdehyde dehydrogenase and semialdehyde hydrolase (Figure 1).

The *Pseudomonas* sp. OX1 genes involved in toluene/*o*-xylene degradation are organized in two large operons, transcribed divergently and about 2 kb apart (Figure 1). The left operon comprises *touABCDEF* genes[8] coding for the subunits of ToMO. The right operon genes appear to be highly similar to and arranged as in many phenol-degrading isolates in which genes coding for the subunits of PH form a large transcription unit with *meta*-cleavage pathway genes[2,12] (see also volume 2, Chapter 16). A well-characterized example of this kind is the *dmp*-system for phenol, cresols and 3,4-dimethylphenol degradation coded by pVI150 catabolic plasmid of *Pseudomonas* sp. CF600[31] where the *dmpKLMNOP* genes for a multicomponent PH are fused to *dmpQBCDEFGHI* genes for *meta*-cleavage pathway enzymes (Figure 1). Downstream of *touABCDEF* gene cluster lies a gene coding for the *tou* operons activator TouR, found to share a high degree of sequence similarity and effector profile (see below) with DmpR,[39] the regulator of the *dmp*-system of *Pseudomonas* sp. CF600, whose gene is located aside the *dmp* operon (Figure 1).

Figure 2. cont'd. the predominant product of PH catalysis with the same substrate. The regioselectivities of the two enzymes are different also in the case of *o*-xylene. ToMO converts *o*-xylene predominantly to 3,4-DMP, whereas PH produces predominantly 2,3-DMP. Both monooxygenases convert 2,3-DMP to 3,4-DMC, but they exhibit different regioselectivities with 3,4-DMP. ToMO oxidizes 3,4-DMP mainly to 4,5-DMC, which cannot be further metabolized.[7] PH converts 3,4-DMP exclusively to 3,4-DMC. Thus, only PH can produce a dimethylcatechol which can be further metabolized through the lower pathway. From a catalytic point of view, ToMO is a more efficient catalyst than PH in the first hydroxylation step. On the contrary, PH shows a greater catalytic efficiency in the second step of hydroxylation. In the case of *o*-xylene, these kinetic features are instrumental in maximizing the production of 3,4-DMC and preventing accumulation of the dead-end product 4,5-DMC.[13]

Thus, *o*-xylene degradation by *Pseudomonas* sp. OX1 is accomplished by the peculiar miscellanea of toluene and phenol catabolic pathways. The gene organization of the *tou* region in *Pseudomonas* sp. OX1 seems to suggest the origins of this heterogeneous pathway. In fact, one such arrangement could have originated from a pre-existing *dmp*-like system for phenol catabolism through the insertion of a toluene monooxygenase-like gene cluster between the PH/*meta*-pathway chimeric operon and the gene for its positive regulator (Figure 1). In principle, the integration event would have been beneficial since it would have given rise to the possibility of a vertical expansion of the phenol catabolic pathway towards hydrocarbons degradation.[2] This hypothesis is supported by fact that ToMO gene cluster has an unusual 50% of GC content compared to 60–66% for *Pseudomonas* species, and is sandwiched between a transposase-like ORF (*orfA*)[8] and IS remnants. This would suggest that ToMO genes were acquired by a transposition event. However, even though this could be the genetic mechanism underlying the present gene arrangement of *tou* system, recent biochemical data[12,13] indicate that the early hypothesis on ToMO acquisition as a strategy to vertically expand a pre-existing phenol-degrading pathway[2] needs to be revisited (see below).

2.2. Substrate Specificity and Regioselectivity of ToMO and PH: A Vertical Synergy?

Early studies on *touABCDEF* gene cluster,[7,8] before the discovery of the *dmp*-like PH genes, assessed that ToMO has a broad range of substrates, being able to hydroxylate not only toluene and *o*-xylene but also the mono- and di-methylphenols arising from its activity on the two hydrocarbons. Therefore, it was assumed that ToMO alone accomplished the task to convert toluene and *o*-xylene into the corresponding methylcatechols through two subsequent hydroxylation steps. When PH genes were discovered and PH activity tested,[2] the apparent redundancy between the hydroxylation of phenols by ToMO and the PH activity was interpreted as collateral effect of the vertical expansion due to fortuitous acquisition of a toluene monooxygenase endowed with a very broad range of substrates. Recent biochemical data[13] indicate that the redundancy between ToMO and PH can be even larger, since also PH is able to hydroxylate both toluene and *o*-xylene. Therefore, independently both enzymes are able to carry out the conversion of the hydrocarbons into methylcatechols through two subsequent hydroxylation reactions. Nonetheless, an elegant comparative analysis of their enzyme activities[13] showed that ToMO and PH act sequentially and that their catalytic efficiency and regioselectivity optimize the degradation of both toluene and *o*-xylene. In particular, for both hydrocarbons, ToMO is a more efficient catalyst than PH in the first

hydroxylation step. On the contrary, PH shows a greater catalytic efficiency in the second step of hydroxylation. Once combined, ToMO and PH convert hydrocarbons to methylcatechols much more efficiently than separately, and yields of methylcatechols are almost identical to values that can be calculated assuming that ToMO catalyses the first and PH the second step of hydroxylation. This suggests that in *Pseudomonas* sp. OX1 ToMO can act prevalently as hydrocarbon-oxidizing enzyme and PH as methylphenol-oxidizing enzyme. In addition, this coupling appears to be very important in the case of *o*-xylene degradation since the sequential activity of the two enzyme activities lead to the prevalent production of 3,4-dimethylcatechol, the sole dimethylcatechol isomer that can enter the *meta*-cleavage pathway, while preventing the accumulation of the dead-end dimethylcatechol isomer 4,5-dimethylcatechol (Figure 2b).

Thus, in light of these findings, was ToMO acquisition a real vertical expansion? The biochemical evidences described above strongly suggest that the concerted use of the two enzymes provided *Pseudomonas* sp. OX1 with the ability to maximize the efficiency of non-hydroxylated hydrocarbons exploitation, a clear selective advantage. On one hand, we can speculate that ToMO acquisition improved synergistically a pre-existing ability of PH to exploit hydrocarbons carrying on both hydroxylation steps. On the other hand, the broadening of the substrate range of PH to hydrocarbons could be a consequence of the co-evolution of the two enzymes towards pathway optimization.

2.3. Degradation of the Other Two Xylene Isomers by *Pseudomonas* sp. OX1: Dealing with a Metabolic Traffic Jam

Pseudomonas sp. OX1 is unable to grow on the *meta* and *para* isomers of xylene. In addition, cells exposed to these compounds die.[6] This lethal effect was traced to the accumulation of dead-end products, such as 3,5- and 3,6-dimethylcatechol, which, like 4,5-dimethylcatechol (see above), cannot enter the *meta*-pathway due to the restriction imposed by the range of substrates of catechol 2,3-dioxygenase. The generation of these intermediates was attributed to the *tou* pathway. Indeed, ToMO can convert *m*- and *p*-xylene to 2,4- and 2,5-dimethylphenol, respectively.[8] No data are available about the ability of PH to attack *m*- and *p*-xylene. However, PH can convert 2,4- and 2,5-dimethylphenol in 3,5- and 3,6-dimethylcatechol, respectively.[2] Consistent with this observation is the fact that many bacterial strains isolated for their ability to grow on *m*- and *p*-xylene degrade these isomers through a TOL-like pathway (see volume 1, Chapter 6), which proceeds through the progressive oxidation of one

methyl group and leads to the formation of monomethylcatechols included in the substrate range of catechol 2,3-dioxygenase.

In spite of the toxic effects of exposure to *m*- and *p*-xylene, *Pseudomonas* sp. OX1 mutants able to grow on these two isomers were isolated.[6] One of these mutants, called M1, was found to carry out *m*- and *p*-xylene degradation through a TOL-like pathway,[6,9] whereas it had lost the ability to grow on *o*-xylene. Subsequently, a M1 revertant, called R1, able to utilize all the three isomers of xylene was isolated.[18] Comparative genetic analysis carried out on *Pseudomonas* sp. OX1 and the derivatives M1 and R1 revealed the presence of genes for *m*- and *p*-xylene degradation, similar to and arranged as the *upper* pathway genes of the archetypal TOL plasmid pWW0. However, in *Pseudomonas* sp. OX1 such genes were found to be inactivated by the presence of a 3-kb insertion sequence, named *ISPs1*. This element was not found in the corresponding DNA region of the mutant M1, while it was detected in the middle of the ToMO gene cluster[9] (Figure 3). Therefore, the efficient growth of M1 on *m*- and *p*-xylene was traced to the simultaneous activation of TOL-like pathway and inactivation of the *tou* pathway to prevent toxic effects resulting from the accumulation of dead-end products (see above). No *IS* element was found in either *tou* or TOL-like regions of the revertant strain R1.[9] Therefore, the ability of R1 to grow on *m*-xylene and *p*-xylene, even retaining a functional *tou* pathway, was attributed to a favourable balance between the

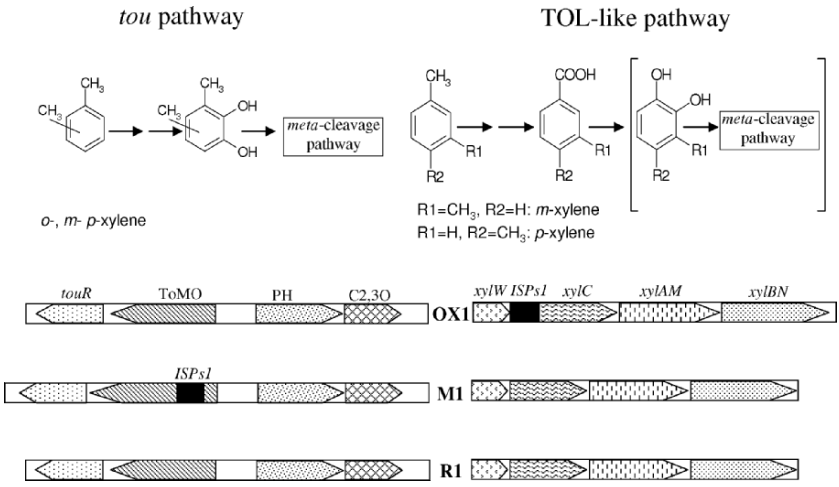

Figure 3. The *tou* and TOL-like pathways for xylene degradation and schematic representation of the *tou* and *xyl* gene arrangement (not shown to scale) in *Pseudomonas* sp. OX1, M1, and R1. The *xyl* genes involved in the reactions shown in parentheses are not reported in the scheme. The black box represents the insertion sequence *ISPs1*. *m*-xylene and *p*-xylene can be only partially oxidized through the *tou* pathway (see the text for further details).

amounts of *m*- and *p*-xylene that are successfully metabolized via the TOL-like pathway and those misrouted through the *tou* pathway.

3. PATHWAY REGULATION

3.1. Catabolic Expansion – Regulatory Conservation

As mentioned above, TouR protein positively controls the expression of both *touABCDEF* and the *tou dmp*-like operons.[2,3] TouR belongs to the aromatic responsive group of the σ^{54}-dependent transcriptional regulators[38] (see also volume 2, Chapter 10, 16 and 18) including members such as XylR, DmpR, HbpR and TbuT, which control pathways for the degradation of toluene/xylene, (methyl)phenols, 2-hydroxybiphenyl and toluene, respectively. *touR* gene is located downstream and in the same direction of transcription of the *touABCDEF* operon[8] (Figure 1). Although this organization resembles the one described for the *tbu* genes coding for toluene-*p*-monooxygenase in *Ralstonia pickettii* PKO1[19] and the regulatory gene *tbuT,*[10,11] in *Pseudomonas* sp. OX1, an additional ORF, *orfA*, putatively coding for a transposase[8] is located on the opposite strand between *touF* and *touR*. Moreover, the regulatory circuits of the *R. pickettii* and the *Pseudomonas* sp. OX1 systems appear to be very different. In the *R. pickettii* system, *tbuT* expression is driven by read-through transcription from the *tbuA1* cognate promoter.[11] In contrast, a *rho*-independent terminator was detected, downstream from *touF*, in the *tou* gene cluster[8] and a typical σ^{70} promoter was mapped upstream the *touR* gene.[3] The translation start of *touR* lies 211 nt downstream of the transcriptional start. The analysis of the sequence between the *touR* + 1 and its ATG did not reveal any significant homology to known catabolic regulatory genes. The functional significance of this region is unclear.

The *Ptou* (formerly P_{ToMO}) promoter of the *touABCDEF* operon, mapped by primer extension approximately 2-kb upstream of *touA*, displays the features of a σ^{54}-dependent promoter: a –12/–24-like sequence, recognized by the σ^{54}-RNA polymerase, and two 15-bp repeats located 77-bp upstream of the –24 position.[3] These latter sequences are similar to UAS (Upstream Activating Sequences) found in other σ^{54}-dependent aromatic catabolic operons, where they were shown to act as the binding site for the cognate transcriptional activators.[30,41] In the leader region between the *Ptou* and *touA*, two putative ORFs, *orf1* and *orf2*, were detected.[3] These two ORFs do not show any homology to known sequences and are clearly non-essential for the enzyme activity. In this respect, the *tou* upper operon resembled the pWW0 *xyl* upper operon, in which two genes, *xylUW*, not essential for xylene catabolism were mapped between the *Pu* promoter and

the genes coding for the catabolic enzymes.[47] A similar organization was also observed in the DNA region cloned from *Pseudomonas mendocina* KR1, in which the *tmo* genes, coding for toluene-4-monoxygenase, are preceded by two ORFs of unknown function.[49] Less information is available for the TouR-responsive promoter of the *tou dmp*-like operon. The transcription start site has not been determined experimentally. However, sequences matching the –12/–24 consensus of σ^{54}-dependent promoters and UAS sites were clearly identified at the 5′-end of the operon.

Compared with the members of the aromatic responsive group of the σ^{54}-dependent transcriptional regulators,[38] TouR was found to be more similar to proteins which regulate phenol catabolism, like DmpR,[39] than to those controlling methylbenzene catabolism, like XylR or TbuT.[11,24] Consistent with this sequence similarity was also the ability to become activated in response to mono- and dimethylphenols and not to non-hydroxylated aromatic hydrocarbons. In this ability to recognize intermediates of the catabolic pathway, and not the primary substrates as effectors, TouR resembles some regulators belonging to the LysR family of transcriptional activators, such as CatR, ClcR and NahR.[27,35] In particular, NahR activates the transcription of both the *nah* (naphthalene degradation) and the *sal* (salicylate degradation) operons in the presence of salicylate, an intermediate of naphthalene catabolism. Low-level transcription and translation of the *nah* operon allow naphthalene to be slowly converted to salicylate, which then triggers the NahR-mediated transcription of both operons.[34]

A similar model has been proposed for the TouR-mediated regulation of the *tou* system (Figure 4). The presence of methylphenols as substrates of the pathway triggers directly the expression of both *touABCDEF* and *tou dmp*-like operons. On the contrary, in the presence of toluene or *o*-xylene, basal levels of ToMO and/or PH would convert first the hydrocarbons into the corresponding phenols to trigger then the TouR-mediated activation of both operons. In this way, a cascade effect is established, which leads to increased synthesis of the monooxygenases.

Thus, the *tou* system seems to conserve the regulatory features of the *dmp*-like genes from which apparently originated. The promiscuity of σ^{54}-dependent promoters,[16] which possibly made the *Ptou* promoter activatable by TouR, could be a factor that contributed to the successful acquisition of the ToMO gene cluster.

3.2. Effector-Independent Activation of the *Tou* Pathway by TouR: a Case-by-Necessity?

According to the model of regulation presented above, responsiveness of the *tou* system to hydrocarbons requires basal effector-independent expression of the monooxygenases. Due to the intrinsic characteristics of

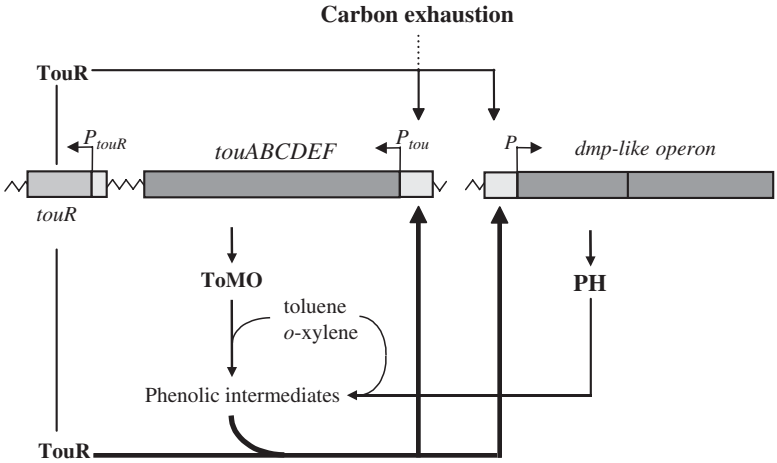

Figure 4. Model of the *tou* regulatory circuit. TouR activates transcription from the σ^{54}-dependent promoters of the left and right *tou* operons either in response to phenolic effectors or, in the absence of effectors, by means of an unknown mechanism upon carbon exhaustion. In the presence of hydrocarbons, gratuitous expression of the *tou* operons would ensure a basal monooxygenase activity sufficient to convert the substrates into the phenolic intermediates, which in turn could be recognized by TouR and stimulate the expression of the enzymatic activities at higher levels by virtue of a positive feedback mechanism.

the σ^{54}-RNA polymerase, which is unable to undergo spontaneously the DNA melting step, the interaction between the RNA polymerase and a σ^{54}-dependent activator is strictly required for transcription initiation.[50] As a consequence, σ^{54}-dependent promoters do not usually display any basal transcription activity in the absence of an activated form of the cognate activators. Therefore, basal effector-independent expression of *tou* operons was in principle unexpected. However, low levels of ToMO genes transcripts were detected in both a *Pseudomonas putida* PaW340 host harbouring *touR* as well as the ToMO-encoding operon *touABCDEF*[3] and in *Pseudomonas* sp. OX1[40] when grown in malate-supplemented mineral medium in the absence of genuine TouR effectors. Interestingly, this effector-independent, "gratuitous" transcription was not constitutive, but appeared to occur at the onset of the stationary phase. A *Ptou-lacZ* transcriptional fusion was used to study the TouR-mediated regulation of *Ptou* promoter in the host *P. putida* PaW340. This confirmed that TouR could activate transcription from the *Ptou* promoter in an effector-independent, growth-phase-dependent manner.[40] Moreover, regulator–promoter swapping experiments demonstrated that the presence of TouR is necessary and sufficient for imposing gratuitous activation on the P*tou* promoter, as well as on other σ^{54}-dependent catabolic promoters.[40] This

capacity of TouR cannot be replaced by the highly homologous DmpR protein.[40] Thus, TouR appears peculiar in its ability to shift from a completely effector-dependent behaviour during exponential growth to a constitutive or semi-constitutive behaviour in stationary phase. Transcriptional activation in the absence of genuine effectors was reported for the PhcR protein of the phenol-degrading strain *Comamonas testosteroni* R5.[43] However, it is not clear whether PhcR is a fully constitutive regulator, and the authors did not investigate if this gratuitous transcription was dependent on the growth phase.

Unfortunately, the mechanism by which TouR is activated at the phase transition remains elusive. No major modifications of the protein were detected upon entry into stationary phase nor the gratuitous activation of the *Ptou* promoter correlates with the TouR levels within the cell. The hypothesis that TouR could be intrinsically endowed with a low constitutive, effector-independent activity somehow masked during exponential growth or enhanced in stationary phase was not supported by *in vitro* transcription experiments, where TouR activity appeared fully dependent on effectors.[1] Since the activity state of the XylR/DmpR-like regulators is dictated by the mode of interaction between the A and C domains,[37,46,50] it may be envisaged that in TouR the intramolecular repression is released or becomes intrinsically weaker when the molecule encounters specific physiological conditions. Although gratuitous activation does not appear to be under the control of quorum sensing, the possibility that in carbon-starved stationary-phase cells intracellular metabolites accumulate and function as TouR effectors cannot be excluded. Alternatively, the A domain–C domain repressive interactions may be somehow relaxed at all growth phases, but the partial release of the repression would result in effective stimulation of transcription only in the stationary phase because in this phase the σ^{54}-bound holoenzyme is more abundant or more active or because the interactions between the holoenzyme and the regulator at the promoter are facilitated. Indeed, the accumulation of the stringent response alarmone (p)ppGpp at the phase transition was shown to favour σ^{54} in the competition with the housekeeping σ^{70} for the limiting core RNAP, thus improving the performance of σ^{54}-dependent promoters in the stationary phase.[26,42] Furthermore, whether an as yet unidentified factor(s) is required to assist the TouR-mediated activation of the *Ptou* promoter in the absence of genuine effectors and in response to carbon starvation remains the subject of speculation.

Other well-studied σ^{54}-dependent catabolic systems are known to be subjected to global regulation. Examples are the glucose-repression of the *Pu* promoter of the *xyl* system and the silencing of the expression of the catabolic operons during rapid growth in rich media, observed in the *xyl* system and in the *dmp* operon of *Pseudomonas* sp. CF600 (see volume 2,

Chapters 13, 16 and 18). The effector-independent activation of TouR was shown to be regulated according to the physiological status of the cell. In particular, the TouR-mediated gratuitous activation of the *Ptou* promoter is triggered at the exponential to stationary phase transition during growth in mineral medium only when the growth arrest is due to carbon, but not to nitrogen, exhaustion. The same profile of transcriptional output from the *Ptou* promoter was observed regardless of which carbon source – malate, succinate, glucose or pyruvate – was used to supplement the growth medium and independently of the repression that each carbon source exerted on effector-induced transcription during exponential growth.[40] These data indicate that the gratuitous activation phenomenon is not influenced by carbon catabolite repression exerted by specific, preferred carbon sources. Instead there seems to be a correlation between the effector-independent activation of TouR and the general metabolic status or the energy flow within the cell.

Whatever the mechanisms, the gratuitous activation phenomenon clearly enables TouR to activate the transcription of the ToMO-encoding operon not only in the presence of phenolic effectors but also in the absence of effectors upon carbon starvation (Figure 4). This mechanism would provide the basal ToMO activity necessary to initially convert toluene and *o*-xylene into the corresponding cresols, which would then bind and activate TouR leading to the full activation of transcription from the *Ptou* promoter and high levels of the ToMO activity. No data are available on the TouR-mediated gratuitous activation of the *tou dmp*-like operon coding for PH and *meta*-cleavage enzymes. According to the regulator–promoter swapping experiments cited above, it is very likely that also the σ^{54}-dependent promoter detected upstream of this operon becomes gratuitously activated by TouR. If this is the case, also PH would participate with ToMO to effectors generation from hydrocarbons (Figure 4).

Whatever the case, without gratuitous activation, *Pseudomonas* sp. OX1 could utilize the hydrocarbons as growth substrates only if phenols are also available. In this scenario the carbon exhaustion control can be envisaged as a global, superimposed regulation to avoid the costly expression of multicomponent enzymes when other carbon source can be found in the environment.

4. FINAL REMARKS

Bacterial adaptation to the degradation of aromatic compounds has to cope with different hindrances (see volume 4, Chapter 7). The onset of novel or broader substrates specificites/regioselectivities, instrumental to progress, can generate metabolic traffic jams because of the conservation or

the rigidity of the rest of the system. Another challenge in "paving the way for progress" is the co-evolution between novel degradation capabilities and optimal regulatory responses. In its progression towards *o*-xylene degradation, it is possible that the *tou* system had to face metabolic traffic jams deriving from the broad substrate range active on the three isomers of xylene. In the case of *o*-xylene, the concerted use of ToMO and PH might have fixed the problem. Apparently, this was not the case for *m*- and *p*-xylene. However, the isolation of the R1 derivative shows that the device was not so rigid. As far as the specificity of regulatory response is concerned, apparently the *tou* system was conservative. However, the onset of the gratuitous activation supported the catabolic expansion. Thus, taken together, the whole features of *tou* system show one more time that bacterial adaptation to degradation of aromatics is a fact of subtle equilibrium between progression and conservation.

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5

CARBAZOLE METABOLISM BY PSEUDOMONADS

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Abbreviations: BDO, biphenyl 2,3-dioxygenase; BDO-F, ferredoxin component of BDO; bp, base pairs; CARDO, carbazole 1,9a-dioxygenase; CARDO-F, ferredoxin component of CARDO; CARDO-O, terminal oxygenase component of CARDO; CARDO-R, reductase component of CARDO; $[2Fe–2S]_P$, plant-type [2Fe–2S] cluster; $[2Fe–2S]_{Pu}$, putidaredoxin-type [2Fe–2S] cluster; $[2Fe–2S]_R$, Rieske [2Fe–2S] cluster; HOADA, 2-hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoic acid; HPD, 2-hydroxypenta-2,4-dienoate; Inc, incompatibility; IR, inverted repeat; IS, insertion sequence; kb, kilobase; NDO, naphthalene 1,2-dioxygenase; NDO-O, terminal oxygenase component of NDO; OMO, 2-oxoquinoline 8-monooxygenase; OMO-O, terminal oxygenase component of OMO; ORF, open reading frame; rmsd, root mean square deviation; ROS, Rieske nonheme iron oxygenase system; TCA, tricarboxylic acid.

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1. INTRODUCTION

Crude oil is a heterogeneous mixture of organic molecules, including alkanes and aromatics, as well as sulfur-, nitrogen-, and oxygen-containing heteroaromatic compounds. The nitrogen-containing compounds found in crude oil can be divided into two classes: "nonbasic" molecules, including pyrroles and indoles, and "basic" molecules, including derivatives of pyridine and quinolines. The total nitrogen content of crude oil averages around 0.3%, of which nonbasic compounds comprise approximately 70–75%. Nonbasic nitrogen-containing heteroaromatics are predominantly mixed alkyl derivatives of carbazole. In contrast, coal-tar creosote is a mixture of 150 to 200 different compounds, of which approximately 85% are polycyclic aromatic hydrocarbons, 10% are phenolic compounds, and 5% are heterocyclic compounds.[72] In the past, creosote was used extensively as a wood preservative on railroad ties, telephone poles, and fence posts.[91] Additionally, creosote is an undesired by-product of coal gasification, and has been sold for asphalt production or stored in underground tanks. Carbazole is the major nitrogen heteroaromatic in coal-tar creosote and is one of the 13 most common heterocyclic compounds in creosote.[72]

Carbazole (dibenzopyrrole diphenylenimine, CAS no. 86-74-8, MW 167.2, $C_{12}H_9N$) is used in the manufacture of various products, including dyes, reagents, explosives, insecticides, and lubricants, and as a color inhibitor in detergents. However, carbazole is also known to be an environmental pollutant, and environmental concerns exist about its release because it is both mutagenic and toxic.[1,56] Even though carbazole itself is not highly toxic, it readily undergoes radical reactions to generate genotoxic hydroxynitrocarbazole.

In the petroleum industry, the removal of nitrogen heteroaromatics from petroleum is important for two reasons. First, their combustion directly causes the formation of nitrogen oxides, which contribute to acid rain. Second, nitrogen-containing aromatic compounds can cause the poisoning of refining catalysts, resulting in a decrease in yield.[20,31,124] Carbazole, which is a major nonbasic species, can be converted into basic derivatives during the cracking process; these can absorb to the active sites of the cracking catalyst.[31] Carbazole is potent as a direct inhibitor of hydrodesulfurization, and is commonly included in the refining process to meet sulfur content criteria.[65,73]

In the last decade, extensive investigations have been performed on the aerobic bacterial degradation of dioxin, a mixture of polychlorinated dibenzo-*p*-dioxins and dibenzofurans, because this class of chemicals is highly toxic and has been widely distributed in the environment.[8,42,80,83,125,126] Carbazole is a structural analog of dioxin; carbazole-degrading enzymes can partly function as dioxin-degrading enzymes.

Thus, bacterial degradation of carbazole has been investigated in several groups of bacteria. About one fourth of the carbazole-degrading bacteria are reported to be *Pseudomonas* species. In this chapter, we summarize the research on carbazole metabolism by *Pseudomonas* and related bacteria.

2. DIVERSITY OF CARBAZOLE-DEGRADING BACTERIA

Various carbazole-using bacteria have been isolated by enrichment culture using carbazole as the sole source of either carbon or both carbon and nitrogen. Except for *Nocardioides aromaticivorans* IC177[48,49] and *Gordonia* sp. F.5.25.8,[22,100] all of the carbazole-degrading aerobic bacteria are Gram-negative (Table 1). Among carbazole degraders isolated from soil, freshwater, and activated sludge samples, about 23 and 39% of the strains were classified into the genera *Pseudomonas* and *Sphingomonas*, respectively. When seawater-based screening medium was used in enrichment culture, carbazole degraders with different features were isolated from seawater or sea sediment.[27,48] Such marine bacteria were found to lack carbazole-degradative genes and enzymes highly similar to those found in various carbazole degraders from soil, freshwater, and activated sludge.

3. DEGRADATION PATHWAY OF CARBAZOLE

Ouchiyama *et al*.[88] first reported that anthranilate and catechol accumulated in the culture medium of *Pseudomonas resinovorans* CA10 as the catabolic intermediates of carbazole. CA10 can also grow on anthranilate as the sole source of carbon and nitrogen. When anthranilate is supplied, CA10 accumulates catechol, suggesting that carbazole is converted to catechol via anthranilate. In addition, in the culture medium of CA10 grown on carbazole, the production of 2′-aminobiphenyl-2,3-diol and its *meta*-cleavage product 2-hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoate (HOADA) was suggested.

In the degradation pathways of dibenzofuran and fluorene, dioxygenolytic attack was found to occur at angular and adjacent positions of dibenzofuran (4 and 4a carbon atoms) and 9-fluorenone (1 and 1a carbon atoms).[23,25,105] In the dibenzofuran degradation pathway, an oxygenation product of dibenzofuran, 4-hydro-4,4a-dihydroxydibenzofuran, is unstable and converts spontaneously to 2,2′,3-trihydroxybiphenyl, which is further converted to salicylic acid via *meta*-cleavage and hydrolysis.[8,80,83,125,126]

Table 1. Carbazole-degrading bacteria.

Bacterial strain	Medium[a]	Products[b]	Genetic analysis[c]	Enzyme analysis	References
Ralstonia sp. RJGII.123	C	Anthranilate	None	None	34, 104
P. resinovorans CA10 (*Pseudomonas* sp.)	CN	Anthranilate, catechol	Cloning: *car* (P), *antR*, *ant*, *cat* Regulation: *car*, *ant* Plasmid: pCAR1	Purification/ characterization: CarAa, CarAc, CarAd, CarBaBb, CarC X-ray crystallography: CarAa, CarAc, CarAd, CarBaBb	3, 4, 37–39, 51, 52, 60, 68, 71, 74–77, 81, 82, 84, 85, 88, 99, 101, 102, 108, 115, 119, 122, 123
P. resinovorans CA06	CN	Anthranilate, catechol	Detection[d]: *car* (P) Plasmid[e]	None	88, 112
P. stutzeri ATCC31258	C	Anthranilate	None	None	47
Pseudomonas sp. LD2	C	Anthranilate	Cloning: *car* (P)	Purification/ characterization: CarBaBb, CarC	28, 29, 93, 94
Burkholderia sp. CB1	CN	Not detected	None	None	113
Xanthomonas sp. CB2	CN	Not detected	None	None	113
Sphingomonas sp. CB3	CN	Not detected	Cloning: *car* (SC) Detection[f]: *car* (SK)	None	48, 107, 113
P. stutzeri OM1	CN	Anthranilate	Cloning: *car* (P)	None	87, 109
Sphingomonas sp. CDH-7	CN	Anthranilate	None	None	61
Sphingomonas sp. GTIN11	N	Anthranilate	Cloning: *car* (SK)	None	59
Sphingomonas sp. KA1	C	None	Cloning: *car*-I (SK), *car*-II (SK), *carDFE*, *fdxI-fdrI*, *fdrII*, *and*, *cat*, *pca* Plasmid: pCAR3	Purification: CarAaI, CarAaII, CarAcII, FdrII X-ray crystallography: CarAaII, FdrII	36, 50, 110, 121
Pseudomonas rhodesiae KK1	C	None	Detection[d]: oxygenase gene[g]	None	127
Neptunomonas naphthovorans CAR-SF	C[h]	None[i]	None	None	27

Pseudomonas sp. C3211[j]	C[k]	None	None	None	55
Pseudomonas sp. XLDN4-9	N[l]	None	Cloning[m]: *carAaAcAd* (P)	None	67, 128
Janthinobacterium sp. J3	CN	None	Cloning: *carR, car* (P) Regulation: *car*	Purification/ characterization: CarAa, CarC X-ray crystallography: CarAa, CarAd, CarC	3, 4, 39, 50, 71, 79
Janthinobacterium sp. J4	CN	None	Detection[d]: *car* (P)	None	50
Pantoea sp. J14	CN	None	Detection[d]: *car* (P)	None	50
Novosphingobium sp. J30	CN	None	Detection[d]: *car* (P) Plasmid[e]	None	50, 112
Pseudomonas sp. J11	CN	None	Detection[d]: *car* (P)	None	50
Pseudomonas sp. K15	CN	None	Detection[d]: *car* (P)	None	50, 112
Pseudomonas sp. K22	CN	None	Detection[d]: *car* (P)	None	50, 112
Pseudomonas sp. K23	CN	None	Cloning: *car* (P)[n] Plasmid[o]	None	50, 112
Sphingomonas sp. M2	CN	None	Detection[d]: *car* (SK)	None	50
Sphingomonas sp. J40	CN	None	None[p]	None	50
P. putida HS01	-[q]	None	Cloning: *car* (P) Plasmid: pCAR2	None	108, 112
Acinetobacter sp. IC001	CN	None	None[p]	None	49
Pseudomonas sp. IC017	CN	None	Detection[d]: *car* (P)	None	49
Sphingomonas sp. IC033	CN	None	Detection[d]: *car* (SK), *car* (SC)	None	49
Burkholderia sp. IC049	CN	None	Detection[d]: *car* (P)	None	49
Achromobacter sp. IC074	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC075	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC081	CN	None	Detection[d]: *car* (SK), *car* (SC)	None	49
Sphingomonas sp. IC097	CN	None	Detection[d]: *car* (SK)	None	49
Erythrobacter sp. IC114	CN	None	None[p]	None	49
Burkholderia sp. IC129	CN	None	None[p]	None	49
Burkholderia sp. IC138	CN	None	None[p]	None	49

(*Continued*)

Table 1. Carbazole-degrading bacteria—cont'd.

Bacterial strain	Medium[a]	Products[b]	Genetic analysis[c]	Enzyme analysis	References
Sphingomonas sp. IC145	CN	None	Detection[d]: *car* (SK)	None	49
Janthinobacterium sp. IC161	CN	None	Detection[d]: *car* (P)	None	49
N. aromaticivorans IC177	CN	Anthranilate	Cloning: *car* (N)	Purification/ characterization: CarAa, CarAc, CarAd X-ray crystallography: CarAa, CarAc	46, 48, 49
Stenotrophomonas sp. IC193	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC209	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC258	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC268	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC273	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC290	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC291	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC300	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC306	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC315	CN	None	Detection[d]: *car* (SK)	None	49
Sphingomonas sp. IC321	CN	None	Detection[d]: *car* (SK)	None	49
Marinobacterium sp. IC961	CN[r]	None	None[p]	None	49
Marinobacterium sp. IC977	CN[r]	None	None[p]	None	49
Gordonia sp. F.5.25.8	-[s]	None	None[t]	None	22, 100
Burkholderia sp. IMP5GC	-[u]	None	Cloning[m]: *carAaAcAd* (P)	None	14

[a] C, N, and CN; carbazole was added to the isolation medium as the carbon and energy source, nitrogen source, and carbon, nitrogen, and energy source, respectively. Hyphen means that enrichment culture using carbazole has not been done, and the detailed information is provided in respective foot notes.
[b] Major metabolic intermediate produced when the bacterium is grown on carbazole.

[c] *car* gene clusters were divided into four groups: *car* (P) is homologous to that found in *P. resinovorans* CA10 and *Janthinobacterium* sp. J3; *car* (SK) is homologous to that found in *Sphingomonas* sp. KA1; *car* (N) is found in *N. aromaticivorans* IC177; and *car* (SC) is homologous to that found in *Sphingomonas* sp. CB3.
[d] Occurrences of the genes were detected by Southern hybridization or PCR analyses.
[e] Occurrence of the plasmid containing the *car* gene cluster was detected.
[f] Homologue of the *car*-I gene cluster from *Sphingomonas* sp. KA1 was detected by Southern hybridization analysis.[49]
[g] No direct evidence was provided showing that the oxygenase detected using PCR was definitely involved in carbazole metabolism.[127]
[h] This strain was isolated using seawater medium containing carbazole as the carbon source.
[i] The *meta*-cleavage product in the carbazole degradation pathway [2-hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoate; HOADA] was tentatively identified based on data from Ouchiyama *et al.*[88]
[j] This strain degrades carbazole at 10°C with acetone as a cosubstrate.
[k] Enrichment culture was performed at 10°C using mineral medium supplemented with carbazole and acetone (as a solvent used for carbazole solubilization).
[l] Dr. Li Li personal communication.
[m] Genes were amplified using PCR and characterized.
[n] Two copies of the *car* gene cluster were cloned and analyzed.
[o] Occurrence of plasmid in this strain just after its isolation was suggested.[112]
[p] Probes prepared from *Pseudomonas* and *Sphingomonas car* gene clusters did not hybridize with the genome.[49,50]
[q] *P. putida* HS01 is a transconjugant generated using filter mating analysis with *Pseudomonas* sp. K23 (donor) and *P. putida* DS1 (recipient).[112]
[r] These strains were isolated using filtered seawater containing carbazole.
[s] This strain was originally isolated as a dibenzothiophene-degrading strain.
[t] Dibenzothiophene-desulfurizing *dsz* genes were analyzed.
[u] A detailed explanation of the conditions of the isolation procedure was not provided,[14] although this strain can grow on carbazole as the sole source of carbon and nitrogen.

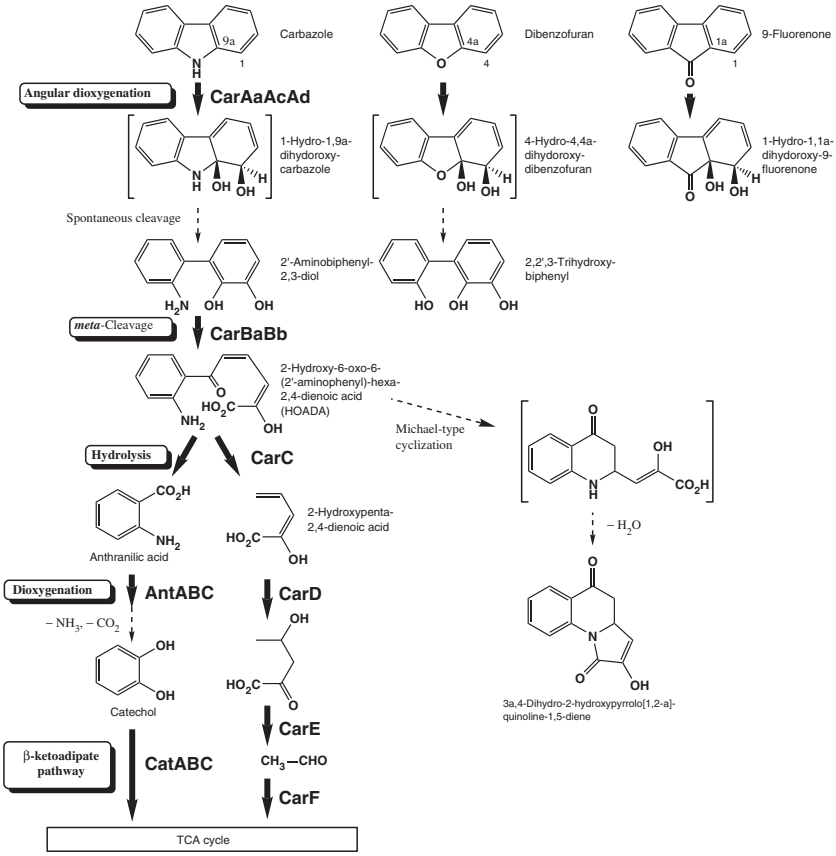

Figure 1. Carbazole degradation pathway and degradative enzymes established in *P. resinovorans* CA10.[88] The spontaneous cyclization dead-end product 3a,4-dihydro-2-hydroxypyrrolo [1,2-a]-quinoline-1,5-diene is formed.[29] Oxygenation reactions of dibenzofuran and 9-fluorenone are also shown. Compounds shown in brackets are unstable, and have not been detected directly. *Solid* and *broken arrows* show enzymatic and spontaneous reactions, respectively.

Based on the analogy of the metabolic intermediates of carbazole with those in the dibenzofuran degradation pathway, the degradation pathway of carbazole was proposed as follows (Figure 1). Carbazole is dioxygenated at angular (9a) and adjacent (1) carbon atoms to produce an unstable hemiaminal (1-hydro-1,9a-dihydroxycarbazole). Its five-member ring is spontaneously cleaved to form 2′-aminobiphenyl-2,3-diol, which is converted to anthranilic acid via *meta*-cleavage and subsequent hydrolysis. Anthranilic acid has been identified as a main metabolite from the culture extract of several carbazole degraders (Table 1),[29,47,49,59,61,87,88,104]

suggesting that these bacteria degrade carbazole through a pathway similar to that in Figure 1.

Anthranilic acid is known as a biotic compound, and is formed by the degradation of tryptophan in various organisms livings.[43] In addition, anthranilic acid is known as an important intermediate in the metabolism of many *N*-heterocyclic compounds, including *Pseudomonas* quinolone signal, which is involved in quorum sensing in *Pseudomonas aeruginosa* cells.[10] In the degradation pathway of carbazole, anthranilic acid is converted to catechol by dioxygenation at the C1 and C2 positions, and spontaneous deamination and decarboxylation (Figure 1).[62] Formed catechol is converted to a tricarboxylic acid (TCA)-cycle intermediate via *ortho*- and/or *meta*-cleavage pathways.[81,87,88]

The initial dioxygenation for carbazole, dibenzofuran, and 9-fluorenone is a distinct reaction because hydroxylation occurs at the ring-fused position (Figures 1 and 2a). This novel type of dioxygenation was termed "angular dioxygenation," which was coined originally by Engesser *et al.*[23] during degradation studies on dibenzofuran. For various aromatic compounds, such as toluene, naphthalene, and biphenyl/polychlorinated biphenyls, initial dioxygenation occurs at the lateral position of the molecules and both oxygen atoms of molecular dioxygen are incorporated into tandemly linked carbon atoms on an aromatic ring as two hydroxyl groups in the *cis*-configuration (Figure 2b). Distinct from angular dioxygenation, this type of dioxygenation is called "lateral dioxygenation." Once angular dioxygenation and subsequent spontaneous ring cleavage occurs for carbazole, the formed 2′-aminobiphenyl-2,3-diol is degraded by analogous biphenyl degradation pathways (Figure 1).[26]

4. CARBAZOLE DEGRADATION GENES

4.1. Carbazole Degradation Genes (*Car* Genes) of *P. resinovorans* CA10

Sato *et al.*[101,102] first succeeded in cloning the genes involved in carbazole conversion to anthranilate from the *P. resinovorans* CA10 genome by shotgun cloning using *meta*-cleavage activity. The resultant gene fragment contained seven degradation genes, one open reading frame (ORF) that encoded a putative protein or unknown function, and two partial possible genes (Figure 3a). A biotransformation experiment using the *Escherichia coli* expression system determined the functions of the degradation genes as follows: two identical copies of *carAa*, *carAc*, and *carAd* encoded terminal oxygenase, ferredoxin, and ferredoxin reductase components of

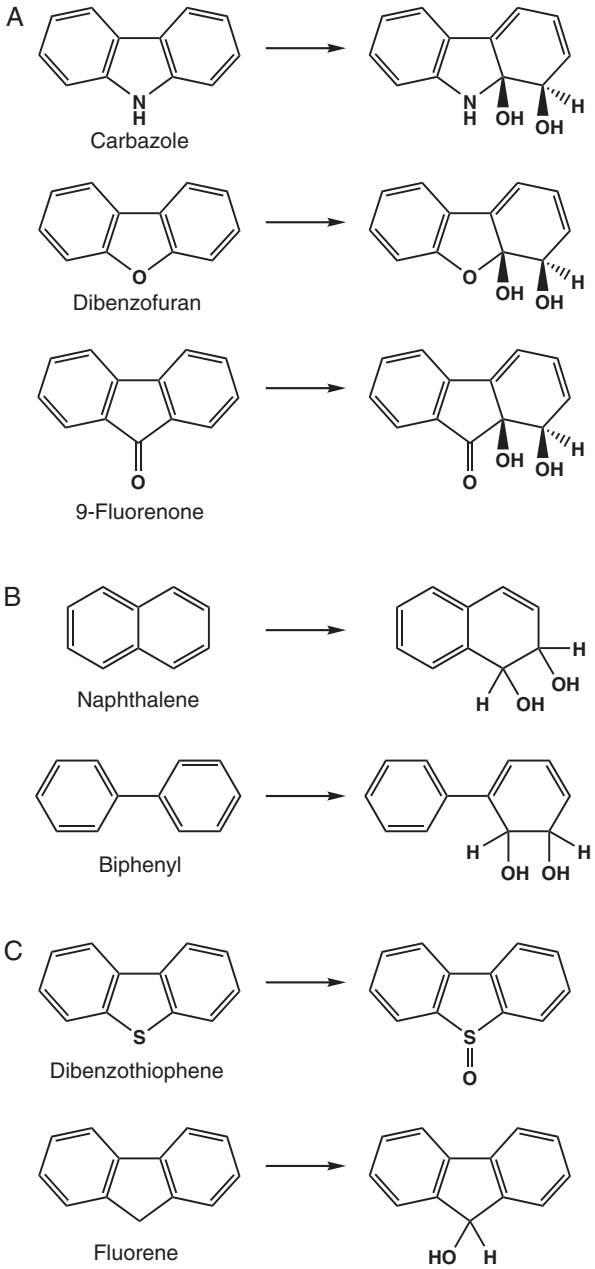

Figure 2. Diverse oxygenation reactions by carbazole 1,9a-dioxygenase (CARDO).[82,115] (a), Shows the angular dioxygenation for carbazole, dibenzofuran, and 9-fluorenone. As shown in (b), CARDO can catalyze lateral dioxygenation for polycyclic aromatic hydrocarbons, such as naphthalene, and for biphenyl. As shown in (c), CARDO also catalyzes monooxygenation for methylene carbon and sulfoxidation for sulfide sulfur. Transformation of the substrates shown in this figure was analyzed by *E. coli* cells having the genes for terminal oxygenase (*carAa*), ferredoxin (*carAc*), and reductase (*carAd*) of CARDO from *P. resinovorans* CA10.

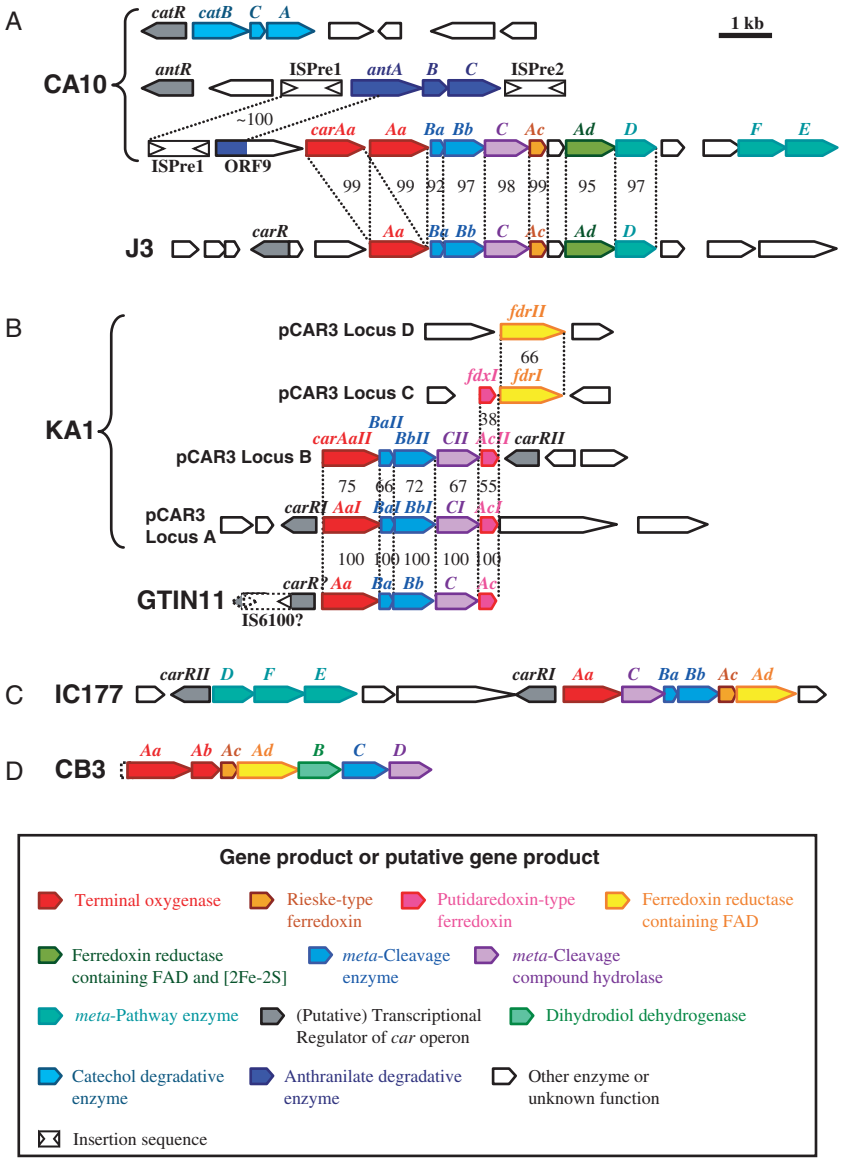

Figure 3. Genetic structures of the gene clusters involved in carbazole degradation by (a) *P. resinovorans* CA10 and *Janthinobacterium* sp. J3, (b) *Sphingomonas* sp. KA1 and *Sphingomonas* sp. GTIN11, (c) *N. aromaticivorans* IC177, and (d) *Sphingomonas* sp. CB3. Amino acid sequence identities between the homologous proteins are also shown.

carbazole 1,9a-dioxygenase (CARDO); *carBa* and *carBb* encoded structural and catalytic subunits of the *meta*-cleavage enzyme (2′-amino-biphenyl-2,3-diol 1,2-dioxygenase); and *carC* encoded the *meta*-cleavage compound (HOADA) hydrolase. Hereafter, the gene cluster from CA10 is designated as the car_{CA10} gene cluster. Previously, a transposon Tn*5* mutant of *P. resinovorans* CA10, which is deficient in carbazole metabolism, was isolated and partially characterized.[60] In strain TD2, Tn*5* was inserted into the car_{CA10} gene cluster, suggesting that the *car* gene cluster is indispensable for carbazole metabolism by CA10. Gene walking around the car_{CA10} gene cluster revealed its entire structure (Figure 3a).[84] Downstream of the *carAd* gene, we found 2-hydroxypenta-2,4-dienoate (HPD) degradative *carDFE* genes (*meta*-cleavage pathway genes). In addition, in the 21-kilobase (kb) region upstream from *carAa*, we found the *antABC* genes encoding anthranilate 1,2-dioxygenase (Figure 3a). This anthranilate degradative gene cluster was flanked by two homologous insertion sequences (ISs), ISPre1 and ISPre2 (formerly IS*car2* and IS*car3*, respectively), resulting in the putative composite transposon. No regulatory gene was detected around the car_{CA10} gene cluster; however, a second copy of ISPre1 was found at about 2-kb upstream of the *carAa* gene. Interestingly, a 698-base pair (bp)-long 5′-portion of ORF9, which was located between the second copy of ISPre1 and *carAa*, corresponded to an 888-bp-long 5′-portion of the *antA* gene, although a deletion and a replacement were found in ORF9 (Figure 3a). Thus, it was suggested that the one-ended transposition of ISPre1 and the 5′-portion of the *antA* resulted in the formation of ORF9, although the target site duplication was not detected at the putative terminal in ORF9.[84] The *antR* gene encoding a transcriptional regulator of the *ant* operon was found outside the putative composite transposon containing *antABC* (Figure 3a),[120] and it was revealed that AntR regulates the inducible expression of the *car* gene cluster (see Section 6.2).

The β-ketoadipate pathway (*ortho*-cleavage pathway) genes involved in catechol mineralization were isolated from the CA10 genome using Tn*5* mutagenesis (Figure 3a).[60,81]

4.2. Distribution of the Car_{CA10} Gene Cluster Homologues in Other Genera

Genetic analysis of carbazole-degrading bacteria shows that many that are classified in the genera *Pseudomonas*, *Burkholderia*, and *Janthinobacterium* have nearly identical carbazole degradation genes with car_{CA10} (Table 1, Figure 3a). A detailed comparison of the gene organization and flanking regions of several *car* gene clusters with different origins suggests the evolutionary scheme establishing the diversity of the *car* gene cluster.[50] In contrast, the copy number of the *car* gene cluster

in the genome differs among carbazole degraders,[50] which may arise because *car* gene clusters are sometimes borne in mobile genetic elements and/or flanked by the ISs (see Section 5).

4.3. The *Car* Gene Clusters in the Genera *Sphingomonas* and *Nocardioides*

Although the homology score is relatively low (<60% identity between counterparts at nucleotide sequence levels), *car* gene cluster homologues showing similarity in gene organization and phylogeny with the car_{CA10} gene cluster have been found in the genera *Sphingomonas* and *Nocardioides* (Table 1). In sphingomonads, *car* gene clusters were first isolated from *Sphingomonas* sp. GTIN11[59] and *Sphingomonas* sp. KA1.[36] Unlike the car_{CA10} gene cluster, car_{GTIN11} and car_{KA1} gene clusters do not contain the NAD(P)H:ferredoxin oxidoreductase gene involved in the initial dioxygenase, but contain the genes for terminal oxygenase (*carAa*) and ferredoxin (*carAc*), the *meta*-cleavage enzyme (*carBaBb*), and HOADA hydrolase (*carC*; Figure 3b). Interestingly, although *Sphingomonas* CarAa shows significant homology with CA10 CarAa (>55% identity), ferredoxin (CarAc) has no relatedness with the Rieske ferredoxin, including $CarAc_{CA10}$, but shows similarity to the putidaredoxin-type ferredoxins. Because $CarAa_{KA1}$ can receive electrons from $CarAc_{KA1}$ and catalyze angular dioxygenation of carbazole, ferredoxin selectivity differs between $CarAa_{CA10/J3}$ and $CarAa_{KA1/GTIN11}$.[50] The car_{KA1} gene cluster (re-designated *car*-I_{KA1}) was found on plasmid pCAR3,[36] and a recent study revealed the presence of an additional copy of the *car* gene cluster (*car*-II_{KA1} gene cluster).[121] In addition, NAD(P)H:ferredoxin oxidoreductase genes (*fdrI* and *fdrII*), and a third putidaredoxin-type ferredoxin gene were found on pCAR3 (Figure 3b).[121] Recent studies by Inoue *et al*. have shown that $car_{KA1/GTIN11}$ gene-cluster homologues occur in various *Sphingomonas* and related strains (Table 1).[49,50]

A Gram-positive *car* gene cluster was isolated and characterized in *N. aromaticivorans* IC177 (formerly *Nocardioides* sp. IC177).[48,49] The *car* genes were clustered in the *carAaCBaBbAcAd* and *carDFE* gene clusters encoding the enzymes responsible for degradation of carbazole to anthranilate and HPD, and HPD to pyruvic acid and acetylcoenzyme A, respectively (Figure 3c).[48] The order of the *carC* and *carBaBb* genes in IC177 was inverted from the above-mentioned *car* gene clusters in Gram-negative bacteria (Figure 3). In the *carAaCBaBbAcAd* and *carDFE* operons in IC177, all of the genes overlap each other by 1 or 4 bp. The *carDFE* genes were closely linked and located upstream of the *carAaCBaBbAcAd* gene cluster in IC177. The carbazole catabolic operons of IC177 were

organized in a more orderly fashion as functional units than those in Gram-negative strains, such as CA10, J3, GTIN11, and KA1.

A distinct carbazole-degradative *car* gene cluster has been isolated from *Sphingomonas* sp. CB3 (Figure 3d).[103] The car_{CB3} did not show relatedness with other *car* gene clusters in terms of gene organization and phylogeny, but showed marked relatedness to the biphenyl degradative *bph* gene cluster. Although carbazole metabolic activity of the enzymes encoded in the car_{CB3} gene cluster has not been confirmed, its transcription was detected when carbazole was used as a sole carbon source by CB3.[103] Recently, it was reported that *Sphingomonas* carbazole degraders of strains IC033 and IC081 had both homologues of *car*-I_{KA1} and car_{CB3}.[49] Moreover, Southern hybridization analysis revealed that CB3 also has a *car*-I_{KA1} homologue in its genome.[49]

5. CARBAZOLE-DEGRADATIVE MOBILE GENETIC ELEMENTS

5.1. Structure of pCAR1 and Tn*4676*

The car_{CA10} gene cluster is located on the circular plasmid pCAR1.[84] The entire nucleotide sequence of pCAR1 was determined to elucidate the mechanism by which the *car* gene cluster may have been distributed in nature.[68] The pCAR1 is a 199,035-bp circular plasmid, and carries 190 ORFs (Figure 4a). The potential origin for replication, *oriV*, and Rep and Par proteins appeared to be closely related to those of plasmid pL6.5 isolated from *Pseudomonas fluorescens* L6.5 (accession no. AJ250853) and pND6-1.[66] The potential tellurite-resistance *klaABC* genes were also related to those in the incompatibility (Inc) P plasmid mainly isolated from pseudomonads. In contrast, the putative transfer genes of pCAR1 showed low, but significant, homology (29–59% identity) with Trh and Tra proteins involved in the conjugative transfer of plasmids or genomic islands from Enterobacteriaceae.

In pCAR1, there were *tnpAcCST* genes that encoded proteins showing >70% overall length-wise identity with transposition machinery proteins borne in the toluene/xylene-degrading transposon Tn*4651*[118] of the TOL plasmid pWW0. Both *car* and *ant* gene clusters were found within a 72.8-kb Tn*4676* sequence defined by flanking *tnpAcC* and *tnpST* genes, and bordered by a 46-bp inverted repeat (IR; Figure 4a). Within Tn*4676* and its flanking region, we found the remnants of numerous mobile genetic elements, such as the duplicated transposase genes that are highly homologous to *tnpR* of Tn*4653*,[119] and the multiple candidates of IRs for Tn*4676* and a Tn*4653*-like element.

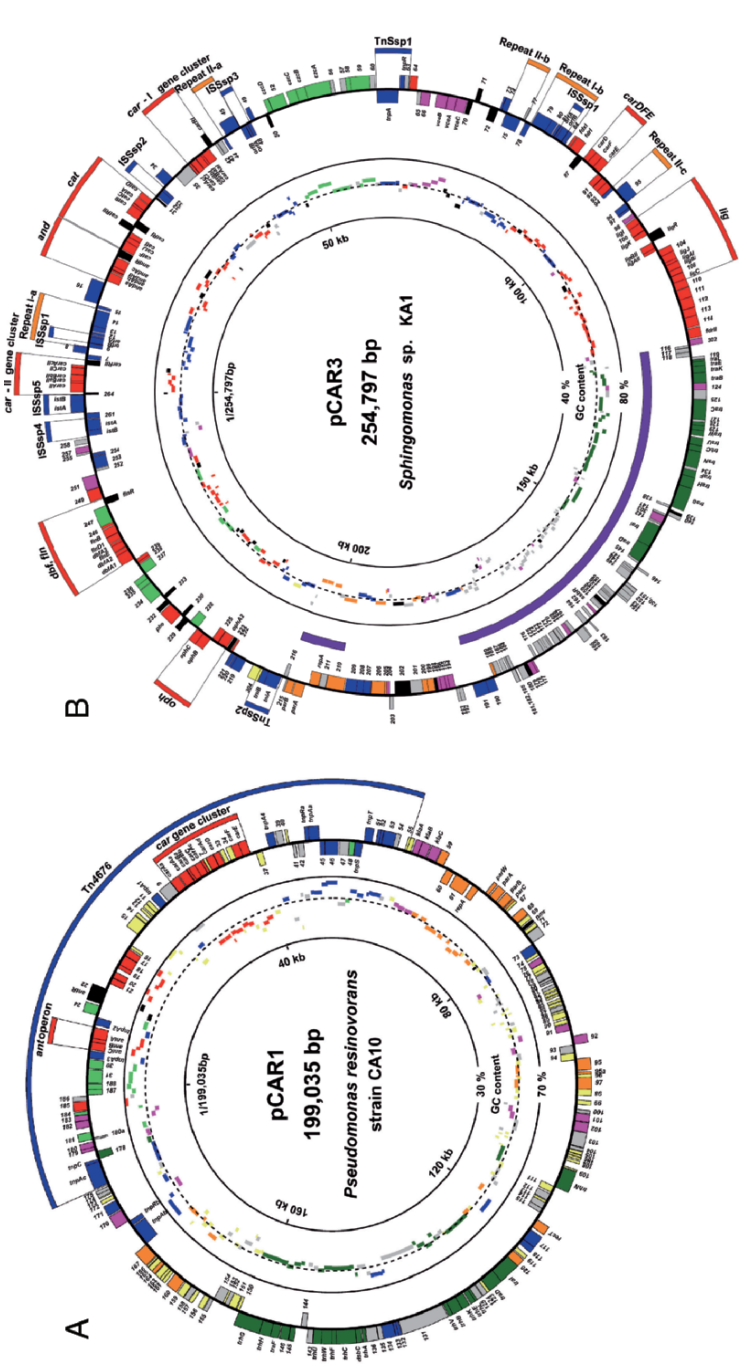

Figure 4. The circular gene map of pCAR1 (a) and pCAR3 (b). Genes or ORFs outside the circle are coded clockwise; those inside are coded counterclockwise. The (putative) functions of genes or ORFs are shown by color as follows: orange, maintenance or DNA processing; dark green, conjugative transfer; blue, transposition or integration; red, degradation; light green, transport; black, regulation; magenta, other known function; gray, unknown function (homologous to hypothetical protein); and yellow, unknown function (no homology). Bars inside the circular gene map indicate the G + C contents of ORFs shown in same color. The *broken* circle indicates the average G + C content of the entire pCAR1 (56%) and pCAR3 (62.5%). In (b), bold purple lines show the regions homologous to pNL1.[97]

5.2. The pCAR1 Function as a Novel *Pseudomonas* Plasmid

5.2.1. Replication Function and Incompatibility Grouping

An approximately 2.7-kb DNA region that contains *repA* and the putative *oriV* is sufficient for the replication of pCAR1 in *Pseudomonas putida* cells (Figure 5).[111] The RepA protein of pCAR1 shows high overall length-wise identity with that of pL6.5 (98%) and pND6-1 (98%), and low-level identity with that of pECB2[17] (43%), pPS10[78] (39%), and pRO1600[54] (31%). The RepA protein of pPS10 binds to repeat sequences, called iterons, in the *oriV* region. AT- or GC-rich regions have also been found, and two copies of an 11-mer repeat sequence occur in an AT-rich region.[78] A DnaA box, which is the binding site of the DnaA protein produced by the host strain, is also found in the *oriV* region of pPS10.[30] In the case of pCAR1, two AT-rich regions (regions I and II) and three copies of a 12-bp repeat sequence (12-mers L, M, and R) were found in AT-rich region I, and another five copies (the 12-mers R1 to R5) were located in region II (Figure 5). These sequences are similar to the three copies of a 13-mer repeat sequence found in the *oriC* of *E. coli*.[106] Two putative DnaA box sequences have been identified; the former sequence is identical to that found in *oriC* of *E. coli*.[7] Fourteen copies of 18-mer repeat sequences were also found from the *oriV* region (Figure 5). Considering the relative locations of the AT-rich regions, DnaA boxes, and 18-mer repeats, these 18-mer repeats are predicted to be the iterons of pCAR1 (Figure 5), although their nucleotide sequences have no similarity to known iteron sequences.[63] Deletion and complementation analyses have revealed that

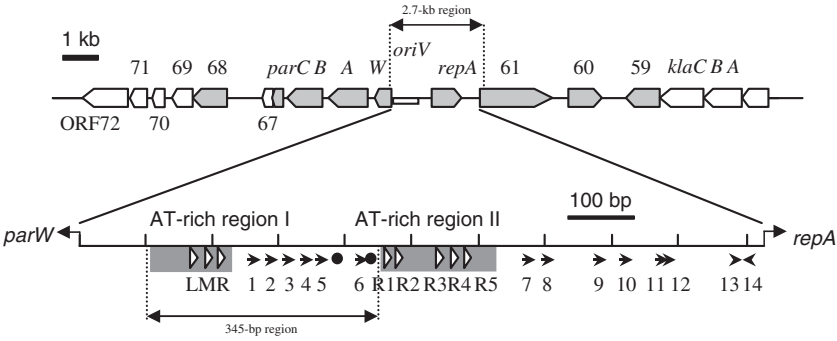

Figure 5. Genetic map of the replication/maintenance region (*parCBAW-oriV-repA* region) of pCAR1. A detailed physical map of the 1005-bp *oriV* region between the *parW* and *repA* initiation codons of pCAR1 is also shown (nucleotides 70,108 to 71,112 in accession no. AB088420). Shaded boxes, AT-rich region; black circles, DnaA boxes; white triangles, 12-bp repeat sequences designated 12-mer L, M, R, and R1 to R5; *black small arrow*s, 18-bp repeat sequences designated iterons.

oriV of pCAR1 is located within a 345-bp DNA region containing AT-rich region I, DnaA boxes, and six iterons (Figure 5).[111] In addition, it was shown that RepA could act in *trans* for the *oriV* region in *P. putida* cells.[111] These findings suggest the importance of the 12-mers L, M, and R, iterons 1–6, and the two DnaA boxes for pCAR1 replication.

In an incompatibility test with the IncP-1 to P-7, P-9, P-12, and P-13 plasmids, it was impossible to obtain a transformant that carried both Rms148 (IncP-7) and pCAR1 mini plasmid, whereas other IncP group plasmids were compatible with the test plasmids.[111] In addition, Southern hybridization analysis using pCAR1 *repA* gene as a probe revealed that a single signal was detected with Rms148.[111] These results definitively indicate that pCAR1 belongs to the IncP-7. Thus, pCAR1 is the first IncP-7 plasmid for which the entire nucleotide sequence has been determined.

5.2.2. IncP-7-Specific Partitioning Apparatus of pCAR1

The *parWABC* genes, which are involved in the partitioning of the plasmid, are located upstream of the *repA* gene of pCAR1 and in the opposite orientation (Figure 5).[68,111] RT-PCR analyses showed that *parWABC* is expressed at the transcriptional level and is transcribed as at least two transcriptional units (*parWABC* and *parABC*) in CA10 cells. Deletion and gene disruption analyses revealed that *parWAB* is sufficient for the stable maintenance of pCAR1. Together with the results of a complementation assay of *par* gene disruptants, it was suggested that the stability of pCAR1 depends on the ParW, ParA, and ParB proteins, but not on the *parC* region.

5.2.3. Conjugal Transfer Function of the IncP-7 Plasmid

The conjugative transfer of pCAR1 was examined using *Burkholderia* sp., *Comamonas testosteroni*, *E. coli*, *P. resinovorans*, and *P. putida* strains as recipients in mating experiments. The transfers of pCAR1 to *Pseudomonas* recipients were detected at frequencies of 3×10^{-1} and 3×10^{-3} per donor cell,[111] suggesting that pCAR1 is a self-transmissible plasmid. Although the conjugal transfer of pCAR1 to non-*Pseudomonas* was not detected, electroporation analyses using a mini-replicon of pCAR1 indicated that the host range for conjugation of pCAR1 into non-*Pseudomonas* hosts is determined by its ability to replicate. In contrast, mating analyses of pCAR2, which is an IncP-7 carbazole-degradative plasmid from *P. putida* HS01 that has a genetic structure highly homologous to that of pCAR1 with various recipient strains, showed that it could transfer from HS01 to *Pseudomonas chlororaphis*, *P. fluorescens*, *P. putida*, *P. resinovorans*, and *Pseudomonas stutzeri*.[108] Therefore, the IncP-7 plasmid has the capability to transfer to at least *Pseudomonas* recipients.

The complete nucleotide sequence of pCAR1 suggested the presence of putative transfer genes, designated *tra* and *trh* genes (Figure 4a).[68] RT-PCR

analysis of *tra/trh* genes of pCAR1 in *P. resinovorans* CA10 and of pCAR2 in *P. putida* HS01 revealed that the *tra/trh* genes were organized into at least four transcriptional units, *trhN*, *traItrhV*, *trhA-trhU*, and *traF-trhG*.[108]

5.3. Structure and Carbazole Metabolic Function of pCAR3

In *Sphingomonas* sp. KA1, carbazole-degradative gene clusters are located on the >250-kb circular plasmid pCAR3.[36,121] The entire nucleotide sequence of pCAR3 has been determined.[110] The annotation of the complete sequence of the 254,797-bp circular plasmid revealed 264 ORFs on pCAR3, including 26 genes or ORFs already reported[49,50,121] (Figure 4b). Two sets of repeat sequences, which were designated as repeat I (5336 bp) and repeat II (2737 bp), respectively, and each nucleotide sequence was completely conserved. In addition, five types of ISs, a Tn*3*-family[33] transposon, and a Tn*501*-subfamily[33] transposon were found on pCAR3.

The gene organization of ORF116 to ORF191 and ORF210 to ORF216 showed homology (43–95% identity at the amino acid sequence level) with those of pNL1 from *N. aromaticivorans* F199.[97] Based on sequence similarity, ORF212, ORF213, and ORF214 are presumed to encode a replication initiator protein and partioning proteins, and were designated as *repA*, *parA*, and *parB*, respectively. The *oriV* on pCAR3 was predicted to be located between *repA* and *parA*, and there was an AT-rich region, six copies of 11-bp repeat sequences, and a DnaA box-like sequence.[7] The deduced amino acid sequences of 16 ORFs (ORF120 to ORF123, ORF126, ORF127 to ORF133, ORF135 to ORF137, and ORF 143 to ORF 144) showed 60–89% homology to *tra* or *trw* genes encoded on pNL1.[97] Because pNL1 was reported to be a conjugative plasmid,[5,97] the transferability of pCAR3 was tested using filter mating with a pCAR3-cured KA1-derivative strain as a recipient. However, no transconjugants were obtained; thus, pCAR3 may be defective in conjugative transfer.[110]

In addition to the multiple gene sets involved in carbazole conversion to anthranilate (Figure 3b), the genes involved in the complete mineralization of anthranilate were identified (Figure 4b). The products of the *and* gene cluster (*andAaAbAdAc-andR*) showed 39–68% identity with And proteins encoding anthranilate 1,2-dioxygenase involved in the anthranilate conversion to catechol by *Burkholderia cepacia* DBO1.[16] The well-established β-ketoadipate pathway genes, the *cat* gene clusters, were found. The *meta*-cleavage pathway genes *carDFE* involved in HPD mineralization were also identified (Figure 4b). These gene clusters were inducibly or constitutively expressed; thus, pCAR3 contains the complete set of genes responsible for carbazole mineralization.

6. TRANSCRIPTIONAL REGULATION OF THE *CAR* OPERONS

6.1. Diversity in the (Putative) Transcriptional Regulatory Mechanisms of the *Car* Gene Clusters

In contrast to the similarity found in the degradation pathways, the gene structures and enzymes, the (putative) regulatory proteins of the *car* gene clusters of CA10, J3, KA1, and IC177, are diverse and are located in different neighboring regions in each *car* gene cluster (Figure 3). The transcriptional regulators of the *car* operons of CA10 and J3 are the AraC/XylS family protein AntR[120] and the GntR family protein CarR.[71] Putative regulators in the car_{IC177} and *car*-I/II$_{KA1}$ operons are the IclR family protein CarRI[48] and the GntR family proteins CarRI (*car*-I gene cluster) and CarRII (*car*-II gene cluster).[50,121] This diversity appears to support the hypothesis that regulatory systems and their target operons do not necessary coevolve, but seem to become associated independently.[12,13,117]

6.2. Transcriptional Regulation of the *Ant* and Car_{CA10} Operons

Through Northern hybridization and RT-PCR analyses, the *antABC* gene cluster, which encodes anthranilate 1,2-dioxygenase, was shown to constitute a single transcriptional unit.[120] The transcription start point of the *ant* operon was mapped at 53 nucleotides upstream of the *antA* translation start point, and the –10 and –35 boxes were homologous to the conserved σ^{72} recognition sequence.[21] This promoter, named P_{ant}, was induced by anthranilate itself, and the region up to at least 70 bp from the transcription start point was necessary for the activation of P_{ant}. AraC/XylS-type regulatory genes, named *antR* and located 3.2-kb upstream of the *antA* gene (Figure 3a), were indispensable for the anthranilate-dependent activation of P_{ant}.[120] ORF9, located in the immediate vicinity of the car_{CA10} gene cluster, is proposed to be formed via the transposition of ISPre1 along with the 5′-portion of the *antA* gene (Figure 6).[84] The intergenic region between ISPre1 and *antA* is identical in nucleotide sequence to that between ISPre1 and ORF9, and the second P_{ant} promoter upstream of ORF9 (about 2.1-kb upstream of the *carAa* gene) is functional. RT-PCR analysis showed that the ~12-kb car_{CA10} gene cluster constitutes an operon, which is transcribed from the P_{ant} promoter.[71] As a result, both *ant* and *car* operons are simultaneously regulated by AntR.

Reporter gene analysis clearly indicated that another promoter for the constitutive transcription of the *car* operon is located within the 1094-bp

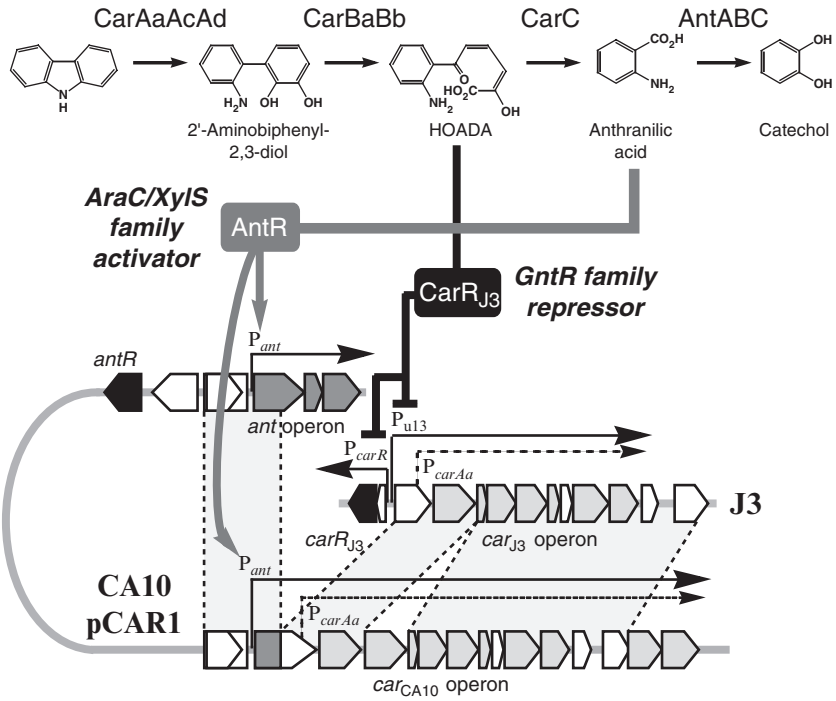

Figure 6. Transcriptional regulation of the car_{CA10} and car_{J3} operons. Both operons have a dual promoter system consisting of an inducible promoter, P_{ant} (car_{CA10}) and P_{u13} (car_{J3}), and a constitutive promoter, P_{carAa}.

3′-portion of ORF9 (Figure 6). The transcriptional start point of the constitutive mRNA of the *car* operon was mapped to a cytosine base 385 nucleotides upstream of the *carAa* translation start point. Putative −35 and −10 promoter sequences preceded the transcription start point, as is typical among *Pseudomonas* σ^{72}-dependent promoters.[21] This result was confirmed by a reporter assay using a deletion series of the 1094-bp region upstream of *carAa*; the promoter required for the constitutive expression of the *car* operon was designated P_{carAa}.[71]

6.3. Transcriptional Regulation of Car_{J3} Operon

The carbazole catabolic *car* operons from CA10 and J3 have nearly identical nucleotide sequences in their structural and intergenic regions, but not in their flanking regions (Figure 3a). Low-level expression in the absence of carbazole was observed, suggesting that a P_{carAa}-like promoter sequence, which is conserved upstream of $carAa_{J3}$ (Figure 6), is also

involved in the constitutive transcription of car_{J3} operon.[71] The transcription start points of car_{J3}, which is induced in the presence of carbazole, were detected at 71 and 66 nucleotides upstream of the ORFU13 translation start point, respectively. Putative –35 and –10 sequences precede the adenine located 71 nucleotides upstream. This promoter was designated the P_{u13} promoter. A GntR family regulatory gene *carR* is divergently located upstream of the car_{J3} operon (Figures 3 and 6). Transcription start points and a P_{carR} promoter, which are involved in the inducible expression of *carR*, were also found upstream of *carR* (Figure 6).

The CarR protein was shown to be a repressor of the P_{u13} promoter. DNase I footprinting with purified CarR and the 276-bp region containing P_{u13} and P_{carR} revealed a protection region containing two operators similar to the consensus sequence of FadR-subfamily members of GntR transcriptional regulators.[96] An electrophoretic mobility shift assay clearly showed that CarR binds to both operators synergistically, but its affinity to the respective operator sequences differed. Although anthranilate is an inducer of P_{ant} regulated by AntR, HOADA is an inducer of P_{u13} under the control CarR.

In both *car* operons, the distal promoter, P_{ant} or P_{u13}, is required for inducible expression. The proximal promoter, P_{carAa}, is conserved in both operons and is involved in constitutive expression. The regulatory circuit of each *car* operon is proposed as follows. First, Car enzymes constitutively expressed mainly from P_{carAa} facilitate the initial degradation of carbazole into the inducer for each operon, that is, anthranilate for car_{CA10} and HOADA for car_{J3}. Then, a pathway-specific regulator, that is, AntR of the AraC/XylS family or CarR of the GntR family, responds to the produced inducer to stimulate or derepress each regulating promoter, which in turn, accelerates carbazole degradation.

7. STRUCTURE AND FUNCTION OF CARBAZOLE 1,9A-DIOXYGENASE

7.1. Enzymatic Features of CARDO

CARDO of *P. resinovorans* CA10 ($CARDO_{CA10}$) is a three-component dioxygenase system[83] that is a member of the Rieske nonheme iron oxygenase system (ROS).[24,70,90] CARDO consists of a terminal oxygenase and electron-transport proteins (Figure 7).[76,101] The terminal oxygenase component of CARDO, hereafter simply referred to as CARDO-O, is a homotrimeric enzyme that contains one Rieske [2Fe–2S] cluster[95] (hereinafter referred as $[2Fe–2S]_R$) and one active-site iron (Fe^{2+}) in a single subunit (CarAa). Similar to a number of mononuclear nonheme iron(II) enzymes,[64,98,114] ROS contains a 2-His-1-carboxylate facial triad motif[45] for

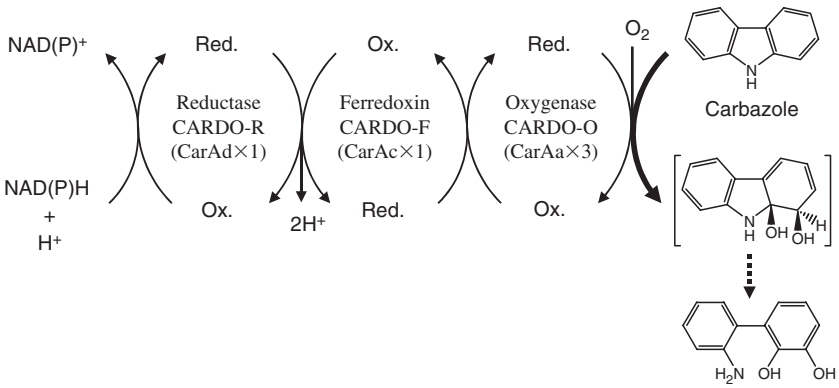

Figure 7. Role of the components of CARDO isolated from *P. resinovorans* CA10. Oxidized and reduced enzyme states are indicated by "Ox" and "Red," respectively.

binding of the active-site mononuclear iron. The electron-transport proteins of CARDO, which mediate electron transport from NAD(P)H to CARDO-O, comprise ferredoxin (CARDO-F; a monomer of CarAc), which contains one $[2Fe–2S]_R$, and ferredoxin reductase (CARDO-R; a monomer of CarAd), which contains one FAD and one plant-type [2Fe–2S] cluster[86] ($[2Fe–2S]_P$).[76,101] ROSs have been classified into three major groups based on the number of constituent components and the nature of the redox center.[6] The characteristics of the electron-transport chain suggest that $CARDO_{CA10}$ should be categorized as class III in Batie's classification.[76] However, phylogenic analysis indicates that the amino acid sequence of $CarAa_{CA10}$ shares rather low-level homology (<19% overall length-wise identity) with almost all known catalytic subunits of ROS terminal oxygenases. In addition, whereas typical class III ROSs contain the terminal oxygenase component consisting of both α and βsubunits with the $\alpha_3\beta_3$ (or $\alpha_2\beta_2$) configuration, CARDO-O consists of only catalytic αsubunit with the α_3 configuration. This homomultimeric structure is typical of class IA ROSs, whose terminal oxygenases are believed to have α_3 configurations.[24]

7.2. Substrate Specificity of CARDO

The most interesting feature of CARDO is its capability to catalyze angular dioxygenation (Figure 2). With very few exceptions, ROSs catalyze the incorporation of both oxygen atoms of molecular dioxygen to tandemly linked carbon atoms on an aromatic ring as two hydroxyl groups in the *cis*-configuration. Distinct from angular dioxygenation, this type

of dioxygenation is called lateral dioxygenation. For example, the lateral dioxygenation of naphthalene and biphenyl is shown in Figure 2b. In addition to lateral dioxygenation, several ROSs have been reported to catalyze other types of oxygenation. Thus, naphthalene 1,2-dioxygenase (NDO) of *Pseudomonas* sp. strain NCIB9816-4 can catalyze lateral dioxygenation, monooxygenation (including sulfoxidation), desaturation, and dealkylation.[92] As the results of the biotransformation experiments using *E. coli* cells having *carAa*, *carAc*, and *carAd* genes, CARDO has also been shown to catalyze diverse oxygenation reactions within a broad substrate range, including angular dioxygenation, lateral dioxygenation, and monooxygenation (Figure 2).[82,115] Although numerous ROSs have been isolated and characterized to date, only a limited number of these can catalyze angular dioxygenation.

Xanthene and phenoxathiin were converted by CARDO to 2,2′,3-trihydroxydiphenylmethane and 2,2′,3-trihydroxydiphenyl sulfide, respectively,[82] suggesting that the oxygenation occurred at the angular carbon bonding to the oxygen atom to yield hetero-ring cleaved compounds. 9-Fluorenone is a possible substrate of angular dioxygenation (C1a and C1 hydroxylation);[23,25,105] however, it is not a preferable substrate for CARDO.[115] Therefore, angular dioxygenation by CARDO occurs effectively at the angular position adjacent to an oxygen or nitrogen atom, but not a sulfur or carbon atom.

7.3. Structural Basis of the Novel Substrate Specificity of CARDO

To clarify the molecular mechanisms governing the capability to catalyze novel angular dioxygenation, the crystal structure of CARDO-O from *Janthinobacterium* sp. J3 was resolved at a resolution of 1.95 Å.[79] The overall shape of CARDO-O resembles that of a 100-Å-wide doughnut, with a 30-Å hole and 45-Å thickness (Figure 8a). The monomeric structure in the asymmetric unit facilitates trimeric interactions with neighboring asymmetric units along the crystallographic threefold axis. The reported structure of the αsubunits of terminal oxygenase of NDO (NDO-O)[58] is shown in Figure 8b. Although some apparent differences exist, such as the size of the subunit and centrally located hole, the subunit interactions in CARDO-O roughly resemble those between the three αsubunits of the heterohexameric NDO-O. Based on the mushroom-like overall structures of NDO-O, it seems likely that this type of structure is common among the terminal oxygenase components of class IB, II, and III ROSs with the $\alpha_3\beta_3$ configuration.[24] Regardless of the quaternary structure (α_3 or $\alpha_3\beta_3$), each (catalytic) subunit can be divided into two distinct domains: the

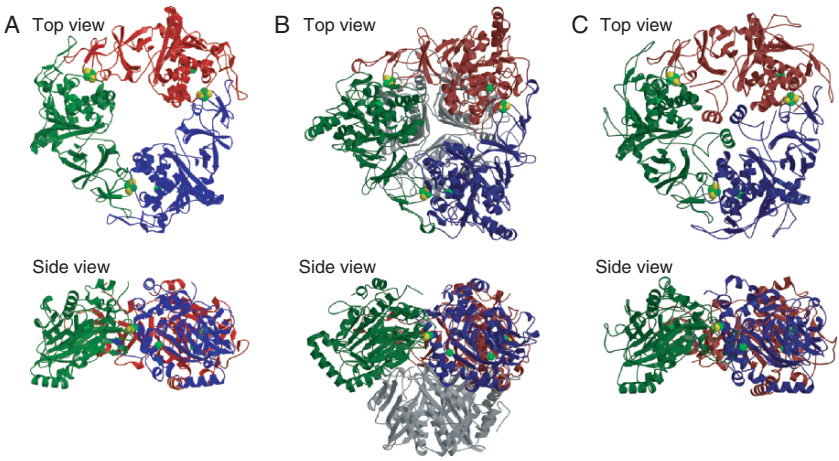

Figure 8. Crystal structures of CARDO-O_{J3} (a),[78] the oxygenase component of NDO (NDO-O) from *Pseudomonas* sp. NCBI9816-4 (b),[58] and the oxygenase component of OMO (OMO-O) from *P. putida* 86 (c).[69]

Rieske domain with a $[2Fe–2S]_R$ and the catalytic domain containing the active-site iron.[24] Recently, the crystal structure of the trimeric oxygenase component of ROS was reported for the terminal oxygenase component of 2-oxoquinoline 8-monooxygenase (OMO-O).[69] As shown in Figure 8c, the overall shape of OMO-O is highly homologous to that of CARDO-O, although OMO-O has an additional small C-terminal trimerization domain consisting of one α helix that forms the contacts in the center of the trimer.[69] Based on the crystal structures of CARDO-O and OMO-O, the doughnut or ring-like structure is likely to be common and typical for terminal oxygenase components in the class IA ROSs having the α_3 configuration. We determined the structural features responsible for the α_3 configuration based on the CARDO-O structure.[79]

Using the CARDO-O crystal, substrate soaking or co-crystallization experiments were performed to clarify the docking manner of the substrate around the active-site Fe^{2+}. However, the trial failed, and the molecular mechanism of angular dioxygenation was only interpreted based on a three-dimensional structure prediction.[79] Recently, we succeeded in determining the carbazole-binding and carbazole/oxygen-binding structures under oxidized and reduced conditions using a CARDO-O:CARDO-F complex crystal (see Section 7.4.3).[4] Upon carbazole binding, amino acid residues Leu202–Thr214 and Asp229–Val238, which were situated near the entrance of the substrate-binding pocket, adopted a different structural conformation (Figure 9a). Both regions moved toward the entrance with shifts of up to approximately 4 Å for the Cα atoms. Especially pronounced were the movements of the side chains

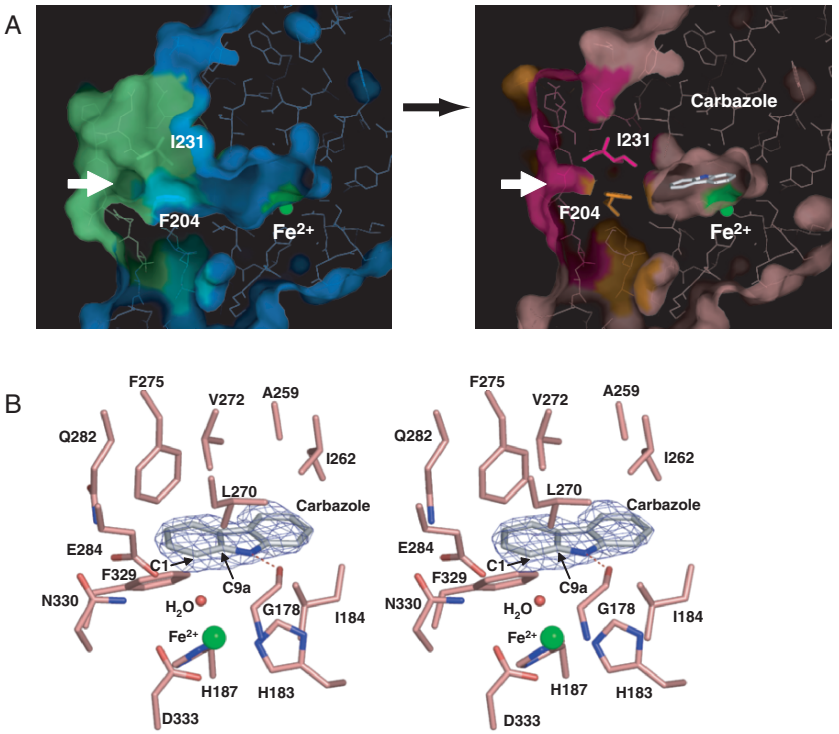

Figure 9. Carbazole binding to the substrate-binding pocket of CARDO-O. (a) Illustrates the closure of the entrance of the substrate-binding pocket (*white arrows*) upon carbazole binding. Conformational changes are obvious for amino acid residues Leu202–Thr214 (shown as cyan [before binding] and orange [after binding]) and Asp229–Val238 (shown as lime [before binding] and magenta [after binding]). Phe204 and Ile231, which had large conformational changes, are shown as stick models. (b) Shows carbazole binding at the substrate-binding pocket under oxidized conditions. The iron ion ligands and amino acid residues that constitute the substrate are shown in stick models and are salmon in color. Carbazole is also shown as a stick model (white for carbon). The iron ions and water ligands are shown as green and small red spheres, respectively. The hydrogen bond interaction between the imino nitrogen of carbazole and the carbonyl oxygen of Gly178 is indicated by a red broken line. The substrate was omitted from the calculation of the difference density map, which is shown in blue and contoured at 3.0 σ.

of Phe204 and Ile231, which were shifted by about 2.8 to 4.2 Å. Such conformational changes resulted in the closure of a lid over the substrate-binding pocket, thereby seemingly trapping carbazole at the substrate-binding site (Figure 9a).

The refinement of the carbazole-bound CARDO-O structure indicated that carbazole is situated above the nonheme iron. The C1 and C9a

carbon atoms were located at a distance of about 4.3 Å from the iron at the active site, and the water or hydroxyl ligand of the nonheme iron was 2.8–2.9 Å from the two carbon atoms, whose binding characteristics were nearly identical to the predicted docking structure (Figure 9b).[79] This binding manner agrees with the fact that CARDO-O catalyzes the angular dioxygenation of carbazole. The wall of the substrate-binding site created a flat and elongated compartment in which carbazole was oriented such that the imino nitrogen of its middle ring was situated within hydrogen-bonding distance of the carbonyl oxygen of Gly178 (approximately 2.9 Å; Figure 9b).

7.4. Electron-Transport Mechanisms in CARDO Systems

7.4.1. Diversity in the Manner of Electron Transport in CARDO

Table 2 summarizes the electron-transport features of the components of CARDOs from *P. resinovorans* CA10, *Janthinobacterium* sp. J3, *Sphingomonas* sp. KA1, and *N. aromaticivorans* IC177.

$CARDO_{CA10}$ and $CARDO_{J3}$ are classified into class III. Two CARDOs of KA1 are classified into class IIA.[121] Class IIA ROS is a three-component oxygenase in which the electron-transfer components comprise a simple flavoprotein and a putidaredoxin-type ferredoxin containing a putidaredoxin-type [2Fe–2S] cluster[19,35] ($[2Fe–2S]_{Pu}$). Almost all of the ROSs are classified into classes IIB or III. Only a few examples have been reported for class IIA ROSs, such as pyrazon dioxygenase from an unidentified bacterium,[103] dioxin dioxygenase from *Sphingomonas wittichii* RW1,[2] and dicamba *O*-demethylase from *Pseudomonas maltophilia* DI-6.[15,45] The conserved amino acid motifs proposed to bind the $[2Fe–2S]_R$ and the mononuclear iron, and the $[2Fe–2S]_R$, and FAD were found in the amino acid sequences of CarAa, CarAc, and CarAd of IC177, respectively. On the basis of these results, $CARDO_{IC177}$ belongs to class IIB.[48]

It is noteworthy that the terminal oxygenase components of the CARDOs from CA10/J3, KA1, and IC177 have substantial homology with each other (>45%), but their electron-transfer components are completely different. Unpublished data (Urata *et al.*) show that electron-transport components (CARDO-F and CARDO-R) can not be replaced between $CARDO_{CA10/J3}$ and $CARDO_{KA1–1}$. It will be interesting to learn how similar terminal oxygenases can accept electrons from specific electron-transfer counterparts.

7.4.2. Crystal Structure of CARDO-F

The crystal structure of the electron-transfer donor of CARDO-$O_{CA10/J3}$, CARDO-F_{CA10},[75] was determined at 1.9-Å resolution by molecular

Table 2. Characteristics of each component of CARDO from CA10, J3, KA1, and IC177.

		CARDO-R		CARDO-F		CARDO-O	
	Batie's class[a]	Protein[b]	Prosthetic group(s)	Protein	Prosthetic group(s)	Protein	Prosthetic group(s)
$CARDO_{CA10}$ $CARDO_{J3}$	III	CarAd (M)[c]	FAD $[2Fe–2S]_P$[d]	CarAc (M)[c, e]	$[2Fe–2S]_R$[d]	CarAa (T)[c, f]	$[2Fe–2S]_R$[d] Fe^{2+}
$CARDO_{KA1–1}$	IIA	FadI, FadII (M)[g]	FAD[h]	CarAcI (M)[g]	$[2Fe–2S]_{Pu}$[d]	CarAaI (T)[g]	$[2Fe–2S]_R$[d] Fe^{2+}
$CARDO_{KA1–2}$	IIA	FadI, FadII (M)[g]	FAD[h]	CarAcII (M)[g]	$[2Fe–2S]_{Pu}$[d]	CarAaII (T)[g, i]	$[2Fe–2S]_R$[d] Fe^{2+}
$CARDO_{IC177}$	IIB	CarAd (M)[g]	FAD[h]	CarAc (M)[g]	$[2Fe–2S]_R$[d]	CarAa (T)[g, j]	$[2Fe–2S]_R$[d] Fe^{2+}

[a] Based on the scheme proposed by Batie *et al.*[6]
[b] The quaternary structure of each component is indicated as: (M), monomer; (T), trimer.
[c] Based on the results of gel-filtration and SDS–PAGE analyses.[76]
[d] Abbreviations are as follows: $[2Fe–2S]_P$, plant-type [2Fe–2S] cluster;[86] $[2Fe–2S]_R$, Rieske-type [2Fe–2S] cluster;[95] $[2Fe–2S]_{Pu}$, putidaredoxin-type [2Fe–2S] cluster.[19,35]
[e] Shown by crystal structure.[75]
[f] Shown by crystal structure.[79]
[g] Based on the results of gel-filtration and SDS–PAGE analyses, although the detailed data have not yet been reported.
[h] Proposed from the amino acid sequence similarity.
[i] Shown by crystal structure (Katsuki *et al.* unpublished result).
[j] Shown by crystal structure.[46]

replacement using the structure of the ferredoxin component (BDO-F; a monomer of the BphF protein) of biphenyl 2,3-dioxygenase (BDO) from *B. cepacia* LB400[18] as a search model. The CARDO-F molecule has a wedge shape, with approximate dimensions of 25 × 30 × 45 Å (Figure 10a). The molecular structure of CARDO-F, which is composed of three antiparallel β-sheets, is also common to other proteins having the $[2Fe–2S]_R$, such as cytochrome bc_1,[52] b_6f,[11] and BDO-F; the structure can be divided into two domains: a cluster-binding and a basal domain. The $[2Fe–2S]_R$ is located at the tip of the cluster-binding domain, where it is exposed to solvent.

The overall length-wise sequence identity between CarAc (107 residues) and BphF (109 residues) was 34%, and the structures could be superimposed with a root mean square deviation (rmsd) of the Cα position of 1.75 Å (Figure 10a). Despite the similarity between the two ferredoxins, their electron-transfer counterparts, ferredoxin reductases, and terminal oxygenases have distinctly different features. The BDO of LB400 is a class IIB oxygenase, and BDO-F receives electrons from the reductase protein BphG, which is a simple flavoprotein lacking an iron–sulfur cluster.[9,41] In BDO, the electrons are transferred from BDO-F to an $\alpha_3\beta_3$ class II oxygenase component consisting of BphA (large subunit) and BphE

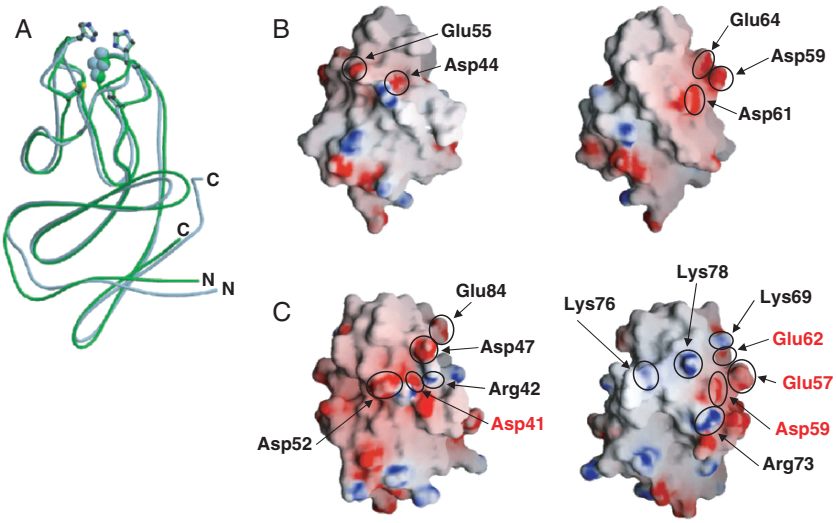

Figure 10. Comparison of (a) the Cα structure, and the surface electrostatic potentials and overall shapes of (b) CARDO-F from *P. resinovorans* strain CA10 and (c) BDO-F from *B. cepacia* strain LB400. In (a), the Cα structure of CARDO-F (green) is superposed with BDO-F (blue). In (b, c), the right view shows the opposite side of the left view. Red indicates negatively charged residues; blue indicates positively charged residues; highlighted ovals indicate the positions of charged residues in the cluster-binding domain of each ferredoxin.

(small subunit).[40] In fact, the molecular surfaces and surface electric potentials of both proteins are very different (Figure 10b, c), which may account for the selectivity of the electron-transport counterpart.

7.4.3. Structure of the Electron-Transfer Complex of CARDO-O: CARDO-F in Class III CARDO

Diverse types of electron transport between proteins have been reported, but few studies have examined the electron-transport mechanisms between freely occurring proteins with respect to their three-dimensional structures. As for the structures of ROS components, the number of reports on the structure of single components is increasing, as reviewed by Ferraro *et al.*,[24] yet there have been no structure-based interpretations of the interactions among components. Very recently, Ashikawa *et al.* succeeded in preparing the crystal of the electron-transfer complex between CARDO-O_{J3} and CARDO-F_{CA10},[3] and in determining its crystal structure[4] (Figure 11a). Three molecules of CARDO-F bind to the subunit boundary of one CARDO-O molecule. The superposition of three molecules of CARDO-F binding to one CARDO-O molecule resulted in a rmsd of 0.73 Å for the cluster-binding domains (45 Cα atoms) and 1.87 Å for the basal domains (59 Cα atoms; Figure 11b). This suggests that the binding of the cluster-binding domain to the CARDO-O subunit boundary is almost identical among the three CARDO-F molecules, although the basal domains of the CARDO-F molecules deviated slightly. Interactions occurred between four regions (residues 11–15, 115–119, 210, and 350–363) of CARDO-O and the cluster-binding domain of CARDO-F. This surface in the binary complex structure consisted of 34 residues (19 of CARDO-O and 15 of CARDO-F), and the accessible area buried in the interface was approximately 1,800 $Å^2$ for each pairing, which covers 12 and 39% of the total surface area of CARDO-O and CARDO-F, respectively. The superposition of the structure in binary complex and in single state crystal reveals that various conformational changes of both CARDO-O and CARDO-F mainly occur at the component boundary. For example, two clear movements were found at Lys12–Trp15 and Asp347–Asn352 in CARDO-O. In addition, two notable shifts in the loop structures of CARDO-F consisting of Pro66–Gly70 and His48–Ala51, which included two respective histidine ligands of the $[2Fe–2S]_R$, were observed. Most of these conformational changes allow the formation of specific hydrophobic interactions, hydrogen bonding, and electrostatic interactions, which may contribute to the stabilization of the complex.

Based on the binary complex structure, we can assume two electron-transfer pathways, partly mediated by hydrogen bonds with water molecules, between the two $[2Fe–2S]_R$ (Figure 11c). After an electron is

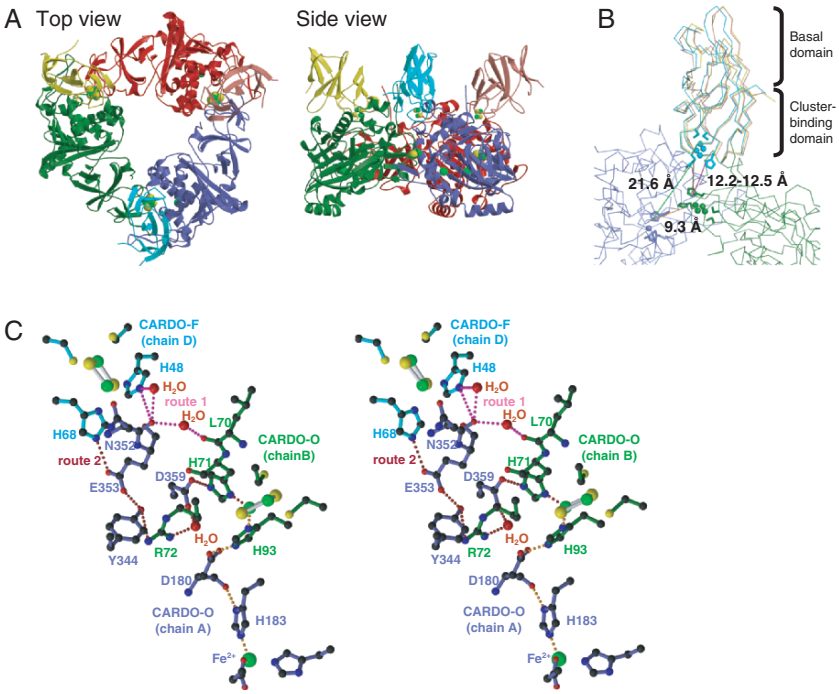

Figure 11. (a) Overall structures of the binary complex of CARDO-O_{J3} with CARDO-F_{CA10}, (b) superposed view of the three CARDO-F_{CA10} molecules, and (c) hypothetical electron-transport routes between CARDO-F_{CA10} and CARDO-O_{J3}. In (a), the three CARDO-O subunits (chain A, slate blue; chain B, forest green; and chain C, red) and the three CARDO-F molecules (chain D, cyan; chain E, yellow; and chain F, salmon) are shown. The $[2Fe–2S]_R$ and the nonheme iron are shown as spheres (iron ions, green; sulfide ions, yellow). In (b), the distances between the electron-transfer centers are shown. In (c), the active-site iron and its coordinated residues of CARDO-O chain A, the $[2Fe–2S]_R$ and the coordinated residues of CARDO-O chain B and CARDO-F, and the amino acid residues and water molecules possibly involved in electron transfer in the complex are shown. Each chain is colored as in (a). The magenta and brown dotted lines indicate the two hypothetical electron-transfer pathways (routes 1 and 2) between the two $[2Fe–2S]_R$. The possible electron-transfer pathway from the $[2Fe–2S]_R$ to the active-site iron in CARDO-O is shown as an orange dotted line.

transferred to the CARDO-O $[2Fe–2S]_R$, it is further transported to the nonheme iron. The distances between the two $[2Fe–2S]_R$ and between $[2Fe–2S]_R$ and the nonheme iron were approximately 12.2–12.5 and 9.3 Å, respectively (Figure 11b), well within the 14-Å threshold.[89] Electron transfer in the CARDO-O molecule was proposed to occur from the $[2Fe–2S]_R$ via Asp180 to the nonheme iron of the neighboring subunit (Figure 11c).[79] A similar electron-transfer pathway in the terminal oxygenase component mediated by Asp residue has been proposed for NDO-O[55] and OMO-O.[68]

8. FUTURE PROSPECTS

In last decade, carbazole metabolism has been investigated extensively. Studies have focused on the novel genetic structures of degradative gene clusters and their diversity, and the molecular mechanisms of the novel substrate specificity of degrading enzymes, especially in CARDO. Screening of additional carbazole-degrading bacteria has revealed the molecular diversity of CARDOs, and has provided good tools for enzymatic analysis, such as X-ray crystallography, to analyze the mechanisms underlying the novel substrate specificity and electron transport.

In terms of the function of CARDO, the manner of substrate recognition during other oxygenation reactions (for example, lateral dioxygenation, monooxygenation, sulfoxidation) needs to be clarified for better understanding the function of CARDO-O. The mechanism of substrate recognition is proposed to be common (or at least homologous) in various ROSs, and the investigation of the function of CARDO will provide useful information for understanding the general mechanism governing the ROS reaction selectivity for aromatic compounds. The structural interpretation of the electron-transfer function between ferredoxin and oxygenase components in CARDO is a breakthrough in ROS research. If the structural analyses of electron-transfer complexes of CARDOs in other Batie's classes are successful, they may provide clues to clarify the functional alternation mechanism that has developed during the diversification of CARDO. Such alternation of the CARDO system should be related to the fact that the CARDO-encoding *car* gene cluster is often located on mobile genetic element(s).

Another future research target related to carbazole metabolism is the investigation of the plasmid function in nature. Together with the 2,4-dichlorophenoxyacetic acid degradative pJP4[116] (IncP-1) and toluene degradative pWW0[32] (IncP-9), pCAR1 has become one of the most well-investigated IncP degradative plasmid. It is important to clarify the detailed plasmid function in the natural environment, such as in soil and water. Because pCAR1 is a self-transmissible plasmid, the alternation of plasmid function under various host-cell backgrounds should be investigated.

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6

THE CATABOLISM OF PHENYLACETIC ACID AND OTHER RELATED MOLECULES IN *PSEUDOMONAS PUTIDA* U

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1. INTRODUCTION

One of the most important causes contributing to the modification of living conditions is the accumulation of many chemical compounds in broad areas of the planet. Among the thousands of new contaminants released to the environment every year, the appearance in many habitats (aquatic, aerial

Although in previous publications some of us have referred to PhAc catabolic genes as *pha*, in order to avoid mistakes in nomenclature and confusing interpretations between these genes and those involved in the polyhydroxyalkanoate biosynthesis and catabolism (*pha* locus), we strongly suggest the use of the prefixes *paa* and Paa to indicate the genes or proteins involved in the aerobic catabolism of PhAc (Luengo *et al. Mol. Microbiol*. 39:1434–1442, 2001).

Juan-Luis Ramos and Alain Filloux (eds.), Pseudomonas, 147–192.

or terrestrial) of quite stable molecules containing aromatic rings in their chemical structures has been observed with increasing frequency. This is the case of certain industrial products such as phenol, toluene, biphenyl, naphthalene, benzene, salicylate, methylbenzene, methoxybenzoic acid, ethylbenzene, phenylacetate, 4-hydroxyphenylacetate, chlorophenoxyacetate, 3-phenyl-2-propenoic acid, styrene, aromatic hydrocarbons and many halogenated compounds, all of which are common constituents of the organic pollutants present in the effluents of factories devoted to the synthesis or transformation of different synthetic products.[21,27,65,70,88,94,112,120] Additionally, other contaminants, which usually appear as pollutants in municipal effluents, has emerged. This group includes steroids and other organic compounds released as a consequence of the pharmacological treatment of a large number of people.[37,64,89]

Furthermore, many of these compounds (or their derivatives) have potent metabolic activities that affect a large number of essential cellular processes, such as cell proliferation, control of the cell cycle, basic mechanisms of intercellular recognition, etc. Thus, their accumulation cannot only cause dangerous effects to human health but may also alter the biological cycles of many different species, affecting the structure and evolution of ecosystems.[37]

While some of these pollutants only require short periods of time to be eliminated, those with stable molecular structures (synthetic organohalogens and other recalcitrant xenobiotics) are not eliminated even after decades.[21,27,88,94,112,120] In fact, however, only a few compounds cannot be eliminated since the microbial world are able to transform or to eliminate most of the chemical compounds generated as a consequence of industrial activities.[16,39,85-86,121,124,127-128]

The broad metabolic versatility of microorganisms, in particular that of bacteria, has led many scientists to study the microbial processes that mineralize such organic compounds. Currently, a considerable body of knowledge about the catabolic pathways involved in the degradation of different molecules has been accrued.[16,21,23,36,56,66,86,94,112,120] All this information is being collected to elaborate authentic catabolic maps where non-conventional enzymatic processes, subject to peculiar regulatory mechanisms, of great academic, environmental and industrial interest are combined with other well-known biochemical routes.[8,23,56,66,81,106]

Thus, among all the bacteria those belonging to the genus *Pseudomonas* are able to mineralize a large number of organic compounds that are toxic or may be dangerous for animals, plants and other microbes.[21,70,112,120] The high catabolic potential of these bacteria seems to have been acquired because along evolution these microbes have been able to organize new catabolic pathways using genetic information collected from different origins.[20] These new acquisitions (which usually represents

certain nutritional or ecological advantages) contribute to the polarization of bacterial catabolism and, hence, to a high metabolic specialization of the recipient microorganism.[3,58,65–66,95]

Most of these special catabolic routes are encoded by genes that usually have quite different organization and enzymes that exhibit different subcellular locations. Thus, whereas in some strains they appear linked in well-organized clusters, in other cases the genes are placed, in an isolated fashion, at different sites in the bacterial chromosome.[47,66] Furthermore, whereas in a given strain a specific route may be encoded by plasmid genes, in others it (or they) may appear in the chromosome or may be partially distributed between plasmids and chromosomes. Although the genetic evolution of such pathways is essentially obscure, it is thought that these catabolic units have not evolved from an ancestral route that diverged, but that they arose as independent events characterizing a particular strain of a bacterial species. The molecular bases of the mechanisms that induce the catabolic specialization of certain species along evolution, and hence their study, is now one of the most exciting challenges.[86,88,94,112,121]

In this chapter we shall describe how *Pseudomonas putida* (CECT 4848, a non-pathogenic member of rRNA group I of the genus *Pseudomonas*), which is a paradigmatic microbe in terms of biodegradation, is able to aerobically assimilate phenylacetic acid, a model aromatic molecule that is formed in nature from different natural and synthetic compounds. Additionally, we shall describe how this bacterium has managed to incorporate several independent degradative pathways that form a complex functional unit, defined by us as the phenylacetyl-CoA catabolon, which allows the degradation of several aromatic compounds (2-phenylethylamine, 2-phenylethanol, phenylacetaldehyde, ethylbenzene, tropic acid, phenylacetaldoxime, phenylacetamide and other esters of phenylacetic acid, *trans*-styrylacetic acid, *n*-phenylalkanoic acids and poly-3-hydroxyphenylalkanoates with an even number of carbon atoms) structurally related to phenylacetic acid.[41,65–67,70,81]

2. THE DEGRADATION OF AROMATIC COMPOUNDS

Early observations of the basic enzymatic mechanisms used by microbes, plants and animals to attack aromatic molecules revealed that most of them involved the participation of mono- and dioxygenases (i.e. cytochrome P450 monooxygenases, 4-hydroxyphenylacetate hydroxylase, homogentisate synthase),[8,88,97] suggesting that these catalytic processes require the direct participation of oxygen. Moreover, the fact that the aromatic ring can only be opened by dioxygenases (through *ortho* or *meta*

cleavage), strongly reinforced this assumption and led many scientists to suspect that the degradation of aromatic compounds would be restricted to aerobic organisms.[17,35,44,46–48,107] However, the elimination of aromatics from natural habitats, even under anoxic conditions, revealed that anaerobic microorganisms were also able to assimilate these compounds.[25-26,51,103] Currently, it is generally accepted that low-molecular weight aromatic compounds such as toluene, chlorophenol, biphenyl and polycyclic aromatic hydrocarbons can be mineralized either aerobically (bacteria, fungi and algae) or anaerobically (certain bacteria) through quite different catabolic pathways.[6,17,44,49,105,107,129]

A common feature that characterizes all these routes is their particular organization. Most of them are integrated by different peripheral pathways that converge in a central route.[3,23,47,51,56,58,66,70,81,95] The peripheral routes are involved in the transformation of many compounds (usually very structurally related molecules) into a small number of intermediates that are later transformed through the central route into general metabolites (Figure 1a and b). The enzymes belonging to the peripheral pathways seem to be quite specific and are only induced by one (or few) substrate. By contrast, the enzymes belonging to the central route can be induced by the convergent products and can catalyze the transformation of these compounds into non-aromatic intermediates.[17,41,47,65–67,103]

Generally, the mechanism of degradation of the aromatic compounds in aerobic conditions (Figure 1a) involves the introduction of one or two hydroxyl groups into the aromatic ring, thus facilitating ring opening by dioxygenases and their transformation into Krebs cycle intermediates through different enzymatic reactions.[17,23,41,44,56,66–67,107] However, taking into account that oxygenases do not operate in the absence of oxygen, anaerobic organisms have resorted to a different mechanism of dearomatization that usually involves a reductive attack of the aromatic rings as well as the synthesis of CoA thioesters, carboxylation, dehydration and other additional chemical reactions (Figure 1b) that have never been observed in aerobic catabolism.[6,24–26,49,51,53,63,92-93,103,105] This is why the aerobic and the anaerobic degradation of the same compound requires the participation of different enzymes and the generation of unrelated catabolic intermediates.[2,6,17-18,23,26,44,49,51,56,103,105,107]

3. THE AEROBIC CATABOLISM OF PHENYLACETIC ACID: FIRST APPROACHES

Initial studies about the aerobic catabolism of phenylacetic acid (PhAc) in microorganisms revealed that the assimilation of this compound requires, in the first steps, the consecutive incorporation of two hydroxyl groups into the aromatic ring, as had been reported for other similar com-

pounds.[7,14,15,19,100,115,125] Depending on the aromatic position first hydroxylated (C2, C3 or C4), three different mechanisms of ring activation have been proposed.

The first one involves the hydroxylation of PhAc to 3-hydroxyphenylacetic acid (3-OH-PhAc) which is then transformed into homogentisic acid (2,5-OH-PhAc) through a reaction catalyzed by an unknown homogentisate synthase, which has been recently identified in *P. putida* U.[8-9] Finally, 2,5-OH-PhAc is converted, through the homogentisate pathway,[7,9,115] into fumarate and acetoacetate (Figure 2). Bacteria belonging to the genus *Flavobacterium*[7] as well the yeast *Trichosporum cutaneum*[115] use this pathway to assimilate PhAc.

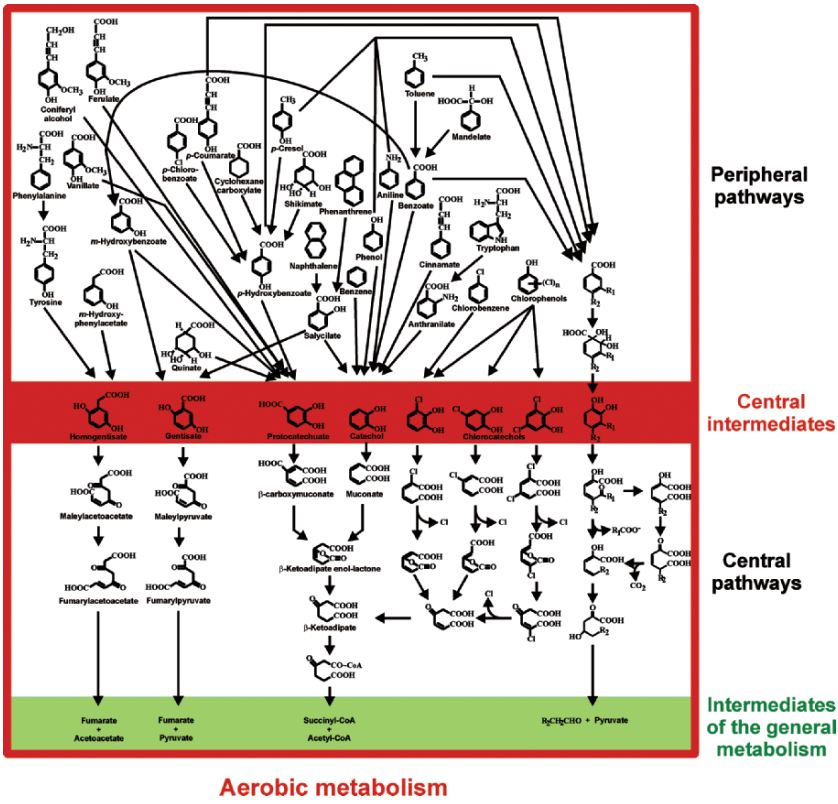

Figure 1. Schematic representation of the main catabolic steps involved in the aerobic (a) and in the anaerobic (b) degradation of some aromatic compounds.

(*Continued*)

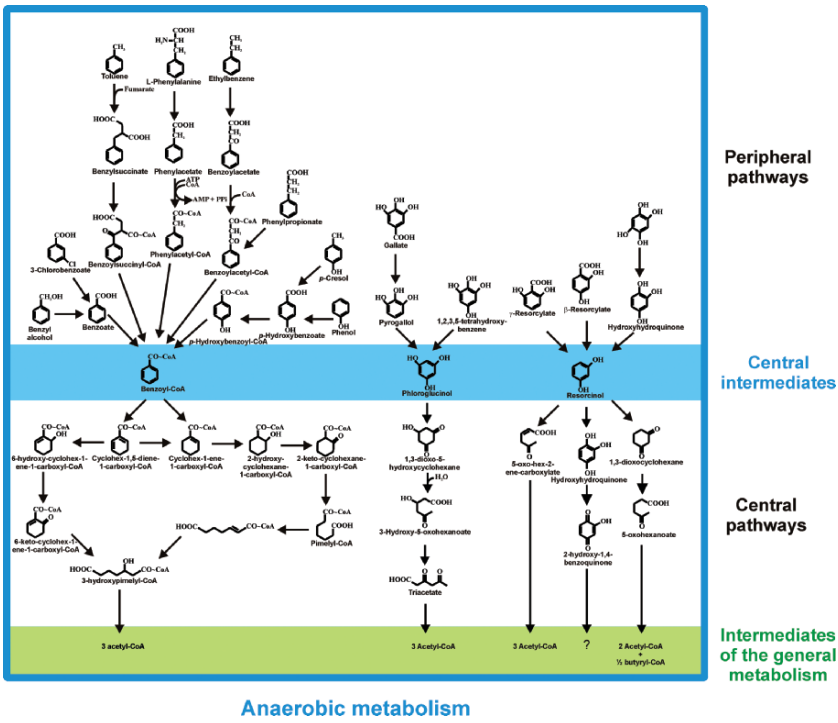

Figure 1. cont'd.

In other microbes, such as *Nocardia salmonicolor*, *Aspergillus niger* and *Penicillium chrysogenum*, the degradation of phenylacetic acid involves its hydroxylation in *ortho* producing 2-OH-PhAc.[100,110] This reaction is performed by a phenylacetate 2-hydroxylase, an enzyme similar to cytochrome P450 monooxygenases.[97] Later, 2-hydroxy-phenylacetic acid is transformed into homogentisic acid. Although this second hydroxylase has not yet been purified, it could be similar to the homogentisate synthase indicated above.[8,64]

The third mechanism would start with a putative reaction catalyzed by a hypothetical phenylacetate hydroxylase that should transform PhAc into 4-OH-PhAc. Later this compound would be hydroxylated to 3,4-OH-PhAc (homoprotocatechuic acid) which should be degraded through a specific catabolic pathway (Figure 2). Although it was assumed that this mechanism would be used by all the bacteria able to catabolise 4-OH-phenylacetic acid (4-OH-PhAc) (*P. putida, Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter* and other unrelated microbes),[7,10,82,100,110,115,125] there was no evidence supporting the hydroxylation of PhAc to 4-OH-PhAc.

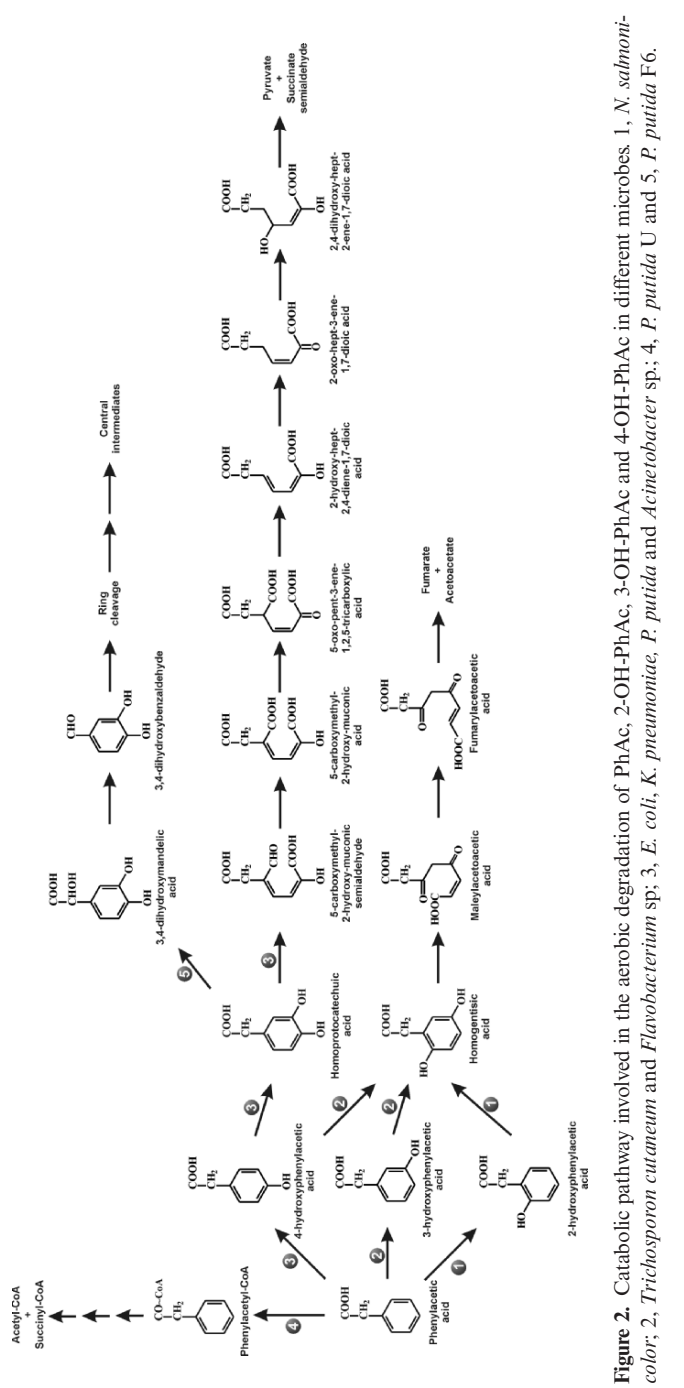

Figure 2. Catabolic pathway involved in the aerobic degradation of PhAc, 2-OH-PhAc, 3-OH-PhAc and 4-OH-PhAc in different microbes. 1, *N. salmonicolor*; 2, *Trichosporon cutaneum* and *Flavobacterium* sp; 3, *E. coli*, *K. pneumoniae*, *P. putida* and *Acinetobacter* sp.; 4, *P. putida* U and 5, *P. putida* F6.

To analyse this latter mechanism, we studied the catabolic pathway involved in the degradation of PhAc and 4-OHPhAc in *P. putida* U. Two independent observations allowed us to conclude that both catabolic pathways should be different.

First, we noticed that when *P. putida* U was cultured in a chemically defined medium containing PhAc as the sole carbon source, an unusual enzyme showing phenylacetyl-CoA ligase activity was induced.[70,81] However, when it was cultured in the same medium, but containing either 4-OH-PhAc or 3-OH-PhAc as the sole carbon source, this enzyme was not found in cell-free extracts.[82]

Second, mutagenesis with the transposon Tn*5* allowed the isolation of different mutants of *P. putida* U that, unlike the parental strain, were unable to grow in chemically defined medium containing phenylacetic acid, 4-OH-phenylacetic acid or 3-OH-phenylacetic acid (3-OH-PhAc) as the carbon source. It was observed that: (i) the mutants unable to catabolise PhAc grew well in media containing either 3-OH-PhAc or 4-OH-PhAc; (ii) the mutants affected in the catabolism of 4-OH-PhAc could assimilate PhAc and 3-OH-PhAc efficiently; and (iii) all the mutants unable to degrade 3-OH-PhAc grew well in minimal media containing either PhAc or 4-OH-PhAc as the carbon source. All these data revealed that the degradation of PhAc, 3-OH-PhAc and 4-OH-PhAc in *P. putida* U is not carried out, as formerly believed, through convergent linear pathways but by three independent routes specifically involved in the assimilation of these aromatic compounds (Figure 2).

It could be argued that *P. putida* U might catalyze the degradation of PhAc through 2-OH-PhAc, as had been seen in *N. salmonicolor*, *A. niger* and *P. chrysogenum*.[7,10,97,100,110,115,125] However, *P. putida* U was unable to grow in chemically defined media containing 2-OH-PhAc as the sole carbon source.[82] Furthermore, when this bacterium was cultured in the same media containing an additional carbon source (glucose, succinic acid, glutamic acid or octanoic acid), 2-OH-PhAc remained unaltered in the broths, suggesting that *P. putida* U was unable to catabolize this compound and therefore that PhAc catabolism cannot be performed through a catabolic pathway that involves the formation of 2-OH-PhAc as an intermediate.[65,67,70,81]

4. A PHENYLACETYL-CoA LIGASE IS DIRECTLY INVOLVED IN THE AEROBIC CATABOLISM OF PhAc

Although the involvement of CoA thioesters as catabolic intermediates in degradative pathways seems to be a common characteristic in the anaerobic metabolism of aromatic compounds,[51,118] some evidences were

obtained in support of the true participation of this enzyme in the aerobic catabolism of PhAc.

First, analysis of the cell-free extracts of *P. putida* U grown in chemically defined media containing phenylacetate as the sole carbon source revealed the existence of an unusual phenylacetyl-CoA ligase (AMP-forming) that seemed to be involved in the aerobic catabolism of PhAc.[70,96,106] This enzyme was only found in cell-free extracts obtained from bacteria cultured in the presence of PhAc, whereas when 3-OH-PhAc, 4-OH-PhAc, 3,4-OH-PhAc or 2,5-OH-PhAc were supplied to the cultures this enzymatic activity was never detected, suggesting that PhAc-CoA ligase (PCL) is specifically induced by PhAc.[70,81,82,96,104] Although this single observation did not in itself imply the direct participation of this enzyme in the aerobic catabolism of PhAc (it could either be induced gratuitously or it could play a different physiological role, PhAc-CoA ligase activity being a secondary function of this enzyme), other additional studies reinforced its involvement.[117]

Second, biochemical analysis of the different mutants unable to catabolize PhAc, revealed that in half of them PhAc-CoA ligase activity was not detected in the cell-free extracts, suggesting that the mutation affecting the expression of its encoding gene hindered these strains from degrading PhAc.[70,81] All these observations reinforced the hypothesis that PhAc-CoA ligase would be an essential enzyme for the aerobic catabolism of PhAc in *P. putida* U.[70]

Additional studies performed in other Pseudomonas species,[1,31,119,122] as well as in *Escherichia coli* W,[23,33] *Azoarcus evansii*,[73,75] *Bacillus halodurans*,[111] *Acinetobacter*, *Providencia rettgeri*,[119] *Klebsiella oxytoca*, *Alcaligenes* sp., *Ralstonia* sp., *Burkholderia* sp., *Paenibacillus chivensis*, *Arthrobacter atrocyaneus*, *Pandoraea* sp., *Flavobacterium breve* and *Stenotrophomonas maltophilia*[1] revealed that phenylacetyl-CoA ligase is an enzyme directly involved in the degradation of PhAc (see below) and that its encoding gene is subject to lateral gene transfer within and across the bacterial phylum, thus accounting for its widespread distribution (β- and γ-Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes) in nature.[1]

Although it has been reported that some aromatic compounds (benzoate, 2-aminobenzoate and 4-cholorobenzoate) are degraded through catabolic pathways that have thioesters of CoA as intermediates,[4-5,61,73] the participation of these intermediates in the aerobic assimilation of aromatics is a rare event. The existence of a single aerobic pathway with a typical biochemical characteristic of anaerobic routes (the CoA esterification)[22,33,66,81] has led the PhAc catabolic pathway to be considered as a new type of mixed pathway.

5. GENETIC AND BIOCHEMICAL DESCRIPTION OF THE PhAc CATABOLIC PATHWAY

5.1. Genetic Organization

The genes encoding the PhAc catabolic pathway in *P. putida* U are located in an 18-kb DNA fragment containing five consecutive operons (*paaABCEF*, *paaGHIJK*, *paaLMN*, *paaY* and *paaX*) that include all the information required for the aerobic degradation of this compound (see Figure 3 and Table 1). Restriction and sequence analyses revealed that these genes encode 13 catabolic enzymes (PaaABCEFGHIJKLMN), a regulatory protein (PaaX) and a different protein (PaaY) whose particular function, although it seems to be required for the efficient catabolism of PhAc, has not yet been clarified.[66,81]

Further analyses revealed that the genes encoding the PhAc catabolic pathway are not rare and restricted to a particular strain of *Pseudomonas* (*P. putida* U) but that they are widely distributed in nature, being present in many unrelated microbes.[56,65,67,122] However, although most of the genes usually show high sequence similarity, their genetic organization (number of clusters, size of the cluster and chromosomal location) seems to be variable, such that it could be considered to be strain-specific (Figure 4). Thus, whereas in most *Pseudomonas* species studied (*P. putida* U, *P. putida* KT2440, *P. putida* CA-3) the genetic organization of the *paa* locus follows the pattern described above (see Figure 4), in some strains (i.e. *Pseudomonas* sp. Y2) duplication of the *paa* cluster has been observed.[13] The close location in *Pseudomonas* sp. Y2 of the genes belonging to *paa1* and to the *sty* clusters, encoding the activities needed for the complete assimilation of styrene, could be explained by assuming that both genetic units have had a common evolutionary history.[13,66,116] Furthermore, preliminary results have revealed the existence of a crossed regulation pattern between *paa* and *sty* genes,[13] suggesting a functional cooperation between both pathways. The high toxicity of the styrene derivatives could be one of the reasons why these genetic units could have been forced to have evolved together.

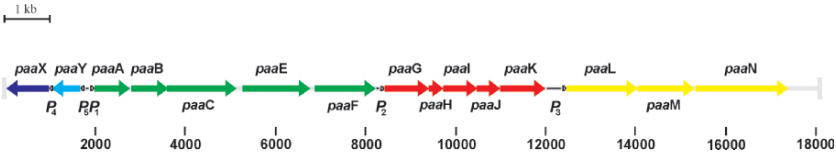

Figure 3. Genetic organization of the phenylacetate catabolic pathway in *P. putida* U.

The *paa* cluster in the model bacterium *E. coli* and in *K. pneumoniae*, contains 14 genes grouped in 3 transcription units: *paaABCDEFGHIJK*, *paaZ* and *paaXY* (later indicated by consensus as *paaGHIJKABCDE*, *paaN* and *paaXY*) (see Table 1). In *E. coli* W, the first enterobacteria in which this genetic organization was reported, the *paa* locus is located downstream from a fragment of DNA containing the *mao* region, which encodes the enzymes necessary to transform 2-phenylethylamine into phenylacetic acid.[33,34] This location suggests a possible physiological cooperation between both pathways, providing new insight for understanding the global catabolism of certain aromatic compounds structurally related to PhAc in bacteria.

In sum, analyses of the sequenced bacterial genomes has revealed the existence of putative gene clusters involved in the degradation of PhAc of many bacterial species,[1] reinforcing our initial hypothesis[70] and suggesting that bacterial strains able to metabolize PhAc through the PhAc-CoA catabolic pathway are widely distributed throughout the eubacteria. Furthermore, the variations observed in the gene arrangement of the *paa* locus in all bacteria analysed (see Figure 4) suggest that the gene transfer processes involved in the acquisition of the genes encoding the PhAc catabolic pathway have been different enough to invoke, in each case, a particular and interesting evolutionary history.

5.2. The PhAc Catabolic Pathway: Biochemical Characterization

As indicated above, the *paa* cluster includes all the genes required for the aerobic catabolism of PhAc in the model bacterium *P. putida* U. This degradative pathway (Figure 5) arose as a result of the biochemical cooperation established between five different functional units, each composed by one or more Paa proteins. Thus, in a first step, PhAc is taken up from the medium by a transport system that involves the participation of a specific channel-forming protein (porin, PaaM) and a permease (PaaL). These two components, which are essential for the transport of phenylacetic acid in this bacterium, seem to be quite specific and they are absent in the *paa* clusters of most of bacteria analysed to date (Figure 4). Later, a phenylacetyl-CoA ligase (PaaF) activates phenylacetic acid to phenylacetyl-CoA (PhAc-CoA) through a reaction that involves the participation of ATP, CoA and Mg^{2+} (see Figure 5). In a following step, a complex system (PaaGHIJK) catalyzes the hydroxylation of the aromatic ring of PhAc-CoA. This compound is now the substrate of an aromatic ring-opening protein (PaaN) that generates an acyclic-CoA intermediate which, finally, is transformed into a tricarboxylic acid (TCA) intermediate (succinyl-CoA)

Table 1. PhAc catabolic pathways in different bacteria: correspondence

Function\Strain	*P. putida* U/KT2440	*Burkholderia pseudomallei* K96243	*Azoarcus evansii* KB740	*R. palustris* CGA009	*E. coli* W/K12	*Klebsiella* PAMU-1.2	*B. pertusis* Tohamal
Phenylacetyl-CoA ligase (*paaF*)	*phaE*	*paaK*	*PacD*	*paaK*	*paaK*	*paaK*	*paaK*
Ring Oxidation Complex. Protein 1 (*paaG*)	*phaF*	*paaA*	*PacE*	*paaA*	*paaA*	*paaA*	*paaA*
Ring Oxidation Complex. Protein 2 (*paaH*)	*phaO*/ *PP3277*	*paaB*	*PacF*	*paaB*	*paaB*	*paaB*	*paaB*
Ring Oxidation Complex. Protein 3 (*paaI*)	*phaG*/ *pp3276*	*paaC*	*PacG*	*paaC*	*paaC*	*paaC*	*paaC*
Ring Oxidation Complex. Protein 4 (*paaJ*)	*phaH*	*paaD*	*PacH*	*paaD*	*paaD*	*paaD*	*paaD*
Ring Oxidation Complex. Protein 5 (*paaK*)	*phaI*	*paaE*	*PacI*	*RPA3764*	*paaE*	*paaE*	*paaE*
Ring Opening enzyme (*paaN*)	*phaL*	*paaZ*	*PacL*	*RPA1725*	*paaZ*	*paaZ*	*BP2678*
Enoyl-CoA hydratase 1 (*paaA*)	*phaA*/ *PP3284*	*paaF*			*paaF*	*paaF*	
Enoyl-CoA hydratase 2 (*paaB*)	*phaB*	*paaG*	*PacA*		*paaG*	*paaG*	*paaG*
3-OH-Acyl-CoA dehydrogenase (*paaC*)	*phaC*/ *paaC*		*PacB*		*paaH*	*paaH*	
Unknown function protein (*paaD*)		*paaI*	*PacC*	*paaI*	*paaI*	*paaI*	*paaI*
Ketothiolase (*paaE*)	*phaD*	*paaJ*			*paaJ*	*paaJ*	
Permease (*paaL*)	*phaJ*/*actP*						
Porin (*paaM*)	*phaK*						
Repressor protein (*paaX*)	*phaN*				*paaX*	*paaX*	*paaX*
Putative regulator (*paaY*)	*phaM*		*PacM*		*pay*	*paaY*	
Source[a]	AF0 29714	NC_ 006350	AJ27 8756	NC_ 005296	X97452/ U00096	AB1 82627	NC_ 002929

[a]Accesion numbers in the GenBank at NCBI.

between original gene denomination and protein function.

B. bronchiseptica RB50	*B. halodurans* C-125	*D. radiodurans* R-1	*S. meliloti* 1021, pSymB	*Rhodococcus* RHA1	*C. efficiens* YS-314	*S. coelicolor* A3(2)	*S. avermitilis* MA-4680
paaK	*phaE*	*DR_A0256*		*paaF*	*CE0663*	*SCO7469*	*paaK*
paaA	*phaF*	*paaA*	*paaA*	*paaG*	*CE0667*	*SCO7471*	*paaA*
paaB	*phaO*	*paaB*	*paaB*	*paaH*	*CE0668*	*SCO7472*	*paaB*
paaC	*phaG*	*paaC*	*paaC*	*paaI*	*CE0669*	*SCO7473*	*paaC*
paaD	*phaH*	*paaD*	*paaD*	*paaJ*	*CE0670*	*SCO7474*	*paaD*
paaE			*paaE*	*paaK*	*CE0671*	*SCO7475*	*paaE*
BB3430	*phaL*	*paaZ*	*paaZ*	*paaN*	*CE0677*	*SCO7139*	*paaZ*
	phaA			*paaA*	*CE0672*	*SCO5459*	
paaG	*phaB*		*paaG*	*paaB*	*CE0674*	*SCO5144*	
	phaC		*SMb21632*	*paaC*	*CE0675*		*paaH*
paaI	*phaI*		*SMb21634*		*CE0666* *CE06761*	*SCO7470*	*paaI*
	phaD			*paaE*	*CE0673*	*SCO6967*	*pcaF1*
paaX	*phaN*		*paaX*				
	phaM						
NC_ 002927	BA 000004	AE 000513 AE001825	NC_ 003078		NC_ 004369	NC_ 003888	NC_ 003155

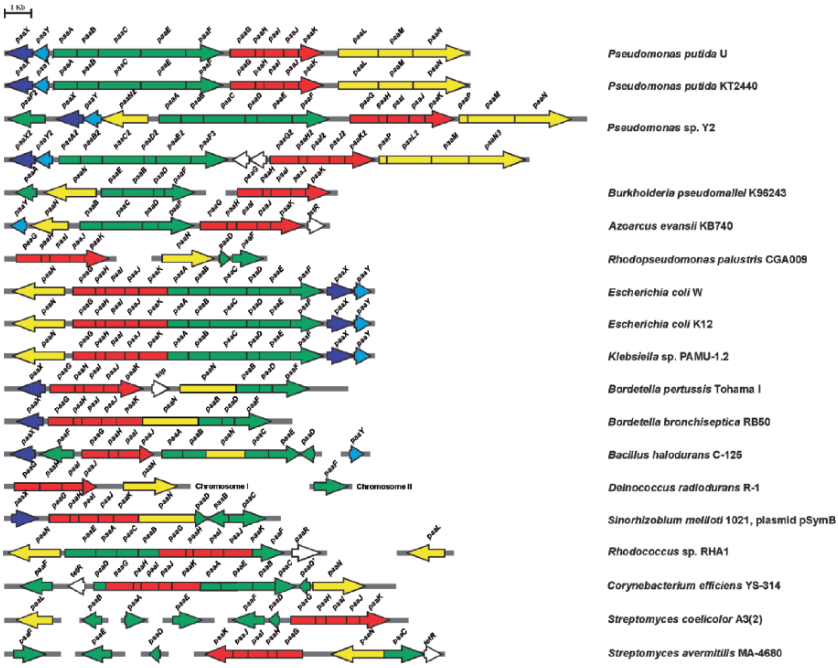

Figure 4. Organization of the gene clusters encoding the phenylacetate catabolic pathway in different microorganisms.

by the β-oxidation-like system PaaABCE (see Figure 5).[65–67,78,81] In sum, four groups of enzymes (PaaML, PaaF, PaaGHIJK and PaaABCE) have catabolic functions while another two (PaaX and PaaY) and several promoters (*P1* to *P5*) have regulatory functions.[66,81]

5.2.1. Functional Catabolic Units

Transport system The phenylacetic acid transport system in *P. putida* is an active mechanism that depends on an energy-rich membrane state that does not use ATP or a phosphorylated compound as a driving force.[104] It consists of a porin (Paa M) and a permease (PaaL), which are integrated in a single functional unit (Figure 5) specifically involved in the uptake of PhAc. Both enzymes are encoded by genes belonging to the third operon (Figure 3). Mutations in *paaL* or in *paaM* prevent these mutants from growing in chemically defined media containing PhAc as the sole carbon source. However, all these mutants (collected either after mutagenesis with the transposon Tn*5* or by specific gene disruption) grow well in similar media containing 3-OH-PhAc, 4-OH-PhAc, L-tyrosine, L-phenylalanine, 2-phenylethylamine, 2-phenylethanol, *n*-phenylalkanoates, *trans*-styrylacetic

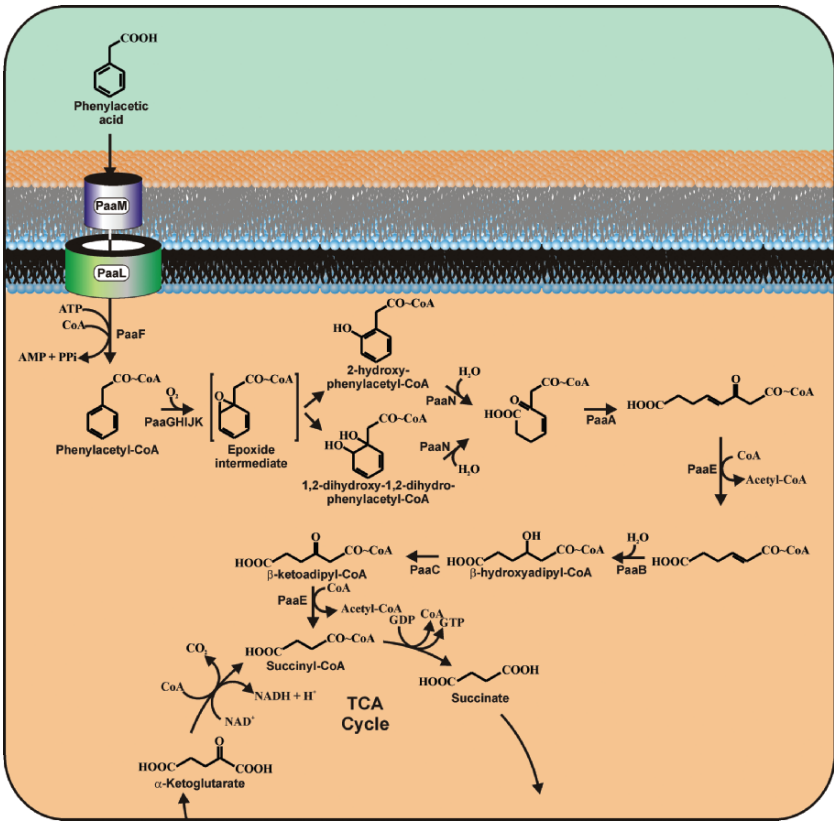

Figure 5. Sequence of reactions of the hypothetical catabolic pathway involved in the aerobic degradation of PhAc in *P. putida* U.

acid, styrene as well as other aromatic compounds structurally related to PhAc as carbon sources. These observations suggest that PaaML are essential for the uptake of PhAc and that they are not required for the transport of other aromatics.[66,81]

The porin PaaM is a protein (417 amino acids, *Mr,* 46,078) that shares certain similarity with the D2 protein of *P. aeruginosa*.[77,114] It has been defined as a model of the specific channel-forming proteins that in bacteria are involved in the uptake of: (i) basic amino acids (L-Lys, L-Orn, L-Arg, L-His); (ii) peptides containing these amino acids; and (iii) in the transport of other compounds (even synthetic ones) whose molecular structure (at least in part) resembles basic amino acids.[77] Surprisingly, in *P. putida* U the lack of PaaM does not affect the uptake of basic amino acids, suggesting that, although similar, these two proteins (D2 and PaaM)

could have different functions. Their functional specificity could be due to the existence in the uncommon sequence of other ligand-binding sites. However, it could also be argued that the specificity of the channel might not only depend on the existence of new ligand-binding sites but on the catabolic pathway coupled to the transport system (i.e. permease, PhAc-CoA ligase, etc). A comparative structural study between PaaM and other well-known proteins revealed that it is related to the porin of *Rhodopseudomonas blastica* DSM2131 as well as to the maltoporin of *Salmonella typhimurium* (Figure 6).[59,71] However, from a phylogenetic point of view these porins do not seem to be related.

The other component of the PhAc transport system is PaaL (520 amino acids, *Mr,* 55,066). This protein, which shows certain similarity with the permeases involved in the symport of proline and sodium and panthotenate and sodium,[65,81] belongs to the Solute/Sodium Symporter Family (SSF) and is related to the permeases responsible for the uptake of different compounds in archea, bacteria and mammals.[99] Structural analysis revealed that PaaL contains 12 putative transmembrane regions as has

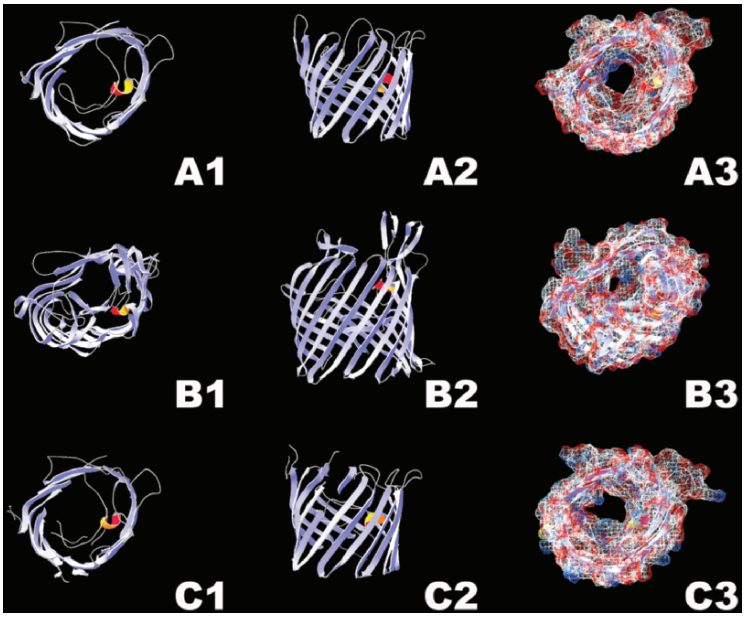

Figure 6. Modelled three-dimensional structures of PaaM (PhAc-specific porin) and other structurally related specific channel-forming proteins. *R. blastica* porin (a); *Salmonella tiphymurium* maltoporin (b) and *P. putida* PaaM (c). A1, B1 and C1 correspond to an apical view; A2, B2 and C2 correspond to lateral view and A3, B3 and C3 correspond to the molecular surface of the pore.

been reported for other members of SSF.[81] The mutation of the gene *paaL* in *P. putida* U prevents this bacterium from growing in chemically defined media containing PhAc.[81] Although the uptake of phenylacetic acid had been studied in different microorganisms (*Penicillium*, *Aspergillus*, *Saccharomyces*, *Escherichia*),[29,30] the data reported by Olivera *et al*.,[81] constituted the first description of the sequence of a permease involved in the transport of this aromatic compound.

The participation of this permease in the PhAc transport in *P. putida* U seems paradoxical since non-hydroxylated aromatic acids should be able to enter the cell (at least at high concentrations) without the help of a specific transport system. However, these results agree with the data reported by Schleissner *et al*.,[104] who demonstrate that an active inducible transport system is required for the uptake of PhAc in this bacterium.

Phenylacetyl-CoA ligase Phenylacetyl-CoA ligase (AMP-forming, PhAc-CoA ligase) is a key enzyme in the PhAc catabolic pathway. This protein, which catalyzes the activation of PhAc to PhAc-CoA in the presence of ATP (*Km* 9.7 mM), CoA (*Km* 1.0 mM), PhAc (*Km* 16.6 mM) and Mg^{2+}, was the first enzyme of the pathway to be identified, purified and characterized in detail.[70] In *P. putida* U PhAc-CoA ligase is encoded by the gene *paaF*, which is located at the *3′*-end of the first operon and is expressed under the control of the promoter *P1* (see Figure 3).[70,81] This gene has been cloned, sequenced and overexpressed in different microorganisms.[72,81] The enzyme (439 amino acids) has an *Mr* of 49,392 and in the active form is a monomer.[70] PhAc-CoA ligase shows maximal catalytic activity at pH 8.2 and at 30°C and it is inhibited by some divalent cations (Zn^{2+}, Cu^{2+} and Hg^{2+}) as well as by the sulfhydryl reagents *N*-ethylmaleimide, 5,5′-dithiobis(2-nitrobenzoic acid) and *p*-chloromercuribenzoate.[70] Taking into account that sulfhydryl groups seem to be essential for the activity of PhAc-CoA ligase, the roles of the seven cysteines present in the enzyme were studied. Mutation analyses revealed that only three of them (Cys52, Cys385 and Cys419) are essential since their modification involves loss of the activity.[67]

PhAc-CoA ligase uses many different aliphatic and aromatic compounds as substrates *in vitro*, although *in vivo* the enzyme seems to be involved only in the activation of PhAc.[31,41,70,81,96] Very recently, we have observed that, when overexpressed (or when it is constitutively expressed), PhAc-CoA ligase is also able to gratuitously activate 3-OH-PhAc, a compound that is catabolized in this bacterium through the homogentisate pathway.[8,9]

PhAc-CoA ligase is synthesized by *P. putida* U when cultured in chemically defined media containing PhAc or all the compounds that are assimilated through the PhAc catabolic pathway (i.e. phenylacetaldehyde,

Table 2. Molecules which are able to induce or do not induce the enzymes involved in the PhAc aerobic catabolic pathway in *P. putida* U.

Inducers	Non-inducers
Phenylacetic acid	L-Phenylalanine
2-Phenylethylamine	L-Tyrosine
2-Phenylethanol	2-hydroxyphenylacetic acid
Phenylacetaldehyde	3-hydroxyphenylacetic acid
Styrene	4-hydroxyphenylacetic acid
Phenylacetamides and other esters of phenylacetic acid	Homoprotocatechuic acid
n-Phenylalkanoic acids with an even number of carbon atoms	Homogentisic acid
Z-Phenylacetaldoxime	*n*-Phenylalkanoic acids with an odd number of carbon atoms

2-phenylethylamine, 2-phenylethanol, styrene, amides and other esters of phenylacetic acid, *trans*-styrylacetic acid, *n*-phenylalkanoates with an even number of carbon atoms, etc.). However, when cultured in similar media containing other structurally related compounds (hydroxyl-PhAc derivatives, *n*-phenylalkanoates with an odd number of carbon atoms, L-phenylalanine and L-tyrosine) PhAc-CoA ligase was not induced[81] (Table 2). It is interesting to note that 3-OH-PhAc, DL-phenyllactate and phenylpyruvate, two compound that do not induce PhAc-CoA ligase in *P. putida* U, do induce it in *Azoarcus evansii*,[98] suggesting that in the absence of specific degradative routes, the microbe has organized (or incorporated) others that catalyze the transformation of these compounds into PhAc-CoA, the first intermediate of the PhAc pathway. The evolutionary mechanisms involved in this particular catabolic specialization are unknown.

Structural analyses of *P. putida* U PhAc-CoA ligase revealed that although it has a conserved AMP-binding sequence,[65,81] this enzyme is quite different from other acyl-CoA ligases reported until now. Thus, it does not show significant homology even with enzymes involved in the activation of aromatic compounds (benzoyl-CoA ligases, coumaroyl-CoA ligases, etc.).[33] Although the collection of PhAc-CoA ligase crystals has been approached using different strategies, until now all attempts have been unsuccessful. Furthermore, this enzyme (even when overexpressed) is particularly unstable and we have observed that in pure preparations it undergoes an autodigestive process that releases several inactive peptides (Luengo and Sanz, unpublished results). For all these reasons, the elaboration of a putative model established either from pure crystals or predicted by comparison with other crystallized and well-known acyl-CoA ligases[45,57] has not yet been possible.

The existence of a PhAc-CoA ligase directly involved in the aerobic catabolism of PhAc[70] was an interesting result since until its description the synthesis of CoA intermediates as catabolites of aromatics was considered a typical event related to the anaerobic catabolism of these compounds.[11,43,48,74] Currently, it is known that phenylacyl-CoA ligase activities are commonly used by different microbes for the aerobic catabolism of PhAc and other aromatic molecules. Thus, *P. putida* CA-3 contains at least three different enzymes showing phenylacyl-CoA ligase activity: a PhAc-CoA ligase, a phenylpropionyl-CoA ligase (acting on phenylpropionate and cinnamate) and a phenylalkanoyl-CoA ligase, which activates aromatic substrates with more than four carbon atoms.[122] In *P. putida* U we have also reported the existence of at least three different phenacyl-CoA ligases: the PhAc-CoA ligase indicated above, and two different ones, involved in the activation of 4-phenylbutyric acid and *n*-phenylalkanoic acid ($n > 4$ carbon atoms), respectively. However, the latter two enzymes, even though they activate aromatic compounds, seem to be involved in the activation of aliphatic acids whose carbon length ranges between C3 and C4 (the one that activates 4-phenylbutyric acid) and between C6 and C12 (the second one).[41]

Some years ago, Vermeij *et al.*[117] reported the existence of two genes encoding two proteins with high homology with PhAc-CoA ligase. Both enzymes, which are homologues, were involved in the biosynthesis of the coenzyme F390.[117] However, neither of these proteins shows PhAc-CoA ligase activity and the PhAc-ligase from *P. putida* U did not catalyze the reaction performed by these enzymes either. These data suggest that the three proteins (FtsaI, its homologue and PhAc-CoA ligase) could have evolved from a common ancestor, which probably had a different catalytic function. This could be the reason why PhAc-CoA ligase is an unusual acyl-CoA synthetase.

The importance of studies of PhAc-CoA ligase is increasing not only because of the interest in the particular reactions catalyzed by this enzyme but also due to the fact that it has important biotechnological applications (see below).[64–67,70] Furthermore, this enzyme is now being used as an evolutionary target to study the transference within and across the microbial world of the genes involved in the degradation of PhAc and other structurally related aromatic compounds.[1]

Aromatic ring hydroxylation complex (ring oxidation complex) Once transported and activated, the phenylacetyl-CoA generated from PhAc undergoes an aromatic ring attack involving the participation of all the proteins encoded by the *paaGHIJK* operon (Figure 3). These genes, which are located downstream from *paaF* in *P. putida* U, belong to the second operon and are expressed under the control of the *P2* promoter.[81] In all

the species studied these five proteins seem to constitute a multifunctional oxygenase complex that catalyzes the incorporation of an oxygen atom onto the aromatic ring.[33,54,81] The mutation in *P. putida* U or in *E. coli* W of any of these genes (*paaGHIJK*) prevents these bacteria for growing in chemically defined media (MM) containing PhAc as the sole carbon source, this compound remaining unaltered in the broth.[33,70,81] However, when all the *paaGHIJK* mutants of *P. putida* U were cultured in MM + 4-OH-PhAc (for supporting bacterial growth) and 2-phenylethanol, 2-phenylethylamine, phenylacetaldehyde or *n*-phenylalkanoates (containing an even number of carbon atoms) were used as sources of catabolites, PhAc was accumulated in the broths.[81] These results showed that all the proteins belonging to this complex are required for the catabolism of PhAc.[66,81] Further experiments revealed that PhAc-CoA, but not PhAc, is the substrate of this enzyme, reinforcing the role of PhAc-CoA ligase in this catabolic pathway. Additionally, we have recently shown that 3-OH-PhAc-CoA is also a substrate of the hydroxylation system.[67]

By means of different genetic and biochemical approaches it has been suggested that PaaGHIJK catalyzes the introduction of an oxygen atom (probably through the formation of an epoxide intermediate, see Figure 5) into the PhAc-CoA molecule, thus generating 2-OH-PhAc-CoA. Although in *P. putida* U this compound has been proposed as a putative intermediate (all the mutants blocked in the *paaABCE* accumulate 2-OH-PhAc in the broths)[81] there is not enough experimental evidences to support its role as a true catabolic intermediate. By contrast in *E. coli* the PhAc catabolic pathway, whose genetic organization was also established by us,[33] seems to be clearer. Thus, it has been suggested that PhAc-CoA is transformed into a *cis*-dihydrodiol derivative (1,2-dihydroxy-1,2-dihydrophenylacetyl-CoA), this compound, and not 2-OH-PhAc, being the catabolic intermediate.[54] However, the existence of an epoxide as a true intermediate (which would generate as secondary products 1,2-dihydroxy-1,2-dihydrophenylacetyl-CoA and 2-OH-PhAc-CoA) before ring opening, cannot be excluded yet.

Comparative analyses of the sequences of the five members of the PaaGHIJK complex with other proteins present in the databases revealed that all of them are enzymes similar to oxidases or other proteins involved in electron transfer processes.[33,66,81] Thus, PaaG (329 amino acids, *Mr*, 37,824) seems to be related to the large oxygenase subunit of the methane, toluene and phenol diiron multimeric monooxygenases.[33,81] PaaH (98 amino acids, *Mr* 11,084) contains the conserved residues found in the dissociable activator protein required for optimal turnover of the oxygenase complex of multicomponent diiron monooxygenases.[33,81] PaaK (358 amino acids, *Mr*, 39,433) represents the first example of a reductase (similar to the class IA-like reductases) subunit from a multicomponent oxygenase that shows an unusual NFNR-like N-terminal domain and a

plant – type ferredoxin C-terminal domain.[33,66,81] In sum, these proteins, together with PaaI (254 amino acids, *Mr*, 28,212) and PaaJ (177 amino acids, *Mr*, 19,461), constitute the different subunits of a diiron multicomponent oxygenase in which PaaH is the effector protein and PaaK is the reductase that catalyzes the transfer of electrons between NAD(P)H and the oxygenase complex (PaaGIJ). Furthermore, the identification of PaaGHIJK (and its equivalent in other microbes) was the first description of a multicomponent oxygenase that uses a CoA derivative of an aromatic compound as substrate.[33,81]

Ring-cleaving enzyme (ring-opening enzyme) The introduction of an oxygen atom into the PhAc moiety of the PhAc-CoA catalyzed by the multicomponent oxygenase complex PaaGHIJK described above facilitates the opening of the benzene ring. This reaction is performed by the enzyme PaaN (688 amino acids, *Mr*, 73,559), which is encoded by the *paaN* gene (Figure 3).[81] This gene, which in *P. putida* U belongs to the third operon and which is expressed under the control of *P3* promoter, is divergently translated in *E. coli* W, constituting a single transcription unit.[33] The specific disruption of *paaN* prevents both *P. putida* and *E. coli* strains from growing in chemically defined media containing PhAc as the sole carbon source.[33,81] Furthermore, when the *paaN* knocked-out mutant of *P. putida* U was cultured in MM containing 4-OH-PhAc (to support bacterial growth) and 2-phenylethylamine, 2-phenylethanol or *n*-penylalkanoates containing an even number, 2-OH-PhAc was excreted and accumulated in the broths 2-OH-PhAc.[81]

Sequence analyses revealed the existence of two different regions in the PaaN protein.[81] The N-terminal domain shows a certain similarity to the NAD(P)-binding sites and the active site motifs of aldehyde dehydrogenases,[33,81] whereas the C-terminal region shows similarities with the *maoC* and *nodN* gene products involved in the catabolism of catecholamines and in nodulation signal production, respectively.[12,109] Although it could be assumed that the product of PaaN is a putative C8 alicyclic-CoA intermediate (see Figure 5), the definitive molecular structure of this compound has not yet been established.

β-oxidation system The last step involved in the aerobic degradation of PhAc involves the transformation of the alicyclic intermediate generated by PaaN into TCA intermediates (see Figure 5). In *P. putida* U these reactions are performed by the proteins encoded in the *paaABCE* genes, which together with *paaF* (the gene encoding the PhAc-CoA ligase) constitute the first *paa* operon. The proteins PaaABCE integrate a complex functional unit with β-oxidation activity that converts the ring-opening product into succinyl-CoA (Figure 5).[65,67,78] PaaA (257 amino acids, *Mr*,

27,352) and PaaB (263 amino acids, *Mr*, 28,648) are proteins which show certain sequence homology with enoyl-CoA hydratases belonging to the enoyl-CoA hydratase/isomerase superfamily.[33,81] These two enzymes seem to be involved in the hydroxylation of double bonds once the ring of the intermediate (2-OH-PhAc-CoA or 1,2-dihydroxy-1,2-dihydrophenylacetyl-CoA) has been opened. Comparative sequence analysis of PaaC (505 amino acids, *Mr*, 53,265) revealed that it contains the signature motifs of 3-OH-acyl-CoA dehydrogenases.[52] This enzyme would be involved in the transformation of β-hydroxyadipyl-CoA into β-ketoadipyl-CoA.[67] Finally, PaaE (497 amino acids, *Mr*, 52,248) is a ketothiolase that shows a certain sequence similarity to the enzymes belonging to the catabolic pathway involved in the degradation of 3-oxoadipate, catechol and protocatechuate.[33,50,65,81] This enzyme catalyzes the hydrolysis of β-ketoadipyl-CoA to actyl-CoA and succinyl-CoA.[78] Very recently, Nogales *et al.*[78] have assayed PaaE in cell-free extracts of *P. putida* KT2442 and *E. coli* W, showing unequivocally that PaaE is a ketothiolase that cleaves β-ketoadipyl-CoA in a similar way to other thiolases that have been characterized in other *Pseudomonas*.[50,60] Furthermore, these authors[78] also showed that succinyl-CoA synthetase mutants of *P. putida* KT2440 were unable to catabolize PhAc, reinforcing the hypothesis that succinyl-CoA is the final product in the catabolism of PhAc (Figure 5). When similar studies were performed in *P. putida* U mutants in which the succinyl-CoA synthetase encoding genes (*sucCD*) had been deleted, it was seen that although their growth was delayed (24 h) they were still able to catabolize PhAc and α-ketoglutarate. This suggests that in this strain, at least, there is an additional enzyme that replaces the function of the succinyl-CoA synthetase encoded by *sucCD* (Olivera *et al.*, unpublished).

In sum, although many interested results have been reported[33,54,78,81] the chemical structure of the PhAc catabolic intermediates is a long way from being fully elucidated, both in *P. putida* and in *E. coli*, and much further work remains to be done to clarify that central pathway on which others responsible for the degradation of many aromatic compounds converge.

5.2.2. Regulation of the Pathway

Analysis of the promoter regions present in the PhAc pathway revealed the existence of different promoters as a function of the microbe studied.[13,33,81] Thus, in *P. putida* U there are three promoter sequences that drive the expression of the three catabolic operons (*P1*, *paaABCEF*; *P2*, *paaGHIJK;* and *P3*, *paaLMN*), and two additional ones (*P4* and *P5*) that control the expression of two genes located at the 5′ end of the *paaABCEF* (*P4* drives the expression of *paaY* and *P5* that of *paaX*) (see Figure 3).[81] However, in *E. coli* W only three promoters were identified (*Pa* located upstream from *paaGHIJKABCDEF*, *Pz* located upstream from the

divergently translated *paaZ*, and *Px*, which drives the expression of *paaXY*).[33] It is interesting to note that whereas in *E. coli paaX* and *paaY* are translated together, in *P. putida* U each of these genes has its own promoter.[33,81] Thus, the analysis of several mutants in which *paaX* or *paaY* and their putative promoter sequences (*P4* or *P5*) had been deleted revealed that PaaX was synthesized in the absence of *paaY* and *P4* and *viceversa* (Arias-Barrau and Luengo, unpublished).

The first studies addressing the regulation of the catabolic pathway involved in the degradation of PhAc were performed in *P. putida* U by Olivera *et al.*,[81] who showed that while the disruption of *paaY* did not (at least appreciably) affect the catabolism of PhAc, when *paaX* was knocked-out the PhAc pathway became constitutive.[81] These results suggested that PaaX (307 amino acids, Mr, 35,100) was a repressor that would control the expression of the three operons integrating the *paa* cluster. Biochemical analyses of some target enzymes (or enzymatic systems) belonging to each of the three *paa* operons reinforced this assumption. Thus, although phenylacetyl-CoA ligase activity was never detected in *P. putida* U when this bacterium was cultured in the absence of PhAc or its precursors (2-phenylethylamine, 2-phenylethanol, *n*-phenylalkanoates, *trans*-styrylacetic acid, etc.), cell-free extracts of the *paaX* mutants showed strong PhAc-CoA ligase activity when they were cultured in different chemically defined media containing several non-inducer carbon sources (octanoic acid, 4-OH-PhAc, 3-OH-PhAc. L-Tyr, glycerol, glutamic acid, succinic acid, etc.).[66,81] Moreover, when mutants containing a disrupted *paaX* gene were cultured in minimal medium with 3-OH-PhAc, they accumulated a dark red product in the broth, which was identified as a quinoid derivative of 2,3-di-OH-PhAc, a compound that requires the presence of PaaF (PhAc-CoA ligase) and the diiron multicomponent oxygenase (PaaGHIJK) to be synthesized.[67] Additionally, those mutants have a constitutive PhAc transport system which efficiently incorporates [1-^{14}C]PhAc. These data indicate that in the absence of PaaX the whole *paa* cluster is expressed, showing that this protein acts as a repressor. Furthermore, sequence analyses revealed that PaaX bears a certain similarity to other transcriptional repressors (GntR and FadR).[33] Ferrández *et al*. showed that PaaX also represses the expression of *paa* genes in *E. coli* W (under the control of the two promoters Pa and Pz) and that PhAc-CoA abolishes the repression caused by this protein whereas it is only alleviated by PhAc.[32,33] These results, confirmed by gel retardation experiments in *E. coli* W[32] and by using other genetic approaches in *P. putida* U,[40] constitute the first description of a transcriptional regulator involved in the catabolism of aromatics that responds to CoA derivatives.

Additionally, recent studies have shown that PaaX also regulates the expression of other genes (or operons) involved in the degradation of

aromatic compounds structurally related to PhAc. In this sense, it has been shown that PaaX represses the transcription of the genes involved in the degradation of styrene (*sty* genes) and the gene encoding the enzyme penicillin G acylase (*pac* gene).[87,90] In both cases PaaX binds to promoter regions that contain the following consensus (underlined) sequence (. . . **T*GATTC***____27 bp apx____***GAATC*A** . . ., in *E. coli* and . . . ***GAT-AC*A**____26 bp apx____**T*GTATC*** . . ., in *Pseudomonas*).[32,87]

Preliminary studies of the three-dimensional structure of PaaX revealed that this protein has two domains, the N-terminal one (particularly a winged-helix motif) being the domain involved in DNA binding. The existence of these two domains is not only deduced from sequence comparisons but chemical denaturing experiments have also confirmed this. Additionally, it has been reported that the four cysteine residues present in the protein (Cys-179, Cys-200, Cys-275 and Cys-323) are in free form (without forming disulfide bridges) and that none of them is found in the DNA-binding domain, since the PaaX protein of the quadruple mutant (C179A, C200A, C275A y C323A) maintains its capacity to bind to DNA. Nevertheles, substitution of Cys-179 by an Ala (C179A) strongly affects its binding to PhAc-CoA, suggesting that it is within or close to the binding zone of this ligand (J. Sanz and V. Hernández, personal communication).

Although the repressor role of PaaX has been well established and is generally accepted,[1,32,33, 40,75,81] it has been reported that this transcriptional regulator could have other important functions. Thus, Miller and co-workers have shown that in *E. coli* PaaX acts as a mutator that could affect DNA replication, DNA recombination or DNA repair.[126]

Furthermore, the genes belonging to the *paa* pathway are also regulated by carbon catabolite repression. Thus, when *P. putida* U was cultured in the presence of glucose, neither PhAc-CoA ligase activity nor [1-^{14}C]PhAc uptake was detected, even in the presence of the inducer (PhAc), showing that this pathway is subject to carbon catabolite repression.[69,81] Additionally, when *E. coli* W was cultured in MM containing PhAc and glucose, the expression of the genes under the control of the promoters *Pa* and *Pz* was not observed. In sum, in both bacteria the operon-specific regulation mediated by PaaX is subordinate to a superimposed regulation caused by global regulators.[32,81,90] Surprisingly, in *P. putida* U a blockage in *paaX* prevents the catabolic repression caused on this pathway by glucose.

On the other hand, PaaY (199 amino acids, *Mr*, 21,049) the product of *paaY,* a gene located upstream from *paaX* in *P. putida* U, is a protein of unknown function. Even though it has been attributed a regulatory function, the role played by this protein needs to be clarified. This protein, which bears a certain sequence similarity to some bacterial transferases (CaiE and ferripyochelin-binding proteins), can catalyze *in vitro* the

hydrolysis of PhAc-CoA and other acyl-CoA derivatives (Olivera and Luengo, unpublished). It could be argued that this enzyme might be involved in the hydrolysis of the PhAc-CoA generated from different precursors (i.e. *n*-phenylalkanoates with an even number of carbon atoms) when the catabolic flux throughout the PhAc pathway decreases. In such conditions, PhAc-CoA would start to be accumulated, and hence PaaY could be needed to restore the intracellular pool of CoA required for attending to other important metabolic functions. If this were the case, the double mutants in which *paaY* has been knocked-out (or deleted) and that contain a mutation in the *paaGHIJK* should not accumulate PhAc (or should do so to a much lower extent) in the broth when cultured in minimal medium with 4-OH-PhAc (to support bacterial growth) and 8-phenyloctanoate (as a source of PhAc-CoA). However, we observed that the double mutants accumulated PhAc in the broth at the same rate as a single mutant lacking a functional hydroxylating system (Arias-Barrau and Luengo, unpublished results), suggesting that the hydrolysis of PhAc-CoA is not the true function of PaaY or that other enzymes could replace this function in the absence of PaaY. Thus, at present the function of PaaY in *P. putida* U is unknown and neither can we be sure of whether it is a *paa* regulator or whether it has a different function.

Recently, very interesting studies addressing the three-dimensional structure and the enzymatic activity of PaaY from *E. coli* have appeared in the literature.[28] Thus, it has been shown that PaaY is a trimeric metaloenzyme related to proteins belonging to the hexapeptide LβH repeat superfamily.[91] It has also been reported[28] that PaaY has thioesterase activity, and that it is able to hydrolyze certain CoA derivatives (acetoacetyl-CoA, lauryl-CoA and phenylacetyl-CoA) whereas other CoA thioesters (acetyl-CoA and succinyl-CoA) are not hydrolyzed or are only very poorly hydrolyzed (decanoyl-CoA).

6. THE PHENYLACETYL-CoA CATABOLON

As indicated above, the aerobic catabolic pathway involved in the assimilation of PhAc is an unusual pathway that it is widely distributed throughout the microbial world.[1,66] Taking into account that PhAc is a compound that is only accumulated freely in the Biosphere in limiting amounts,[55] it is striking that this route is present in so many microorganisms. It could be argued that in the past PhAc could have been accumulated in certain ecosystems and hence that those microorganisms that had acquired this catabolic pathway would have had some metabolic advantage for their survival in such ecological niches. However, it has been observed that most of the microorganisms in which *paa* genes have been

found (comparing their genome *in silico*) have a functional PhAc catabolic pathway. If this pathway were a metabolic ancestor (which would have been used only during a particular period of time) it would be expected that at present the *paa* genes would have accumulated many mutations, such that the old *paa* genes would encode non-functional proteins. Taking into account these assumptions a different explanation should be proposed to justify the widespread distribution of this pathway throughout the eubacteria and the frequent lateral *paa* gene transfer within and across bacteria.[1,56,65–66,70,76,81,119]

Analysis of the molecules that are able to induce the PhAc catabolic pathway in *P. putida* U revealed that in addition to PhAc other compounds such as 2-phenylethylamine, 2-phenylethanol, *trans*-styrylacetic acid, *n*-phenylalkanoic acids (*n* being an even number of carbon atoms), phenylacetaldehyde, several amides and other PhAc esters (see Table 2) were also able to induce the Paa enzymes. However, other structural analogues of PhAc (2-OH-PhAc, 3-OH-PhAc, 4-OH-PhAc, 3,4-diOH-PhAc, 2,5-diOH-PhAc, 2-, 3- or 4-tolylacetic acid, phenoxyacetic acid, tyramine, L-Phe, L-Tyr, L-phenyllactate, phenylpyruvate, phenylglyoxylic acid, α-methyl, α-ethyl, α-amino or α-hydroxy-PhAc and -mandelic acid-), as well as *n*-phenylalkanoic acids with an odd number of carbon atoms were unable to do so (see Table 2).[66,70,81]

These data allowed us to conclude that all these molecules that can be transformed through different catabolic pathways into phenylacetic acid or into its CoA thioester (PhAc-CoA) induce the PhAc catabolic pathway. However, all the other structurally related compounds (see above) that are catabolized by means of degradative pathways that do not generate either PhAc or PhAc-CoA as an end product (or as catabolic intermediates), and that therefore do not need the PhAc pathway to be assimilated, do not induce the Paa enzymes.

In sum, the PhAc catabolic pathway constitutes the route of convergence (*core*) of a complex functional unit (defined by us as *catabolon*) integrated by several routes that catalyze the transformation of structurally related molecules into a common intermediate (phenylacetyl-CoA, which gives the name of the catabolon) and that are, or could be, coordinately regulated.[81] Thus, the phenylacetyl-CoA catabolon encompasses all the routes involved in the transformation of 2-phenylethylamine, 2-phenylethanol, *trans*-styrylacetic acid, *n*-phenylalkanoic acids (*n* being an even number of carbon atoms), phenylacetaldehyde, styrene, phenylacetamide and other PhAc esters, tropic acid, ethylbenzene, benzylpenicillin (phenylacetyl-6-APA) and its derivatives, phenylacetonitrile and Z-phenyacetaldoxime (see Figure 7). These convergent pathways that transform all these compounds into PhAc or PhAc-CoA are commonly referred to as the upper pathways, whereas

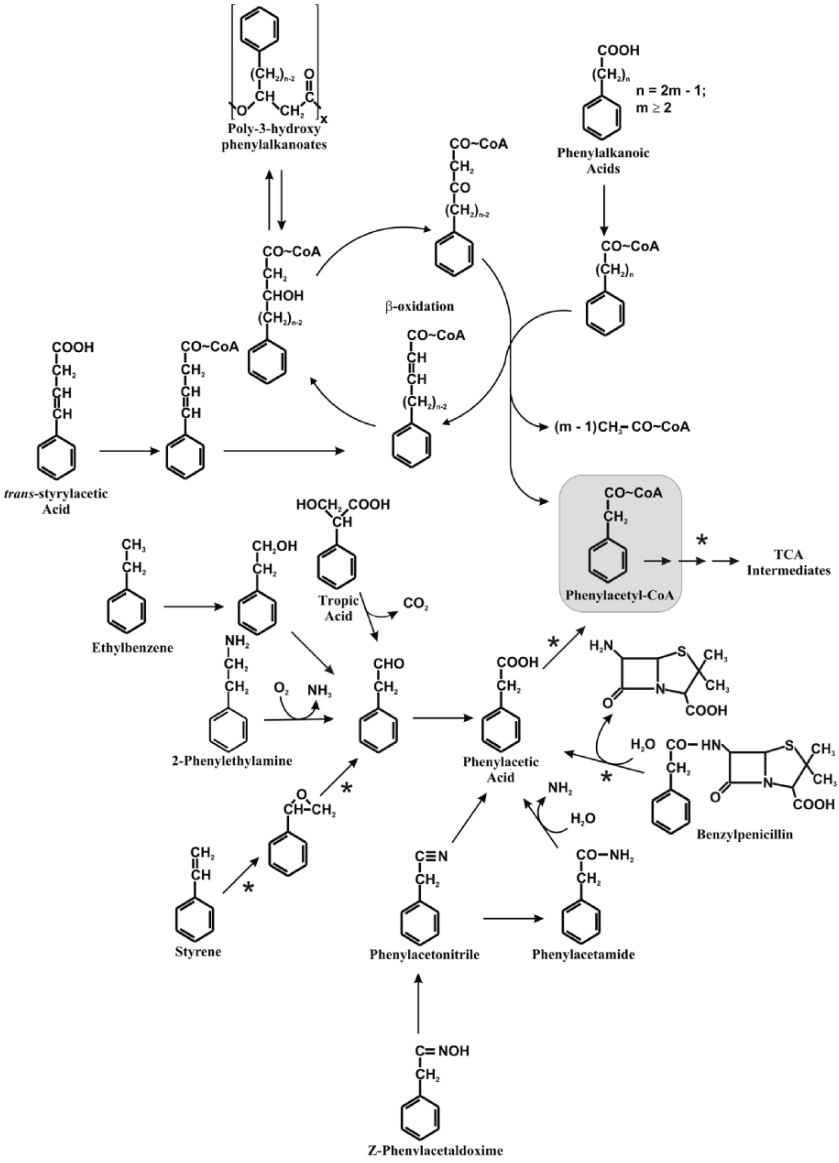

Figure 7. The phenylacetyl-CoA catabolon: Biochemical organization. TCA, tricarboxylic acid cycle. The convergent intermediate (PhAc-CoA) is boxed. Asterisks indicate those routes whose encoding genes are repressed by PaaX.

the PhAc route is called the central route or the PhAc-CoA catabolon core, which ensures the transformation of the common catabolite into TCA intermediates or into other general metabolites.[65–67,70,81]

Taking into account that the PhAc pathway is involved in the degradation of a large number of compounds that could be generated in nature by the action of microorganisms on many synthetic and natural compounds (lignin and other complex structural polymers, amino acids, etc.)[14,19,23,56,66,73–75,100] its broad distribution throughout the microbial world is fully justified.[1,66]

The general structure of the PhAc catabolon (and probably that of other analogous catabolic units) is defined by the independent routes (those involved in the degradation of PhAc precursors or structurally related compounds, and called the upper pathways), by the convergent catabolite (PhAc-CoA, which gives the name of the catabolon), and by the central route (*catabolon core*), which constitutes the connexion between the upper pathways and the central metabolic ones. Furthermore, the upper catabolic routes, which were originally independent routes regulated by quite specific mechanisms, have undergone – during their integration into the catabolon – a hierarchical organization that involves their interdependence and their co-ordinated regulation. The role of PaaX (a repressor of the central route) in the control of the different upper pathways (see above and Figure 7), strongly reinforces this assumption, indicating that the initial definition of the catabolon as a complex catabolic unit integrated by different pathways that are, or could be, co-ordinately regulated,[66,81] is correct.

In microbes, the organization of the degradative pathways in catabolons would facilitate the biochemical and regulatory evolution of the catabolic routes, broadening the range of products that can be assimilated by a given organism. In sum, the existence of catabolic units such as the PhAc-CoA catabolon that have probably arisen as a requirement of microbes to adapt to special habitats could be considered an important evolutionary tool.[65]

7. BIOTECHNOLOGICAL APPLICATIONS OF THE PhAc-CoA CATABOLON

Taking into account the larger number of biotechnological applications of the genes, enzymes, functional units or regulators belonging either to the central PhAc catabolic pathway or to the peripheral routes (upper pathways), in the following section we shall describe the most relevant ones in terms of their industrial interest. However, more detailed information can be obtained from other previously published articles.[41,64–67,70,72]

7.1. β-Lactam Antibiotic Biosynthesis: Enzymatic Synthesis of Penicillins and Genetic Improvement of *P. chrysogenum*

The biosynthesis of penicillins in the fungus *P. chrysogenum* is performed by several well-known enzymes, including a peptide synthetase (L-α-aminoadipoyl-L-cysteinyl-D-valine synthetase, ACVS); an isopenicillin synthase (IPNS) that cyclizes the moieties of cysteine and valine of the tripeptide, generating the β-lactam antibiotic called isopenicillin N (IPN); an acyl-CoA: IPN (6-aminopenicillanic acid, 6-APA) acyltransferase (AT) which catalyzes the replacement of the L-α-aminoadipoyl moiety of IPN by several molecules, thus generating hydrophobic penicillins.[64–66] The biosynthesis of the most important industrial penicillin (penicillin G, or so called benzylpenicillin) requires two additional enzymes: a transport system (PTS) involved in the uptake of phenylacetic acid, and a different one, a putative phenylacetyl-CoA ligase, which synthesises PhAc-CoA, the substrate of AT.[64,66] The first four enzymes (ACVS, IPNS, AT and PTS) have been studied exhaustively, but PhAc-CoA ligase has never been purified or characterized.

To reproduce the last enzymatic steps involved in the synthesis of benzylpenicillin *in vitro*, IPNS and AT from *P. chrysogenum* and PhAc-CoA ligase from *P. putida* U were coupled in a single reaction.[64,66,67] For the first time, this enzymatic system allowed the synthesis of more than 64 different penicillins (including penicillin G) *in vitro*.[64–67] The use of different enzymatic systems involving the participation of other acyl-CoA ligases and acyl-CoA: β-lactam nucleus acyltransferases could lead to the synthesis of new penicillins and, probably, other β-lactam antibiotics.[64]

Furthermore, the transformation of *P. chrysogenum* Wis 54-1255 with the *paaF* gene (encoding the PhAc-CoA ligase from *P. putida* U) improves the rate of benzylpenicillin biosynthesis in this fungus, this being the first achievement of a significant strain improvement carried out using genetic engineering manipulations (at least in this strain).[72]

7.2. Hydrolysis of Phenylacetyl Esters and Amides: Penicillin Acylases

The catabolism of phenylacetyl esters and amides involves the enzymatic hydrolysis of these compounds, releasing PhAc and amines or alcohols (Figure 7). These reactions are usually catalyzed by a large family of enzymes known as β-lactam penicillin G acylases (Pac) which have been identified in different microorganisms, the enzyme of *E. coli* being the one that has been studied best. Its encoding gene has been sequenced, cloned and expressed in different microbes. It is induced by PhAc and its gene

expression is also under the control of the repressor PaaX,[38,90] again suggesting the existence of a coordinated regulation among the central and the peripheral routes belonging to the catabolon of PhAc-CoA.[66,81]

Conversely, *P. putida* U does not contain the *pac* gene in its genome. However, cell-free extracts of this bacterium are able to hydrolyze different esters of PhAc very efficiently (Luengo, unpublished). Taking into account that these proteins can be used for the semi-synthesis of different β-lactam antibiotics and that many of them usually have a broad substrate range (different esters and amides of PhAc, 4-OH-PhAc or of aliphatic and other aromatic compounds),[38,65,81,90] a description of new enzymes would broaden the number of β-lactam compounds whose chains can be hydrolyzed enzymatically. This is why these proteins are of great industrial interest.

7.3. Bacterial Biotransformations: Synthesis of Hydroxyl-PhAc Derivatives

Some hydroxyl-PhAc derivatives (2-OH-PhAc and 2,3-diOH-PhAc) can be used as precursors in the chemical synthesis of many organic products (herbicides, insecticides and other molecules involved in biological control). These compounds, which are routinely obtained by chemical synthesis, can be also produced by fermentation,[81] thus enlarging the collection of these or other similar compounds.

By using a *P. putida* U mutant in which *paaN* had been deleted (Figure 2), 2-OH-PhAc was collected. This strain accumulated 2-OH-PhAc-CoA intracellularly, and since it could not be further catabolized it was hydrolyzed to 2-OH-PhAc and excreted to the culture broth. This manipulated strain transformed PhAc into 2-OH-PhAc very efficiently (more than 90%).

Additionally, cloning of the *paaF* (encoding PhAc-CoA ligase) together with *paaGHIJK* (the multicomponent ring-hydroxylating system) genes into a single plasmid allowed the transformation of different strains of *E. coli*, with the observation that some recombinant strains are able to transform more than 80% of the PhAc added to 2-OH-PhAc, and that this transformation does not occur if some of the *paaFGHIJK* genes are absent.[65–67,81]

The biotechnological interest of these studies lies in the fact that the replacement of some of the *paaFGHIJK* genes by others analogues or the alteration of the regulatory mechanisms that control the expression of these catabolic genes, could allow the collection of different mutants able to synthesize new or modified molecules. Thus, the incubation of a *P. putida* U mutant in which the gene *paaX* had been deleted (thus

containing a constitutive PhAc pathway) in a MM containing 3-OH-PhAc allowed the accumulation in the broth of a compound identified as 2,3-OH-PhAc.[8–9,67]

Furthermore, it could be speculated that many other important compounds, such as salicylic acid, could be obtained by using genetically engineered microbes harbouring some of the *paa* genes (i.e. expression in *E. coli* of the gene encoding benzoyl-CoA ligase from *Rhodopseudomonas palustris* and the operon *paaGHIJK* from *P. putida*).[42,43]

7.4. Bioremediation: Use of Genetically Manipulated Strains in PhAc Elimination

Taking into account that some fermentation industries (particularly those involved in the synthesis of benzylpenicillin) may release waste materials containing certain amounts of PhAc into the environment a complete removal of this compound from industrial residues should be achieved.

It has been reported that the gene *paaX* encodes a transcriptional repressor (PaaX) that in the absence of the inducer (PhAc-CoA) suppresses the expression of the catabolic enzymes involved in the aerobic catabolism of PhAc (see above). Although in *E. coli* W the operon-specific regulation mediated by PaaX is subordinate to a superimposed regulation caused by global regulators,[32,38,81] in *P. putida* U a blockade in *paaX* prevents the catabolic repression of this pathway caused by glucose. However, even though no carbon catabolite repression is caused on the PhAc pathway, in order to prevent any further effect on the catabolism of their end products, a *P. putida* mutant in which the genes *paaX* and *crc* (encoding the carbon catabolite repressor protein, CRC) was obtained. This double mutant (*P. putida* U Δ*paaX*Δ*crc*) is able to efficiently catabolize the phenylacetic acid accumulated in the residual waters of industrial fermentations even in the presence of easily metabolizable nutrients.

7.5. Biotechnological Applications of the Styrene Catabolic Pathway

One of the upper pathways belonging to the PhAc catabolon is involved in the transformation of styrene (*via* styrene epoxide) into phenylacetaldehyde, which is later oxidized to PhAc (see Figure 7). The first reaction is catalyzed by a two-component monooxygenase (StyAB) that catalyzes the transformation of styrene into styrene epoxide. This compound is later isomerized to phenylacetaldehyde by StyC, and finally this molecule is oxidized to PhAc by an aldehyde dehydrogenase (StyD).[116] Two

additional genes (*stySR*), integrated in a different operon, regulate the expression of *styABCD*.[66,78]

Styrene, styrene epoxide and their derivatives (phenylacetaldehyde and phenylethanol) are produced as waste materials by many industries devoted to the production of plastics.[116] These extremely toxic compounds are usually released to the environment as contaminants.[42,65–66,84,116] Their inhalation in industrial settings can be avoided by using warning systems (biosensors) that allow the rapid identification of these compounds in the air,[123] thus avoiding the risks caused by prolonged exposure. Industrial air purification is performed by using special chemical filters (biofilters) containing a trapped bacterial population that can be used for the elimination or styrene or its derivatives.

Knowledge of the genes and enzymes involved in the degradation of styrene has allowed the collection of genetically manipulated bacteria (supracatabolic strains) that eliminate this product (or its derivatives) from different habitats very efficiently.[65–66,84,108] Thus, *P. putida* U, a strain that cannot grow in chemically defined medium containing styrene, when transformed with a plasmid containing *stySRABCD* is able to degrade this compound even when it is added directly to the medium at 5 mM concentration.

Moreover, other recombinant microbes have been used not as warning or decontaminant systems, but to synthesize styrene derivatives of commercial interest. Thus, a recombinant strain of *E. coli* carrying the *styAB* genes from *Pseudomonas* is now being used to convert styrene to chiral (*S*)-styrene.[84,108]

7.6. Biotechnological Applications of the 2-phenylethylamine, Ethylbenzene and 2-phenylethanol Catabolic Pathways

The upper pathways involved in the assimilation of 2-phenylethylamine, ethylbenzene and 2-phenylethanol degrade these three compounds through two different routes (Figure 7), which, as occurs in the route of styrene, lead to phenylacetaldehyde. Later, this common intermediate is oxidized to PhAc in a reaction catalyzed by a phenylacetaldehyde dehydrogenase.

Very recently the degradation of 2-phenylethylamine in *P. putida* U has started to be clarified (Figure 8). Using mutagenesis with the transposon Tn*5*, different mutants specifically disrupted in the 2-phenylethylamine catabolic pathway were isolated. Analysis of these strains revealed that this compound is catabolized through a new catabolic pathway, located in a DNA fragment of 16.6 kb that contains 11ORFs under the control of different promoters (Figure 8). In sum, the degradation of 2-phenylethylamine in *P. putida* U, and probably in other pseudomonads,

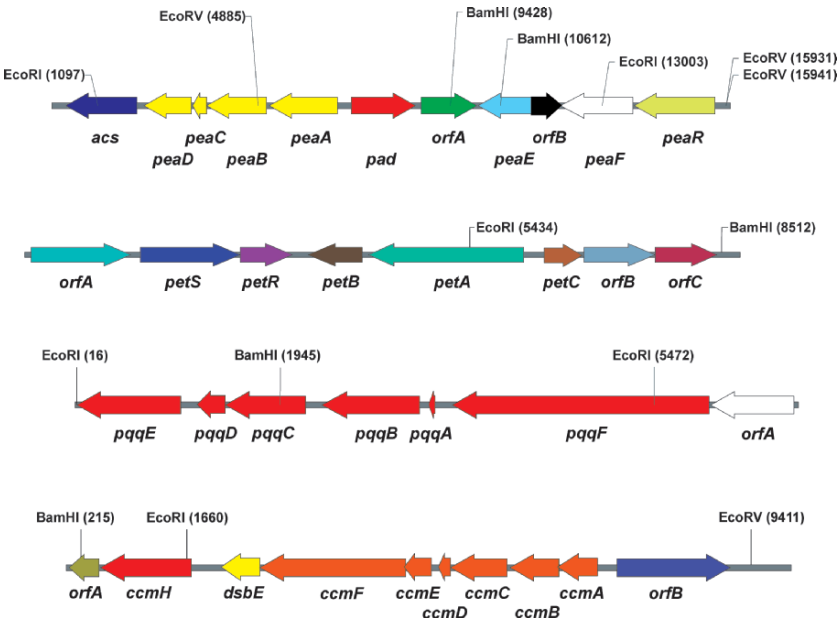

Figure 8. Genetic organization of the gene clusters required for the degradation of 2-phenylethylamine (*pea*) and 2-phenylethanol (*pet*). Two additional clusters involved in the biosynthesis of the cofactor pyrroloquinoline quinone (PQQ) (*pqq*) and in the maturation of the cytochrome c (*ccm*) are required for the degradation of these aromatic compounds in *P. putida* U (Arias and Luengo, unpublished).

is carried out by a complex catabolic pathway that is quite different from that reported in *E. coli*[33] and that involves the participation of several proteins, including an ABC transport system, a deaminase system, a phenylacetaldehyde dehydrogenase and different proteins of unknown functions (Arias and Luengo, manuscript in preparation).

The degradation of ethylbenzene, a compound that is widely used as solvent and as a styrene precursor,[66] requires the oxidation of the methyl group, thus generating 2-phenylethanol which is later oxidized to phenylacetaldehyde (see Figure 7).

Studies addressing the catabolic pathways involved in the degradation of 2-phenylethanol have been approached in *P. putida* U only very recently. Arias *et al*., (manuscript in preparation) showed that the catabolism of this aromatic compound is more complex than formerly believed, since it involves the participation of a two-component signal transduction system, a 2-phenylethanol dehydrogenase, a cytochrome c and an electron transfer protein (Figure 8).

Additionally, two secondary routes, required for the maturation of cytochrome c and for the synthesis of the coenzyme pyrroloquinoline quinone (PQQ), seem to be necessary for the catabolism of 2-phenylethylamine, ethylbenzene and 2-phenylethanol (Figure 8), showing that the degradation of these compounds is more complex than initially believed and that more than 30 different proteins are needed for the transformation of these compounds into PhAc (Arias and Luengo, manuscript in preparation).

The biotechnological interest of these compounds derives from their physiological, environmental and industrial importance. Thus, 2-phenylethylamine is a neurotransmitter that stimulates many physiological processes.[65] An excess of 2-phenylethylamine is related to paranoid schizophrenia, whereas a deficit in the synthesis of the molecule is associated with attention loss.[83] Currently, 2-phenylethylamine and some of its derivatives are being successfully employed in the pharmaceutical treatment of psychiatric illness, especially those coursing with acute depression, as well as in the therapy of patients with suicidal tendencies.[101] In light of this, study of the catabolic pathway involved in the degradation of 2-phenylethylamine in bacteria could contribute to the identification of unknown genes, new enzymes, specific enzyme-inhibitors and modified catabolites that might be used in the near future in gene therapy, in pharmacology or in other applications of biotechnological interest. Additionally, knowledge of the regulatory mechanisms involved in the biosynthesis of this molecule could facilitate the synthesis (or the design) or new drugs useful for the treatment of schizophrenias, Parkinson and Alzheimer diseases.

7.7. Tropic Acid Pathway: Biotechnological Applications

The alkaloid atropine is a natural product obtained from *Atropa belladonna*.[62] It comprises a racemic mixture of D- and L-hyoscyamine, the latter being the active drug. The catabolic pathway involved in the assimilation of atropine in *Pseudomonas* involves its transformation to tropic acid and tropine.[62] Tropic acid is oxidized to phenylmalonic semi-aldehyde and then decarboxylated to phenylacetaldehyde, which is finally transformed into PhAc (Figure 7).

Atropine and its derivatives have many biotechnological applications since they are widely used in medicine and in pharmacy. The drug is a competitive agonist of muscarinic cholinergic receptors[113] that causes an increase in heart rate, inhibits salivary secretion and relaxes the smooth muscle in the gut, biliary tree and in the urinary tract. Thus, the synthesis of atropine derivatives (new or modified molecules) using genetically manipulated bacteria is a research field with a high biotechnological interest.

7.8. *n*-Phenylalkanoate Catabolic Pathway: Accumulation of Bioplastics

Study of the assimilation of *n*-phenylalkanoates (PhAs) in *P. putida* U revealed that this occurs through an independent route belonging to the PhAc-CoA catabolon.[41,81] The catabolism of these compounds requires the same enzymatic system (FadDEBA) that is involved in the β-oxidation of medium- and long-chain *n*-alkanoic acids (As), reinforcing the central catabolic role of the *catabolon core* in *P. putida* U.[79] When the *fadBA* cluster was specifically disrupted (or deleted), these mutants were unable to catabolize As and PhAs.[41] However, the genetic and metabolic versatility of *P. putida* U leads to the induction of other analogous enzymes. In this sense, when the constitutive β-oxidation (β_I-oxidation) is not functional, another β-oxidation pathway (β_{II}-oxidation) is induced.[79] Furthermore, the substrate specificities of both systems (in particular the 3-ketothiolases) are quite different; thus, whereas β_I degrades As and PhAs, the enzymes in β_{II} have difficulty in catabolizing phenylalkanoates.[79]

The degradation of other PhA structurally related compounds such as *trans*-styrylacetic acid (StyAc) in *P. putida* U is not carried out through the same pathway but requires a new catabolic route that leads to the transformation of this compound into PhAc (Figure 7). It was initially believed that the degradation of this compound required activation to *trans*-styrylacetyl-CoA, isomerization of the double bond from position 3 to position 2 (4-phenyl-2-butenoyl-CoA) and its β-oxidation.[70] However, the recent isolation of certain mutants suggests that the degradation of this compound to PhAc-CoA requires a set of enzymes different from FadB and FadA (Giménez and Luengo, unpublished).

The collection of different mutants of *P. putida* U affected in β_I-oxidation revealed that those strains could intracellularly accumulate high amounts of polyhydroxyalkanoates (PHAs).[41,126] These biomaterials (generally called bioplastics) are biodegradable and biocompatible.[2,68,80] Bearing in mind the biotechnological interest of these biomaterials, genetic and biochemical studies have been performed, allowing identification of the different enzymes involved in the biosynthesis, structuring and catabolism of these polymers, as well as the organization of their encoding genes.[41,79] The synthesis of PHAs requires the participation of two polymerases (PhaC1 and PhaC2) as well as three additional proteins (PhaD, PhaF and PhaI) involved in granule structuring and in regulation (see the specific article about this topic also published in this book). Mobilization of the polymer requires the participation of a PHA depolymerase (PhaZ) that catalyzes the release of different monomers (3-OH-*n*-(phenyl)alkanoates) from the polymer accumulated.[102] All these enzymes are encoded by genes belonging to the *pha* cluster.[41,68]

Deletion of the *fadBA* gene cluster encoding the β_I-oxidation pathway, as well the expression *in trans* of the gene encoding the phasin PhaF, elicits a strong intracellular accumulation of unusual homo- and heteropolymers that alters the morphology of these overproducer mutants. In these bacteria, more than the 98% of the cytoplasm is occupied by these bioplastics (Figure 9) and they can be used to synthesize polymers other than those accumulated by the wild-type regarding both monomer size and relative percentages. The design of these genetically manipulated strains has allowed the collection (by single-culture variations) of a huge variety of bioplastics with different characteristic and applications.[2,68,80]

8. CONCLUDING REMARKS AND FUTURE OUTLOOK

The aerobic degradation of PhAc in *P. putida* U and in other bacteria is carried out through a catabolic pathway that involves the activation of PhAc to PhAc-CoA, the introduction of an oxygen atom (probably

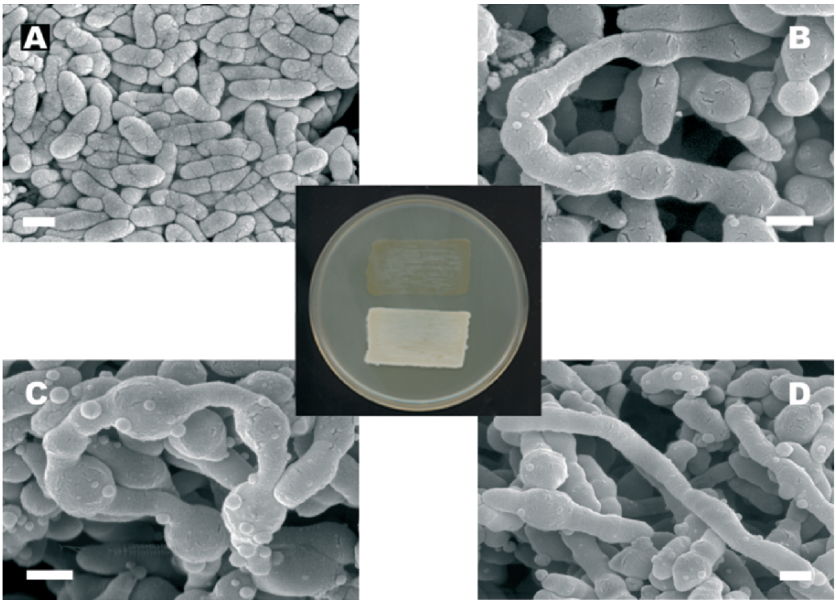

Figure 9. Scanning micrographs of *P. putida* U (a) and its Δ*fadBA* β-oxidation mutant (a bioplastic overproducer mutant) (b–d) cultured in a chemically defined medium containing 4-OH-PhAc (10 mM) and 7-phenylheptanoic acid (10 mM). In the central plate the morphological aspect of two cultures of *P. putida* U (*top*) and its mutant (*bottom*) is shown. Bar indicates 1 μm.

requiring the formation of an epoxide intermediate) into the benzene moiety of PhAc-CoA, and the generation, after ring opening, of an alicyclic intermediate that is converted into succinyl-CoA and acetyl-CoA through a special β-oxidation pathway.

This catabolic pathway represents an interesting model of study not only due to its contribution to basic knowledge about the catabolism of aromatics, but also to certain characteristics that make it a special and very attractive pathway. Thus, in this route, even though it occur under aerobic conditions, all the intermediates are CoA derivatives, and, moreover, ring opening does not appear to involve a typical oxygenase. For these reasons the PhAc catabolic pathway has been considered a hybrid (aerobic/anaerobic) degradative route.

Additionally, this pathway has been shown to be the central route (also called route of convergence) of a more complex functional unit (phenylacetyl-CoA catabolon) integrated by different independent routes (upper or peripheral pathways) involved in the degradation of several PhAc structurally related compounds (styrene, 2-phenylethylamine, 2-phenylethanol, phenylacetaldehyde, ethylbenzene, tropic acid, phenylacetaldoxime, phenylacetamide and other esters of phenylacetic acid, *trans*-styrylacetic acid, *n*-phenylalkanoic acids and poly-3-hydroxyphenylalkanoates with an even number of carbon atoms). We refer to it as the *PhAc-CoA catabolon* because it is integrated by different catabolic routes (*catabolon*) and because the common intermediate of all the upper routes (*convergent catabolite*) is PhAc-CoA.

Regulatory analyses of the central route reveal that it is under the control of PaaX, a repressor protein whose function has been analysed in detail. PaaX binds to different promoter regions involved in the transcription of the *paa* locus and this effect is counteracted by PhAc-CoA, this being the first description of the participation of a CoA derivative in the regulation of genes encoding the enzymes responsible for the aerobic degradation of an aromatic compound. Furthermore, it has been observed that certain peripheral pathways involved in the degradation of PhAc analogues are also under the control of PaaX, suggesting that at least some of the upper pathways could be co-ordinately regulated, as we initially proposed.

Moreover, the genetic characterization of *paa* clusters in different microorganisms reveals that the PhAc catabolic pathway is not a metabolic particularity restricted to a single bacterial strain, but that it is an important route that is broadly distributed throughout the microbial world. Comparative analyses of the genetic variants of the PhAc pathway could contribute to clarifying how the different operons that integrate the *paa* clusters have arisen and to determining how different biochemical functions (PhAc activation, PhAc-CoA hydroxylation, etc) have evolved together in a single operon.

Bearing in mind the large number of peripheral pathways belonging to the phenylacetyl-CoA catabolon, the evolutionary study of the whole catabolic unit seems essential to understand how the original regulatory mechanisms that control each independent route have been hierarchized to facilitate the functionality of the complex unit. Accordingly, the biochemical and genetic characterization of catabolons in single cells, such as bacteria, could be important for understanding how complex catabolic pathways have evolved, and how their degradative potential has been polarized or specialized.

In sum, study of basic models such as the PhAc-CoA catabolon could not only contribute in the near future to establishing the basic molecular mechanisms involved in the hierarchization of the originally independent catabolic pathways, but also to increasing the catabolic potential of this complex unit, that is by introducing new genes. Thus, the cloning in *P. putida* U of the genes involved in the degradation of cinnamic acid or 3-phenylpropionic acid in different bacteria could allow the complete assimilation of *n*-phenylalkanoates with an odd number of carbon atoms, thus expanding the degradative possibilities of this bacterium. Furthermore, cloning of the genes encoding different hydroxylases (PhAc hydroxylases) may be able to interconnect different independent catabolic pathways (or catabolons) in such a way that, by means of metabolic engineering, supracatabolic bacteria could be obtained. In this sense, the identification of the catalytic role played by the different proteins or by the enzymatic complex belonging to the central or peripheral pathway of catabolons could facilitate the collection of genetically engineered microbes containing specific catabolic pathways that have been designed specifically to degrade a particular compound (or a family of xenobiotics) even before they start to be accumulated in the Biosphere. The identification and the cloning of the genes involved in certain pathways (i.e. *paa*, *sty* and *pha* clusters) has allowed their transference to other bacteria, enlarging the degradative capacities of the recombinant strains.

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7

ROLE OF MEMBRANE STRUCTURE DURING STRESS SIGNALLING AND ADAPTATION IN *PSEUDOMONAS*

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1. INTRODUCTION

Bacteria are exposed to a wide range of stress-inducing fluctuating conditions in the environment. These stresses include extreme temperatures, changes in osmotic pressure, the presence of toxic compounds, desiccation and nutrient fluctuation. A quick adaptive response by the bacteria is required for survival. Ubiquitous microorganisms such as *Pseudomonas* are able to modulate their gene expression in response to a wide range of environmental stressors enabling successful physiological/biochemical adaptation. While stress responses in bacteria are well studied, the sequence of events leading to cell death or adaptation, and in particularly the primary sensor(s) involved, remain to be elucidated.

The bacterial membrane has emerged as an initial target for dealing with stresses.[109] Bacterial membranes act as a selective barrier between the

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external and internal environment and are the one of the first components to be exposed to changing conditions affecting instantly their biochemical properties including structure and fluidity.[23] Microbial membranes are responsible for a plethora of physiological processes such as regulating the movement of substances in and out the cells, stabilising protein structure for a correct functioning of membrane embedded enzymes and providing a matrix for many biological reactions. Therefore, the precise regulation of membrane structure and fluidity, known as homeoviscous adaptation, in the face of a constantly changing environment is an important challenge for all bacteria.[115,138]

Interestingly, recent data point towards bacterial membranes not only as a stress targets, but also as the first sensors in activating a stress response. Subtle alterations in the bacterial membrane composition have been shown to transform environmental signals into the transcriptional activation of stress genes, involved in both membranous and non-membranous cellular events.[76]

2. IMPACT OF ENVIRONMENTAL STRESSORS ON MEMBRANE PROPERTIES

The bacterial membrane forms a protective layer around the cells in which the phospholipid bilayer constitutes the structural framework. Phospholipids consist of a single molecule of glycerol, where one OH group is bounded to a phosphate group (hydrophilic head) and two OH groups of the glycerol molecule are bounded to the two fatty acid chains (hydrophobic tail). The membrane phospholipids form a bilayer with the hydrophilic ends towards the outer surfaces and the hydrophobic ends buried in the interior (Figure 1).

The outer membrane bilayer is asymmetric, with an outer leaflet composed mainly of lipopolysaccharide (LPS) and an inner leaflet that is composed primarily of phospholipids.[25,97] A number of proteins, such as porins, are also embedded in the outer membrane. Outer membrane proteins (OMPs) have several functions which include specific recognition/transport of external molecules, formation of pores that facilitate the passage of hydrophilic molecules and efflux mechanism for antimicrobial compounds.[65, 71] The inner membrane is a phospholipid bilayer containing proteins which facilitate membrane-associated transport and biochemical processes required for cell growth and division.[25,26,97] Other lipid-derived compounds found within the cell envelope include membrane-derived oligosaccharides (MDOs) of the periplasm, lipid A of LPS and lipoproteins of the outer membrane.[27]

Pseudomonas possesses a relatively simple phospholipid composition with phosphatidylethanolamine (PE; 69%), phosphatidylglycerol (PG; 15%) and cardiolipin (CL; 11%) being the three major phospholipids found in the cell envelope[6,136] (Figure 1).

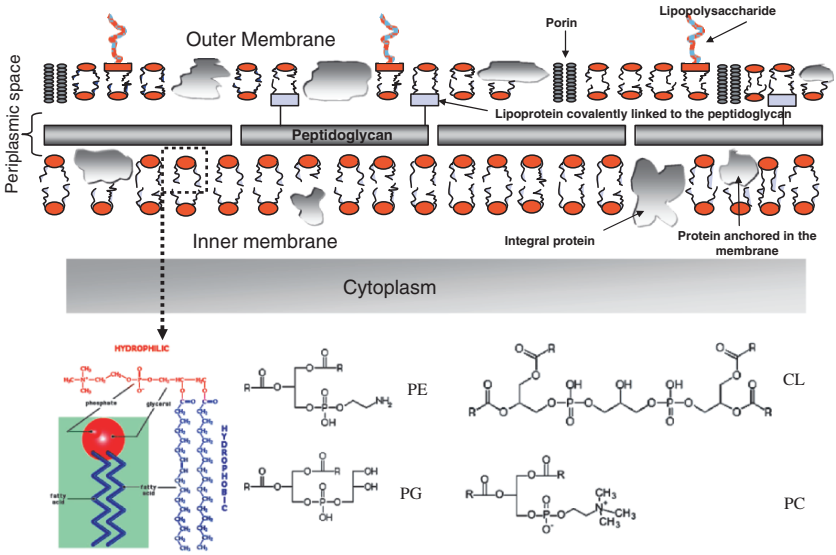

Figure 1. Gram-negative cell wall, phospholipid structure and major phospholipid components. PE, phosphatidylethanolamine; CL, Cardiolipin; PG, phosphatidylglycerol; PC, phosphatidylcholine.

Phosphatidylcholine (PC) is the major membrane forming phospholipid for most eukaryotes and is also present in both outer and inner membrane of *Pseudomonas aeruginosa* at appreciatively 4%.[6,136] It is thought that more than 10% of bacteria contain PC. It seems that PC is frequently found in the membranes of prokaryotes that interact closely with eukaryotic hosts in a symbiotic or pathogenic relationship.[75,116] Under phosphate-limiting conditions, which are frequently encountered by free-living organisms, some bacteria replace their membrane phospholipids with lipids which do not contain any phosphorus such as ornithine containing lipid OL.[34] This phenomenon has been demonstrated in several *Pseudomonas* strains.[21,59,86]

Environmental changes directly affect the structural characteristics and fluidity of membranes by altering the biochemical properties of the acyl components of membrane lipids (Figure 2). Phospholipids can form different phases, for example a liquid-crystalline phase and a gel phase. Interconversions (or "phase transition") between these phases can occur depending on the prevailing conditions (Figure 2). In functional membranes at physiological temperature, the lipids are in a fluid-crystalline lamellar (planar bilayer) phase. The term fluidity encompasses the lateral diffusion, molecular wobbling and chain flexing of molecules within the membrane. Microbial fatty acids are typically 12–24 carbons long and acyl chains of this length correctly balance the fluidity of the membrane.[23] Branched-chain fatty acids, a structure commonly found in *Bacillus*,

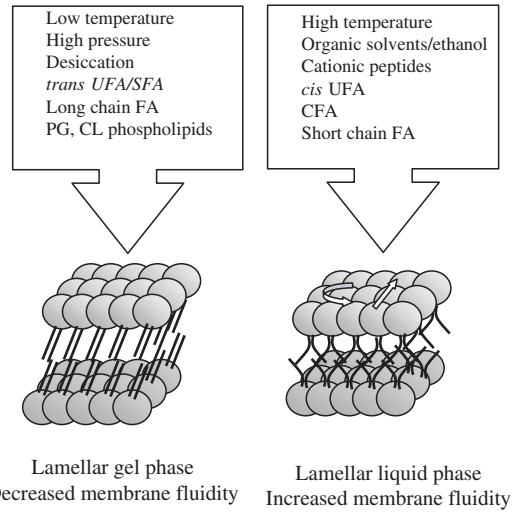

Figure 2. Impact of external stresses and fatty acid composition on bacterial membrane structure. In the lamellar liquid-crystalline state, the lipid molecules are melted and disordered. Upon transition to the gel state, the phospholipids become ordered and the frequency of rotation and lateral movement is reduced.

Micrococcus and *Staphylococcus* genera,[69] predominate in Gram-positive bacteria and some psychrophilic bacteria such *Listeria monocytogenes*.[4] The bacterial cytoplasmic membrane is also characterised by the co-existence of lipid enriched and proteo-lipid domains due to a heterogeneous distribution of membrane constituents.[127] These factors must be maintained for proper functioning.

The physical properties of membranes can be changed upon exposure to biological, chemical, or physical stressors, through alteration of (i) the lipid packing within the membrane, (ii) the phase of membrane lipid by transition between a liquid-crystalline phase to a gel-like phase, (iii) the ratio of bilayer to non-bilayer lipid forming structure and (iv) the membrane–protein interaction ratio which if it is increased can negatively impact on the amplitude of acyl chain motion.

2.1. Impact of Physical Factors on Membrane Properties

Amongst the physical stressors that bacteria will face in the environment are extremes in temperature, osmotic pressure and desiccation. Increases in temperature cause the acyl chain to melt and the transition to the liquid state to occur while low temperature causes a transition towards the gel-phase or crystalline phase state. As temperature increases, the acyl

chains also become disordered and rearranged into the non-lamellar phase.[41] Therefore, high temperature acts to increase membrane fluidity. The melting temperature at which the phase transition occurs depends on the melting temperature of the glycerophospholipid acyl chains and is commonly lower than the optimum growth temperature.[124] Increases in pressure cause the membrane lipid to pack more tightly, promoting the transition towards a gel state membrane.[41,78]

Desiccation, a common stress for soil microorganisms, causes an increase in the melting temperature of the cell membrane.[101] Moreover, on liposome models, the removal of water from the phospholipid hydration shell diminishes the effective size of the polar head group, leading to enhanced lateral packing of the phospholipid acyl chains.[68] Thus desiccation induces a transition to the less fluid gel phase.

2.2. Impact of Chemical Factors on Membrane Properties

Membranes respond differently to chemical toxicants, based on their hydrophobicity and structure. Organic solvents such as toluene, xylene and cyclohexane bind to the cells and disturb the structure and functioning of the membrane.[43,135] The organisation of the glycerophospholipids within the membrane can be disturbed by the accumulation of hydrophobic chemicals. This can alter the hydrogen bonding and dipole–dipole interaction of the acyl chains in the lipid bilayer. In addition, amphipathic molecules affect the bilayer volume by intercalating between the lipid acyl chains.[114]

More hydrophilic chemicals such as ethanol interact with the hydrophilic heads of the phospholipids; the hydroxy group is capable of hydrogen bonding with polar groups at the membrane–water interface. At the same time, the hydrophobic ethyl group can interact with the acyl chain, disordering them and thus increasing the membrane fluidity.[5]

The presence of divalent cations can also induce a phase state transition of the membrane lipid. This results mainly from diminished electrostatic repulsion between the negatively charged polar head groups of the phospholipids.[23,79]

2.3. Impact of Biological Factors on Membrane Properties

Due to interactions with many hosts, bacteria such as *Pseudomonas* may be confronted with biological compounds possessing membrane-altering properties. Antimicrobial host defence peptides are widely distributed in animals and plants. Most of these peptides are positively charged in order to interact with the bacterial membrane comprising negatively charged phospholipids and lipid A from the LPS components of outer membrane.[11]

Recently it has been demonstrated by *in vitro* experiment on membrane models that the α helical peptide NK2 enhanced the fluidity of PE acyl chains and negatively altered the transition temperature of lipid from the gel to the liquid-crystalline phase.[137] In a similar way, using model membranes, it was reported that high concentrations of the tryptophane-rich antimicrobial peptide indolicidin lowers the ratio of the gel-phase fluid domains.[111]

Antibiotics in environment also frequently challenge bacterial viability. Tetracycline was reported to affect the fluidity of *Bacillus cereus* and *Pseudomonas putida* membranes. Whereas it has a positive effect on the cytoplasmic membrane fluidity of *B. cereus*, the converse was observed with *P. putida*, which may reflect the difference in stress-induced behaviour between different bacteria species.[130] Using *Escherichia coli* as model, it was shown that chloramphenicol can also trigger a decrease in the membrane fluidity due to a dissipation of the proteo-lipid domains and a decrease in the phase transition temperature.[127]

In the rhizosphere, microorganisms are in contact with root and seed exudates containing potentially membrane-active toxic compounds. For example, seedling alkylresorcinols composed of long unsaturated chain resorcinols are able to integrate into the phospholipid layers. Therefore they have the potential to affect the structural properties of bacterial membranes.[82]

2.4. Impact of the Cell Physiological Status on Membrane Properties

It is not only external factors that have an impact on membrane characteristics. The growth phase and the nutrient status are also known to modulate the lipid–protein ratio within the membranes. Stationary phase and nutrient starvation are associated with an increased lipid/protein ratio and with increased interaction levels between proteins and lipids. In *E. coli*, while cells progress to stationary phase the CL/PG ratio increases.[25,118] As CL has a higher transition temperature than PE this alters membrane fluidity. Together these alterations result in less mobile lipids within the phospholipid bilayer and a more rigid structure.

3. CELL MEMBRANES AS SENSORS FOR HOMEOPHASIC ADAPTATION

As a response to physical and chemical challenges in the cell environment, adaptive mechanisms have evolved. They lead to the activation of gene expression or protein activity required to cope with a given stress. In this section, we will review the adaptive mechanisms that bacteria use to respond to environmental stressors with the aim of maintaining the fluidity of membranes at levels suitable for cell survival.

Disruption of membrane integrity compromises the ability of the membrane to function as a barrier, energy transducer and matrix for enzymes, and also affects cell division and DNA replication. Therefore, bacteria need to actively keep the fluidity of their membrane at a level suitable for growth irrespective of the environmental conditions. At the level of membrane lipids, the bacterial processes activated as a response to physical and chemical changes in the environment are often referred as homeoviscous adaptation. However, since microorganisms are unable to recover 100% of the initial fluidity and since lipids coexist in separated micro-domains with different behaviour states within the membrane, the term homeophasic adaptation was subsequently proposed.[40]

3.1. How Changes in Membrane Composition Affect Fluidity

The degree of saturation of fatty acids has been initially linked to membrane fluidity.[115] The ratio of *cis*/*trans* monounsaturated fatty acids (MUFAs) was later used as an indicator of membrane fluidity.[24] Using *P. putida* as model, it was demonstrated that an increase of this ratio lowers the membrane fluidity.[73] Saturated fatty acids (SFAs) and *trans* isomers improve the lipid order (i.e. the hydrocarbon chains are tightly packed) while *cis* isomers increase the fluidity of the membrane. The *cis* double bond introduces a kink of 30° into the acyl chain, disturbing the highly ordered packing of SFA and *trans* UFAs.[18,45] This disorder decreases the transition temperature, increasing the fluidity of the membrane.[18] More recently, a membrane viscosity index (MVI) was proposed which integrates the degree of saturation and configuration of fatty acids as well as cyclopropane derivatives (cyclopropane fatty acid, CFAs) that also confer fluidity upon the membranes.[81] Moreover, the extent of the *trans* isomerisation depends on the amount of CFAs already formed on the *cis* UFAs during the transition from exponential to stationary growth phase.[40] Therefore using only the ratio *cis*/*trans* to monitor environmental stresses is questionable.

The index was defined as follows:

$$\text{MVI} = (\text{SFAs} + \text{MUFAs } \textit{trans}/\text{MUFAs } \textit{cis} + \text{CFAs})$$

A low value using this index indicates high membrane fluidity. This index does not, however, consider the length of the acyl chains although it is known that chain shortening increases the membrane fluidity.[109] Longer chains more easily span the width of the bilayer promoting acyl chain packing. Moreover, the melting point of short chain fatty acids is lower allowing a greater fluidity at low temperature.

Changes in the composition of phospholipid head groups have also been associated with homeophasic adaptation. These changes affect the physico-chemical properties of membranes due to differences in melting

temperature between phospholipids and by controlling the bilayer/non-bilayer phase preference of lipids.[135]

Another index, the phospholipid response index (PRI), was formulated to correlate lipid ordering, fluidity and changes in phospholipid composition, including head groups size, chain length and degree of fatty acid saturation. It was used to characterise the bacterial response of five *Pseudomonas* archetypes to toluene exposure.[30] PRI is arbitrarily defined as:

$$\mathrm{PRI} = \frac{(N_{\mathrm{CL1}} \times \mathrm{DS}_1 + N_{\mathrm{CL2}} \times \mathrm{DS}_2)}{2} + N_{\mathrm{HG,}}$$

where N_{CL} is the chain length numbers of the fatty acids on *sn*-1/*sn*-2 positions and is equal to 20 minus the length of the corresponding fatty acid chain; DS constant represents the degree of saturation of these fatty acids, fixed as 1 for SFAs, 5 for MUFAs and 10 for PUFAs (polyunsaturated fatty acids); N_{HG} is the head group number, assigned as 10 for PG, 2 for PE and PDME (phosphatidylmethylethanolamine). It is assumed that the larger the PRI, the less ordering in the membrane phospholipids and the more fluid the cell membrane.

MVI and PRI are convenient indices to monitor changes in membrane composition. However, these arbitrary values have yet to be experimentally correlated and validated with *in vivo* measurements of bacterial membrane fluidity.

Lateral diffusion of the fatty acyl chains can be measured by the intermolecular excimerization of pyrene. This fluorescent probe, when excited by light, can form a complex with an identical unexcited probe molecule. Such a complex is called an excimer and is recognized by the production of a new fluorescent band at a longer wavelength than the usual emission spectrum of the monomer. The rate of excimer formation depends on the lateral diffusion ability of the molecules inside the lipid bilayer. The ratio of excimer/monomer of pyrene formed in the lipid phase is therefore proportional to the lipid bilayer fluidity.[33]

The rotational diffusion of the fatty acyl chains in the membrane interior can be measured by fluorescence anisotropy using a hydrophobic fluorescent probe. The magnitude of the rotational Brownian motion of the probe inside the lipid bilayer, and hence the fluorescence polarisation value, depends on the size and shape of the probe molecule and its surrounding microviscosity.[125,138]

3.2. Biological Processes for Homeophasic Adaptation

Bacteria belonging to *Pseudomonas* genus have been intensively used as models to study homeophasic adaptation in response to environmental stresses because they are widely distributed in ecological niches and

because of their high versatility with respect to nutrient and growth conditions. Strains of *Pseudomonas* are also of ecophysiological interest due to their ability to degrade solvents (*P. putida*; *Pseudomonas mendocina*), a wide range of noxious organic compounds including chaotropic[123] and aromatic pollutants (*Pseudomonas vesicularis*, *Pseudomonas stutzeri*),[88] and due to their capacity to tolerate low temperatures (*Pseudomonas fluorescens*, *Pseudomonas syringae*, *Pseudomonas fragi*).

Homeophasic adaptation mechanisms can be classified in two types: (i) post-synthesis modification processes are the most rapid mechanisms of adjusting membrane fluidity. They occur *in situ* and involved the activity of desaturases, *cis–trans* isomerases and CFA synthases. These short term *in situ* mechanisms may represent the first line of defence of bacteria against membrane-altering stresses; (ii) *de novo* synthesis modification processes that alter the acyl chains and can only be conducted on newly forming lipids. These modifications concern the chain length, branching, saturation degree of acyl components, as well as the ratio of phospholipid head groups. The efficiency of these adaptive processes to ensure immediate survival is rather limited since they can only be performed on growing cells.

3.2.1. In Situ Modification of Fatty Acids

Cis–trans isomerisation Up to late 1980s, the *cis* configuration (hydrogen atoms located on the same side of the bond) was thought to be the only naturally occurring isomer in bacterial fatty acids. *Trans* isomers were further found in a restricted number of bacteria, including Pseudomonads and *Vibrio*.[18,87,90] *Cis–trans* isomerisation is facilitated after phospholipid synthesis by a constitutively expressed periplasmic isomerase Cti, a cytochrome c type protein.[51,131]

Many conditions known to disturb membrane fluidity such as increased temperature, osmotic stress, low pH, exposure to organic solvents and heavy metal activate the *cti* system.[57,100] A plethora of studies concerning the modulation of the *cis*/*trans* fatty acid ratio as a stress adaptive mechanism in *Pseudomonas* strains has been performed. Examples are presented in Table 1.

Recent data reveal that, in *P. putida*, *trans* fatty acids are predominantly formed under non-growth conditions and upon shock impact.[40] It is not clear how the activity of Cti is regulated since the gene encoding Cti appears not to be controlled at the transcriptional level.[62] The decrease in acyl chain ordering may facilitate the penetration of the enzyme into the membrane bilayer and thus, offer a better access to the substrate.[62,132] Post-translational modifications such as phosphorylation and glycosylation are also considered as possible mechanisms for controlling Cti activity.[62]

Cyclpropane fatty acid synthesis CFAs are formed by methylenation of the double bond of UFA using S-adenosyl methionine (SAM) as the donor for

Table 1. Changes in fatty acid component of phospholipids in response to stress exposure in *Pseudomonas.*

Strains	Conditions	*cis*/*trans* ratio	CFA ratio	UFA/SFA ratio	Membrane fluidity	Reference
P. aeruginosa ATCC 15442	Growth 15 to 40°C	nd	nd	D	nd	[29]
P. putida NCTC 10936	Growth 20 to 35°C	NC	NC	D	D*	[74]
	Incubation 20 to 35°C	D	NC	NC	D*	
	Incubation + 4-chlorophenol	D	NC	NC	D*	
P. stutzeri	Growth + naphthalene	nd	D	D	nd	[88]
P. vesicularis		nd	D	I	nd	
P. sp. JS150		nd	D	D	nd	
P. nautica IP617	Growth 13 to 20°C	nd	nd	D	nd	[28]
	Growth + n-eicosane	nd	nd	D	nd	
P. syringae Lz4W	Growth 5 to 28°C	D	NC	D	D**	[63]
	Growth + toluene	D	nd	nd	D**	
P. putida CN-T9	Growth + toluene	D	nd	NC	NC*	[61]
	Incubation + toluene	nd	nd	nd	I*	
	Growth 30 to 37°C	D	nd	NC	nd	
P. putida KT2440	Growth expo. to stat. phase	I	I	D	D*	[89]
P. putida mt-2	Growth + PEG 8000	D	nd	NC	nd	[38]
P. fluorescens SF1	Growth expo. to stat. phase	nd	I	D	nd	[32]
	Growth 16 to 36°C	nd	NC	D	nd	

D: Decreased, I: Increased, NC: No change, nd: Not determined, * Membrane fluidity measured by fluorescence polarization assay, ** Membrane fluidity measured by intermolecular excimerization of pyrene (see Chapter 3.1, Page 200).

the methylene group. The reaction is catalysed by the cytosolic CFA synthase encoded by the *cfa* gene.[18,31,122] Cyclopropane rings stabilise the acyl chains into a *cis* conformation; CFAs pack more poorly into the phospholipid bilayer than do *trans* UFAs.

The link between the activation of *cfa* expression or CFA synthase activity and a change in membrane fluidity is not yet established. *cfa* expression seems to be activated by conditions that provoke a growth slow-down as described for *P. putida* KT2440[89] and *P. fluorescens* SF1[32] during entry to stationary phase. Conversely, growth of *P. fluorescens* at different temperatures ranging from 16 to 36°C barely affected the percentage of CFA in the membranes.[32] Examples of modulation of *Pseudomonas* CFA content in responses to stresses are presented in Table 1.

The sigma factor RpoS, which controls the expression of stress genes and secondary metabolism in various bacteria,[72] is reported as a major regulator of *cfa* expression in *Salmonella enteritica*[60] and in *E. coli*.[134] Consequently, the levels of this enzyme increase upon entry to the stationary phase. This increase is transient since the protein is subject to rapid proteolytic degradation.[16]

3.2.2. De Novo Modification of Fatty Acids

Changes in saturation degree Fatty acid biosynthesis is coordinately regulated according to changes in the environment. When increased fluidity is needed, more unsaturated fatty acids (UFAs) and, in the case of Gram-positive bacteria, branched-chain fatty acids are produced. Aerobic desaturation of existing fatty acids by transmembrane desaturase is only found in bacilli,[1] in cyanobacteria[77] and in mycobacteria.[95]

Other bacteria use *de novo* desaturation processes and *P. aeruginosa* has been the first bacterium identified as having more than one pathway for UFA synthesis.[140]

The FabA-FabB pathway was first described in *E. coli*[42] and is also used by *P. aeruginosa* for UFA synthesis under all growth conditions.[50,140] The *fabA* and *fabB* genes are found in the Gram-negative alpha- and gamma-proteobacteria. This enzymatic pathway involves a dehydratation of β-hydroxydecanoyl-ACP by FabA to produce *cis*-3-decenoyl-ACP. The nascent acyl chain is therefore diverted into the UFA synthesis pathway. Then the *cis*3-enoyl is elongated subsequently by FabB and FabF to produce palmitoleoyl-ACP (C16:1 *cis*Δ9) and vaccenoyl-ACP (C18:1 *cis*Δ11).

DesA is another enzyme of *P. aeruginosa* found to be involved in UFA synthesis. DesA is a Δ9 fatty acid desaturase that introduces a double bond into existing fatty acyl chains esterified to lipids, exclusively at the *sn*-2 position of phospholipids.[140] DesA is thought to supplement the FabAB pathway under aerobic conditions to allow faster growth. DesA-like desaturase sequences are found in many beta- and gamma-proteobacteria species, but not in *E. coli* and *Salmonella*.

Finally, *P. aeruginosa* possesses an oxygen-dependent desaturase, DesB (acyl-CoA Δ9-desaturase) that works by inserting a double bond

into full length SFAs probably using their CoA-thioesther form as substrate. The data suggest that DesB selectively desaturates exogenously imported fatty acids. *P. aeruginosa* genomes contain several *fadD* homologues encoding Acyl-CoA synthetase (FadD), involved in fatty acid uptake as a first step for the assimilation and metabolism of exogenous fatty acids in *E. coli*.[9,103] Interestingly, the substrate of DesB may result from the transformation of phospholipids found in pulmonary surfactant, explaining why, amongst the *Pseudomonas* genus, the gene was only found in the human opportunistic pathogen species *P. aeruginosa*.[140] The *desB* gene is in an operon with *desC*, a gene encoding an oxidoreductase. DesC may play a role in the electron transport coupled with the DesB desaturation reaction. DesBC is only present in actinobacteria and a few proteobacteria species. DesB homologues are not present in other *Pseudomonas* species, such as *P. putida*, *P. syringae* and *P. fluorescens*.

The regulation of UFAs synthesis in *Pseudomonas* is not well understood. *E. coli* has been the main model to study FA synthesis in prokaryotes. In *E. coli*, *fabA* and *fabB* are activated by the transcriptional regulator FadR.[14,48] FadR is a member of the FadR/GntR family of H–T–H proteins and shares amino acid identities and similarities with other members of this protein family. FadR-DNA binding is inhibited by long chain acyl-CoA molecules which may accumulate during a growth deceleration (e.g. nutrient starvation, stationary phase) when phospholipid synthesis but not FA synthesis is repressed.[26] The role of FadR is also to modulate the fatty acid metabolism in response to available fatty acids. In absence of external fatty acids it represses the transcription of genes for fatty acid transport and degradation and activates fatty acid synthesis.[20] So far, from *in silico* analysis of completed bacterial genomes, FadR was only found in γ-proteobacteria colonising vertebrates and plants.[56] In addition to FadR, FabR a member of a TetR family of transcriptional regulators represses *fabA* and *fabB* expression in *E. coli*.[139] However, the regulatory ligand or the mechanisms leading to the activation of FabR are not known. It is interesting to note that the temperature control of UFAs in *E. coli*, and the increase in the amounts of UFAs at low growth temperatures is carried out by a direct effect of temperature on the activity of the FabF condensing enzyme.[22] In *E. coli*, strains carrying *fabA*'::*lux* fusions are responsive to a large variety of environmental pollutants (alcohol, phenol derivatives, halomethanes, aromatics, detergents) and may serve as microbial toxicity biosensors.[7]

In *P. aeruginosa*, the activity of DesA is thought to be controlled negatively by high UFA content in membrane phospholipids, and it is elevated in a *fabA* mutant which displays high SFA C16:0 content. The expression of *desA* is also regulated by changes in oxygen availability, by an as yet uncharacterised mechanism.[140]

The expression of *desB* and its adjacent gene *desC* is controlled by the newly identified repressor DesT. It is postulated that DesT may function as a sensor for exogenous fatty acids (Figure 3).[140]

DesBC introduce double bonds into the acyl-coA (C16:0-CoA, palmitoyl coenzyme A; C18:CoA, octanoyl coenzyme A) resulting of the activity of FadD on exogenously acquired fatty acids. The expression of *desBC* is negatively controlled by the transcriptional regulator DesT. The type II Fatty Acid bioSynthetic pathway (FASII) is the main source of endogenous UFAs via the activity of FabA, FabB and FabF enzymes on *cis*-3-decenoyl-ACP (C10:1-ACP; ACP, acyl carrier protein). Acyl chains are then incorporated to the glycerol backbone or the glycerol-3-phosphate molecule by two acyl transferases, PlsB and LptA (Figure 6), to give the Phosphatidic Acid (PA), the principal intermediate in the phospholipid biosynthetic pathway. DesA introduces a double bond into intact phospholipids. The expression of *desA* is repressed by high oxygen level.

Although little is known about the precise mechanisms for the regulation of UFA synthesis in *Pseudomonas*, many studies have shown that ratio of UFAs/SFAs changes significantly in *Pseudomonas* strains exposed to membrane-altering conditions (Table 1). Examples include the increase in UFAs in membrane phospholipids for *P. aeruginosa* ATCC 15442 and *Pseudomona nautica* exposed to low temperatures,[28,29] for *P. putida* grown in the presence of ethanol,[43] and for *Pseudomona oleovorans* incubated with light hydrocarbons,[117] and a decrease of this ratio for *P. putida* S12 exposed to toluene.[43]

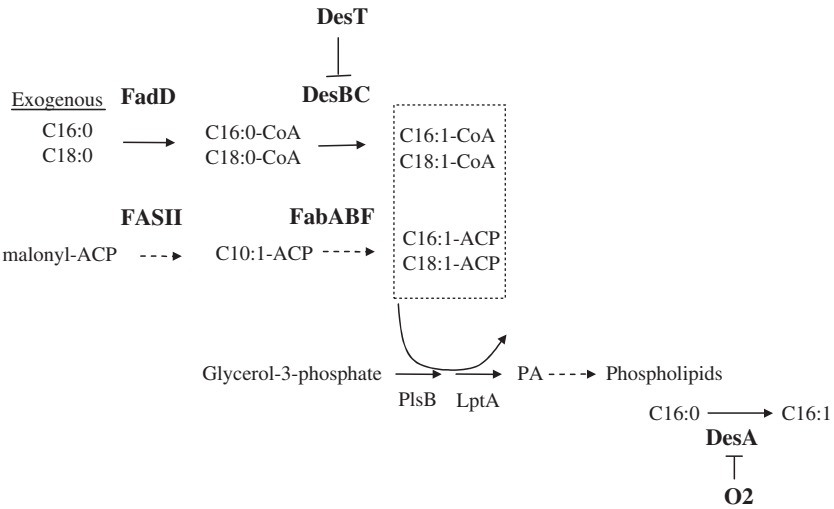

Figure 3. Model for fatty acid desaturation in *P. aeruginosa*.

Interestingly, by proteome analysis of *P. putida* KT2440, FabB was shown to be overexpressed under phenol stress.[110] This is in contradiction with the data from Heipeipper *et al*.[44] on the percentage of UFA in *P. putida* P8 exposed to phenol, and with the notion that bacteria counteract the deleterious action of hydrophobic compounds by decreasing their membrane fluidity.[99] This serves merely to demonstrate the complexity of UFA synthesis regulations as part of a stress response.

Changes in phospholipid head groups Less data are available on head group composition than on fatty acid composition of stress exposed bacteria. Solvent tolerance studies in *P. putida* DOT-T1 have shown a decrease in zwiterionic PE molecule concomitant with an increase in acidic PG and CL levels in solvent exposed (toluene) bacteria. This modification is thought to lower the membrane fluidity since PG and CL have a higher transition temperature than PE.[99,100] Similar results were found for *P. putida* S12 exposed to toluene[135] and for *P. putida* strain Idaho exposed to *o*-xylene.[96] In this last case, the total amount of membrane phospholipid fatty acids also increased in solvent exposed bacteria.

Changes in head groups in response to osmotic stress have been reported,[39,109] possibly as a mean for bacteria to increase the negative charge of their membrane (i.e. increase the proportion of anionic phospholipids) and consequently attract cations to stabilise the membrane structure.[23,126] The regulatory mechanisms used by bacteria to modulate the ratio of each phospholipid are not yet known.

There is still a paucity of information on the molecular mechanisms by which *Pseudomonas* strains, and prokaryotes in general, sense the alterations in their membrane physical properties to activate the expression of genes involved in homeophasic adaptation.

Moreover, few studies have correlating *in vivo* measurements of membrane fluidity with PLFA profiles (Table 1). Therefore, for most studies it is difficult to state conclusively that the changes in PLFA observed in response to membrane-altering conditions really impact on homeophasic adaptation. However, the composition of membrane lipid is a useful biomarker for the analysis of the dynamics of microbial communities,[49,129,133] the assessment for toxicity of organic compound,[46] and to monitor the pollution in specific environments.[58,119]

4. MEMBRANE AS SENSOR FOR GENERAL STRESS RESPONSE

Stress responses in bacteria have been intensively studied, however the primary sensors are still poorly defined. There are significant overlaps amongst bacterial adaptive mechanisms to various stresses.[80] This may result in part

from redundancies in regulatory networks. However, this may also reflects the activation of multi-stress sensors. The membrane, whose fluidity is affected by many different environmental stressors, appears as a likely candidate to sense and transduce the stress signal(s).

Subtle membrane alterations were shown to be critically involved in the conversion of signals from the environment into the transcriptional activation of stress genes. More importantly, changes in phospholipid composition or in membrane properties affect not only various membrane functions (such as the homeophasic adaptive mechanisms described previously) but also non-membranous cellular events.

The study of stress-related regulatory networks in relation to membrane signalling is difficult since multiple cell targets are affected by one single external stressor. The best example is the changes in temperature that affect not only the membrane fluidity, but also the supercoiling of DNA, the mRNA conformation and the protein folding, all playing a role in gene regulation and thermoregulation processes.[53] Therefore, the approach consisting of applying external stress to access the impact of membrane alterations in gene regulatory adaptive mechanisms is questionable and studies involving genetically modified membrane mutants have evolved.

Moreover, cell membranes are involved in a variety of processes such as carrier-mediated transport, activity of membrane-bound enzyme, cell division, DNA replication and respiration.[19] Modification of the membrane lipid composition may affect a number of cellular functions independently of changes in gene transcription and combined global approaches such as transcriptomic will be needed to identify common altered regulatory networks in response to various membrane-altered conditions.

4.1. Membrane as Stress Sensor in Bacteria Other than *Pseudomonas*

A phospholipid specific stress signal was first described in *E. coli* for which imbalanced phospholipid compositions was obtained by genetic manipulation (*pgsA3* and *pssA* mutants deficient in CL/PG and PE, respectively, Figure 4) and led to a decrease in the levels of transcripts (*fhlD*, *fliA* and *fliC*) involved in the flagellin chemotaxis regulon,[64,112] in outer membrane protein OmpF synthesis, in manganese containing superoxide dismutase SodA synthesis and in suppression of SOS-induced filamentation via the expression of *sulA*.[55] Flagellin gene expression was also inhibited by the mutation in *psd* gene encoding a phosphatidylserine decarboxylase, leading to a partial substitution of PE with phosphatidylserine.[112] It was demonstrated that the phospholipid imbalance resulted in an increased expression of the antisense RNA *micF* leading to a decrease in *ompF*, *fhlD*, *fliC* and *fliA* expression. However, the precise molecular mechanisms underlying these phenomena are still not understood.[55]

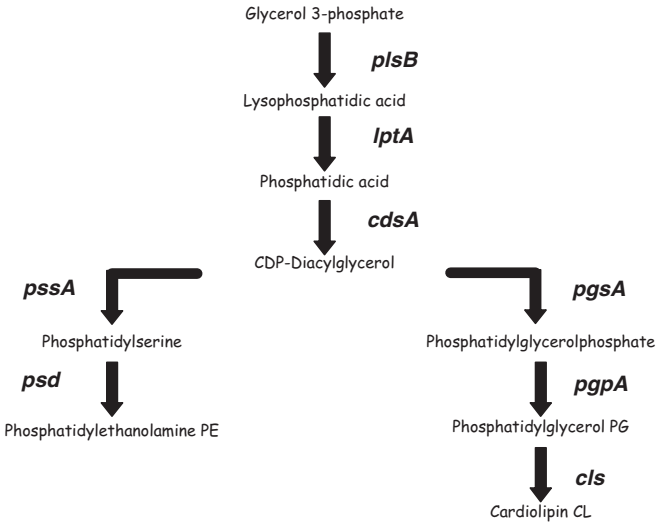

Figure 4. Genes involved in the biosynthesis of phospholipids and metabolic intermediates in *E. coli*. The sequenced genomes of *P. aeruginosa* PAO1 and PA14, *P. fluorescens* Pf0–1 and Pf-5, *P. putida* KT2440, *P. syringae* pv. *phaseolicola* 1448A, *P. syringae* pv. *tomato* str. DC3000 and *P. syringae* pv. *syringae* str. B728a possess homologues of all genes involved in this process with the exception of *pgpA* involved in the dephosphorylation of PGP to form the anionic phospholipid PG, for which significant homologues are found only in *P. aeruginosa* PAO1 and PA14, in *P. fluorescens* Pf-5 and in *P. putida* KT2440. (M. Cullinane and C. Baysse, unpublished observation). However bacteria, such as *E. coli*, may possess several phosphatidylglycerophosphatases.[17]
PlsB, glycerol-3-phosphate acyltransferase; LptA, lysophosphatidic acid acyltransferase; CdsA, CDP-diglyceride synthase; PssA, phosphatidylserine synthase; Psd, phosphatidylserine decarboxylase; PgsA, phosphatidylglycerophosphate synthase; PgpA, phosphatidylglycerophosphatase A; Cls, phosphatidylserine/phosphatidylglycerophosphate/cardiolipin synthase.

In *E. coli*, the absence of PE was also correlated with the activation of *degP* expression, encoding a stress inducible periplasmic protease.[85] Interestingly, it was demonstrated that this effect was the result of an activation of an envelope stress response pathway. The best characterised of these envelope stress response systems are the two-component systems CpxAR and BaeSR, and the extracytoplasmic sigma factor σE of *E. coli*, which are induced by envelope perturbations such as adhesion to abiotic surface or alteration in membrane protein folding and biogenesis.[106] The absence of PE activates the CpxAR system where CpxA is a membrane-localised sensor histidine kinase and CpxR the cognate response regulator. CpxRA homologues exist in many Gram-negative bacterial pathogens, but not in *Pseudomonas* strains. The primary function of the CpxRA two-component signal transduction system is the response to extracytoplasmic stress, also called "envelope stress response". It is involved in the mainte-

nance and adaptation of the bacterial envelope in response to a variety of stressors, such as alkaline pH, overexpression of the lipoprotein NlpE, misfolded pilus subunits and alteration in membrane lipid composition. CpxR activates a subset of target genes encoding envelope protein folding and degrading factors such as the major disulphite oxidase DsbA, and two peptidyl-prolyl-isomerases, PPiA and PPiD.[98,107] The mechanism of induction of CpxA is not understood. One hypothesis that has been proposed is that the alteration in cell envelope structure, such as the lack of PE, leads to an activation of CpxA.[85]

The imbalance in membrane phospholipids in *E. coli* also influences the synthesis of alkaline phosphatase at the level of gene transcription.[84] This effect is underpinned by changes in membrane sensor functioning. In *pssA* and *pgsA* mutants, the P_{PHO} promoter activity is significantly inhibited.[36] The expression of the genes with a P_{PHO} promoter (PHO regulon) is normally controlled by a two-component system implicated in the inorganic phosphate (Pi) signal transduction and which consists of the transmembrane sensor PhoR and the transcription regulator PhoB.

It is therefore possible that the activity of the transmembrane sensors such as CpxA and PhoR is affected by their lipid environment which impacts on their topology and assembly. Two examples of transmembrane sensors directly affected by the membrane fluidity for their topology and activity have been reported for cyanobacteria and *Bacillus subtilis*.

The histidine kinase Hik33 of *Synechocystis* sp PCC6803 was identified as a multifunctional sensor able to perceive a variety of signals.[83] The replacement of polyunsaturated lipids by monounsaturated lipids in the membrane of *Synechocystis*, obtained by mutation of *desA* and *desD* genes encoding a Δ12 and Δ16 desaturases, respectively, provokes a measurable decrease in the membrane fluidity.[121] Interestingly, these mutations induce the expression of cold-inducible genes, except in a *hik33* mutant background.[54] Hik33 possesses two transmembrane domains in the amino acid terminal region and experimental data strongly suggest that Hik33 might recognise the rigidification of the membrane as an activating signal.[54] Hik33 is also involved in sensing hyper-osmotic stress[54] and salt stress,[83] both negatively affecting the membrane fluidity. A hypothetical scheme for the activation of Hik33 postulates that the decrease in membrane fluidity leads to a compression of the membrane bilayer that forces the membrane spanning domains to move close together with a final dimerization and autophosphorylation of the histidine kinase domains (Figure 5).[76]

Resembling Hik33, DesK is a membrane-bound sensor of *B. subtilis* that regulates the *des* gene encoding a Δ5 acyl lipid desaturase. DesK works in tandem with the response regulator DesR. The DesKR system is inhibited by addition of exogenous UFAs that enhance the fluidity of the membrane (see below) when incorporated into the lipid bilayer.[2]

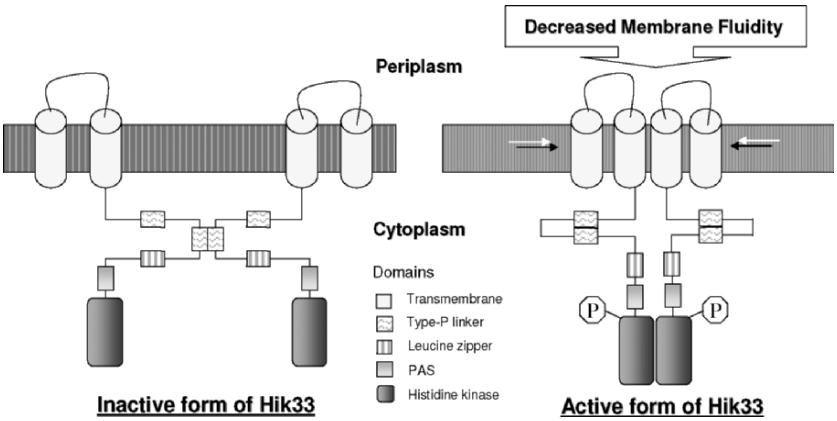

Figure 5. Hypothetical model for the activation of the prokaryotic multi-stress sensor Hik33 by decreased membrane fluidity and subsequent dimerization of histidine kinase cytoplasmic domains. See text for details. From Los and Murata, 2004.[76]

It was demonstrated that the C-terminal fragment of DesK acts as an autokinase and that the transmembrane segment is essential to sense change in membrane fluidity and to regulate the ratio of kinase to phosphatase activity. The phosphorylation may occur upon rigidification of the cytoplasmic membrane and subsequent movement of the transmembrane domains close together, as hypothesised in the case of Hik33.[3]

When fatty acids are provided exogenously, they are incorporated into phospholipids, via the fatty acid transporter encoded by *fadL*[103] and the *sn*-glycerol-3-phosphate acyltransferase reaction.[52] Therefore, when cells are exposed to UFAs or some branched-chain fatty acids (or their precursors), membrane fluidity is increased while exogenous SFAs are decreasing the membrane fluidity. In *Serratia marcescens* and in *Proteus mirabilis*, two transmembrane sensors, RssA and RsbA, respectively, were found to inhibit the swarming motility in presence of exogenous SFAs. It is likely that these fatty acids affect negatively the membrane fluidity. This change may activate the transmembrane sensors and initiate a regulatory cascade of gene expression leading to an inhibition of the swarming motility.[67,70] It is intriguing indeed that RssAB also regulates cellular fatty acid composition. The *rssA* and *rssB* mutants display more UFA and branched-chain fatty acids than wild type strain in their membranes.[67] Like DesK and Hik33, RssA may be involved both in sensing and restoring the membrane fluidity.

4.2. Membrane Composition and Gene Regulation in *Pseudomonas*

It is known that the production of active metabolites by Gram-negative bacteria is mainly regulated by two-component systems. Interestingly a study performed with *P. fluorescens* strains as model organisms has shown a strong correlation between the PLFA profile and the presence of metabolites in the medium, both parameters varying accordingly to the growth conditions that is temperature, pH and growth phase.[32]

Most bacterial species whose genome has been sequenced contain less than 30 two-component systems. However, certain *Pseudomonas* genomes revealed an unexpected high numbers of two-component regulatory systems that could explain the ubiquity of these organisms and reflect the need for rapid adaptation to the diverse environments. For example, an *in silico* analysis of *P. aeruginosa* PAO1 genome revealed 63 histidine kinases and 64 response regulators.[104] *P. fluorescens* Pf-5 is a plant commensal bacterium that inhabits the rhizosphere and produces secondary metabolites that suppress soilborne plant pathogens. *P. fluorescens* Pf-5 has a complex array of regulatory systems including 32 predicted sigma factors, more than 300 genes encoding predicted transcriptional regulators and a variety of two-component signal transduction systems consisting of 82 histidine kinase domains and 120 response regulator domains.[93] Therefore it is tempting to consider that amongst all these sensors, specific multi-stress sensor(s) able to respond to a wide range of environmental stimuli by sensing a change in the membrane fluidity may exist.

In order to assess the impact of changes in the membrane phospholipid composition in signalling and gene regulation in *P. aeruginosa*, we have constructed by genetic manipulation a mutant with a reduced membrane fluidity. We have first demonstrated that LptA (PA0005) is the main LPA acyltransferase of *P. aeruginosa* and that *P. aeruginosa* possesses alternate acyltransferase to produce glycerophospholipid in absence of LptA.[6] These enzymes transfer the acyl chain from either acyl-coenzyme A (CoA) or acyl–acyl carrier protein (ACP) to lysophosphatidic acid (LPA). The product, phosphatidic acid is subsequently converted into the major membrane phospholipids (Figures 4 and 6). Like in the *Neisseria meningitidis nlaA* mutant, also deficient for an LPA acyltransferase synthesis, the implementation of an alternative enzyme result in alteration of the phospholipid composition probably due to difference in substrate specificity for acyl-ACP with different acyl chain lengths.[113]

We have shown that mutation of *lptA* changes the fatty acid profile of *P. aeruginosa* to increase the proportion of longer chain fatty acids C18 with a parallel decrease in C16 fatty acids.[6] Such modifications

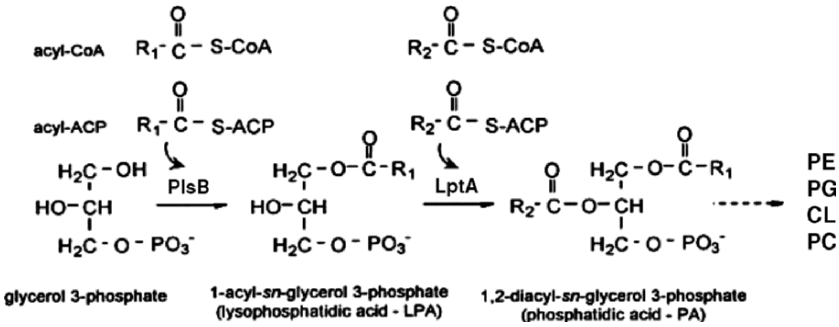

Figure 6. Enzymatic steps catalysed by the acyl transferases PlsB and LptA in *P. aeruginosa*.[6] PE, phosphatidylethanolamine; CL, Cardiolipin; PG, phosphatidylglycerol; PC, phosphatidylcholine.

decreased the membrane fluidity, as measured by membrane polarisation assay, consistent with the fact that longer chain fatty acids promote acyl chain packing by more easily spanning the width of the bilayer, conferring a more gel-like structure to the membrane.[108]

Interestingly, the *nlaA* inactivation in *N. meningitidis* led to pleiotropic effect on cell surface components, altering capsule and piliation.[120] We provided the first documented evidence that genetically modifying the phospholipid composition in *P. aeruginosa* not only alters membrane-associated phenotypes, such as flagella function, or growth at low temperature, but also plays a crucial role in gene regulation via the activation of the Quorum Sensing system and the stringent response mediated by RelA, the GTP pyrophosphokinase.[6] RelA drives the synthesis of (p)ppGpp when activated via the binding of uncharged tRNA to the A site of ribosome.[10] The alarmone (p)ppGpp is considered to be a major signal in response to many kinds of growth perturbations. It was originally identified in cells starved for amino acids[15] and subsequent studies correlated the production of this molecule with various stress exposures. We have observed a transcriptional and translational activation of *relA* in response to the induced alteration of phospholipid biosynthesis. This activation was independent of nutrient starvation and cell density since it was observed in rich medium and in early growth phase.

Inactivation of *lptA* led to a cell density-independent *relA*-dependant premature expression of the Quorum Sensing signals *N*-butanoyl- and *N*-hexanoyl-homoserine lactone (C4-HSL and C6-HSL), and to a *relA*-independent decrease in the production of Pseudomonas Quinolone

Signal (PQS) with accumulation of the metabolic precursor anthranilate.[128] This expression of signal molecules at low cell density was correlated with the up-regulation of transcripts encoding the *N*-AHL synthases RhlI and LasI and a down-regulation of *pqsA* and *pqsC*, both involved in PQS synthesis. In addition, a *relA*-independant increase in the expression of the regulatory RNA RsmZ (formely called RsmB) was observed in the *P. aeruginosa lptA* mutant compared to wild type (Baysse *et al*., unpublished data). RsmZ binds and inactivates the post-transcriptional repressor RsmA, a small RNA binding protein that controls secondary metabolite production and Quorum Sensing in *Pseudomonas*.[12,94] We further observed that the production of PQS is also altered in the RsmA mutant.[13] Therefore the inactivation of RsmA via the RsmZ overexpression in the *lptA* mutant may be responsible for the deficient production of PQS.

Only a few studies have reported an up-regulation of *relA* at the transcriptional or translational level. This effect has been shown by transcriptome analysis for *Mycobacterium tuberculosis* exposed to starvation[8] and by proteome analysis for *P. aeruginosa* under magnesium limitation.[37] It is known that membrane fluidity can be altered by the presence of ions such as Ca^{2+}, Mg^{2+} and Sr^{2+}.[102] It is tempting, therefore, to postulate that magnesium limitation and induced-phospholipid alterations increase the level of RelA via a similar mechanism related to membrane fluidity. The key question is what links alterations in the membrane to activation of RelA and of RelA-independent gene regulation processes? Interestingly, as we described previously for *P. mirabilis* and *S. marcescens*,[67,70] the swarming motility of *P. aeruginosa* PAO1 wild type is reduced to *lptA* mutant levels by the addition of exogenous SFAs (Baysse *et al*., unpublished). Thus, it is possible that transmembrane sensor(s) such as RssA and RsbA may negatively control the swarming motility in response to membrane alterations. This hypothesis provides an experimental strategy to investigate the presence of such sensor(s) in *P. aeruginosa*.

The comparative transcriptome analysis of *P. aeruginosa* wild type and the membrane-modified *lptA* mutant revealed the differential regulation of a number of genes involved in Quorum Sensing, in general stress responses and in the stringent response. In total, the expression of 137 genes was more than twofold up-regulated and the expression of 160 genes was more than twofold down-regulated in the *lptA* mutant compared to wild type (Baysse *et al*., unpublished). The extent of the transcriptional programme modification that resulted from the genetically induced membrane perturbation is consistent with a possible role of the membrane fluidity as an upstream signal for several stress adaptation (Figure 7).

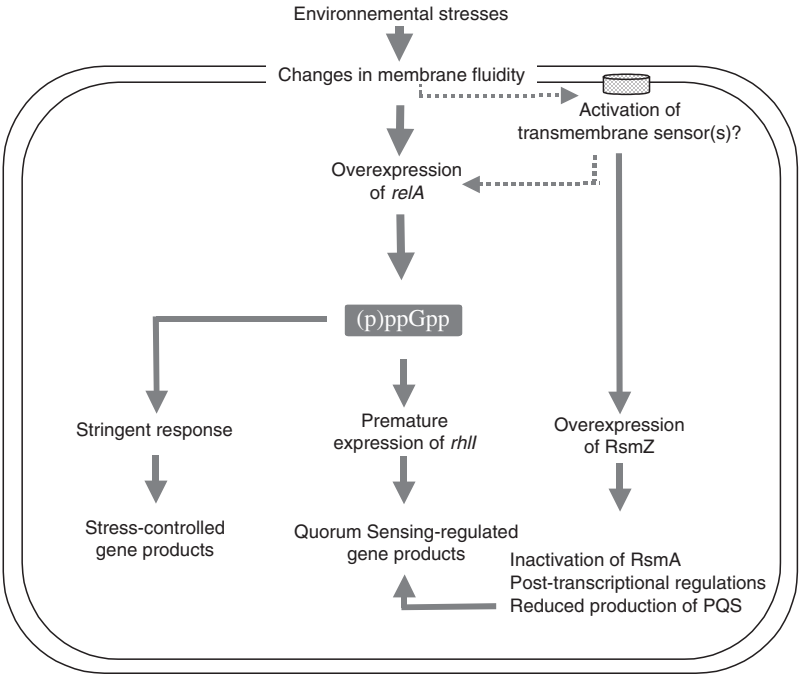

Figure 7. Model for the activation of a global adaptive response via the modification of the membrane fluidity in *P. aeruginosa*.[6] (p)ppGpp, guanosine 3′,5′-bis(diphosphate) (ppGpp) and guanosine 3′-diphosphate,5′-triphosphate (pppGpp); RelA, GTP pyrophosphokinase; PQS, 2-hydroxy-3-heptyl-4-quinolone; RhlI, *N*-butyrylhomoserine lactone synthase. Dashed lines indicate hypothetical events. Plain lines indicate experimentally verified events. See text for details.

5. CONCLUSION AND FUTURE PERSPECTIVES

The response of bacteria to different environmental stresses contains considerable overlaps. Examples of these overlaps include co-ordinately regulated products such as alternative sigma factors, heat shock proteins, cold shock proteins and universal stress proteins. The homephasic adaptation processes, which modulates the bacterial membrane fluidity, are also activated in response to many environmental stresses. In combination these responses correlate with an acquired cross-protection against several stresses by bacteria which have adapted to specific environmental conditions.[35,92] These overlaps in stress responses are the result of a parallel activation of multiple branched systems and of crosstalk between pathways.[47] Emergent data suggest that transmembrane sensor kinases that are

activated by changes in the inner membrane fluidity may also play a role in the overlapping of stress regulons.[3,83]

However, it is not yet established that membrane-fluidity sensing multi-stress sensors are widely distributed in microorganisms, and so far they have only been described in cyanobacteria.[76,91] Current research will attempt to establish the mechanistic link between the alterations in membrane composition/fluidity of *P. aeruginosa* and the resulting activation of global adaptive responses.[6] This will shed further light on how ubiquitous bacteria, such as *Pseudomonas,* are able to adapt their physiology and life cycle to allow growth in diverse environments.

Histidine kinase sensors, paired with specific response regulators, have been shown to react to various stresses by inducing distinct set of genes. Amongst them is the sensor Hik33 (with its cognate regulator Rre31) of *Synechocystis,* a multi-stress sensor reacting to changes in the membrane fluidity and previously described in this chapter.[54,83] These recent concepts emerging from the work in *Synechocystis* are at variance with the currently accepted model for two-component regulatory systems since distinct sets of genes are regulated by the specific two-component system couple in response to different types of stress. This phenomenon suggests that the specificity of a multi-stress sensor, reacting to a universal stress signal such as change of membrane fluidity, is mediated by some unknown factor(s). The identification of these factors will be crucial for a better understanding of the genetic flexibility and adaptability of bacteria in environments and may unravel new conserved prokaryotic processes for stress survival. As an example, recent data show that the messenger bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP), is used by various bacteria, including *Pseudomonas* strains, to control multicellular behaviour (such as biofilm formation, motility and virulence traits) in combination with sensory/regulatory modules.[66,105] c-di-GMP levels are controlled by widely distributed diguanyl cyclases, which share a conserved sequence motif (GGDEF domain) and by c-di-GMP phosphodiesterases that contain a conserved EAL domain. Interestingly, a number of these enzymes are anchored to the membrane via transmembrane segments. Amongst the future strategies that may evolve in the field of multiple stress adaptation study in *Pseudomonas*, the role of c-di-GMP as possible regulatory effectors in modulating membrane fluidity-mediated stress sensing may be an emerging thematic area worthy of investigation in the coming years.

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DATABASES

8

TOWARDS A GENOME-WIDE MUTANT LIBRARY OF *PSEUDOMONAS PUTIDA* STRAIN KT2440

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1. INTRODUCTION

Microbiology is experiencing exciting times thanks to the current explosion of knowledge. About 25 years after Watson and Crick resolved the structure of DNA,[104] Sanger's and Maxam's laboratories[56,86] developed easy ways to determine the nucleotide sequence of a segment of DNA. This in turn led to the development of new technologies that now make it possible not only to decipher the complete genome sequence of an organism, but also to analyze the global patterns of expression of genes based on genomic microarrays or the results of proteomic assays. Nonetheless, although transcriptional arrays and proteomic techniques can identify large numbers of genes expressed under particular conditions, the biological meaning of these correlations is generally unclear without further analysis.

Juan-Luis Ramos and Alain Filloux (eds.), Pseudomonas, 227–251.

The availability of the *Pseudomonas putida* KT2440 genome sequence has made transcriptomic[11,21,22,39,81,82,93,107] of this microorganism possible. These approaches focus on the monitoring of RNA, protein, and metabolite levels. The creation of large libraries of mutants defined by site-directed or random mutagenesis meant another step forward in our functional understanding of the *P. putida* genome. Such mutant libraries can be used to study each mutant's phenotype under defined conditions.[42]

With the use of transposons for mutagenesis, a link can be established between the insertion site and the phenotype, which can help to elucidate the biochemistry and the physiology of processes under study.[19,27,33,40,47,48,81,84,90] However, the generation of mutations that inactivate candidate genes is often time-consuming and commonly limits the number of genes that can be examined. Moreover, all of us who have worked in the laboratory have expressed countless times how much we would like to have a particular mutant to proceed with the analysis of our favorite organism. Indeed, this has been the case for us and for others working in our laboratory, and was one of the reasons that lead us to construct a large mini-Tn*5* transposon mutant library of *P. putida* KT2440, a saprophytic bacterium used as a model system to study biodegradation and interactions of a nonsymbiotic microorganism with plants. We determined the site of insertion of the mini-transposon by sequencing the DNA adjacent to the transposon border. The *P. putida* KT2440 mutant collection includes multiple insertions in a significant fraction of nonessential genes of the bacterium, and can be used systematically to examine, with other approaches, the phenotypes of inactivated candidate genes that have been associated with a biological process of interest. Alternatively, the mutant collection in itself can be used to screen for specific properties, that is, mutants unable to use a carbon or nitrogen source, identification of auxotrophs, identification of mutants tolerant or sensitive to a particular stress, and basically whatever phenotypic marker one might be interested in.

P. putida KT2440 is a plasmid-free derivative of a 3-methylbenzoate-degrading bacterium isolated from garden soils in Japan, which was originally designated *Pseudomonas arvilla* strain mt-2[68] and subsequently reclassified as *P. putida* mt-2.[65,80,105] This strain grows rapidly in minimal salt medium as well as in complex media such as Luria–Bertani (LB). Prof. Kenneth N. Timmis defined this strain as a nutritional opportunist *par excellence* and a paradigm of metabolically versatile microorganisms that recycle organic wastes in aerobic and microaerophilic compartments of the environment.[96] *P. putida* KT2440 is probably the best-characterized saprophytic laboratory pseudomonad. This strain was the first Gram-negative soil bacterium to be certified by the Recombinant DNA Advisory Committee of the United States Institute of Health as the host strain of a host-vector biosafety system for gene cloning in Gram-negative soil bacteria.[30] This, in turn, has facilitated work with recombinant derivatives in the

laboratory and in field assays – work which revealed that the strain retains its ability to survive and function in the environment when reintroduced in soils and waters.[5,25,57,58,60,61,62,69,73,74,76,77,80,83,85,103]

An extensive spectrum of versatile genetic tools developed in the last three decades has been particularly important for these analyses.[7,19,20,40,59,85,106] Indeed, the early genetic characterization of *P. putida* KT2440 and analyses of the pattern of gene expression of a good number of genes were based on the development of wide-host-range vectors for gene cloning, and promoter probe vectors for gene expression analysis.[6,59,75] Later, mini-transposons were developed for (i) mutagenic analysis, (ii) introduction of useful markers for environmental monitoring, and (iii) analysis of the heterologous expression of cloned genes from other soil organisms.[7,19,20,40,85]

Sequencing the KT2440 genome, analysis of mutants and BLAST comparisons of the genomes of KT2440 with those of other *Pseudomonas*, for example, *Pseudomonas aeruginosa* strain PAO1,[94] *Pseudomonas fluorescens* Pf5,[71] several *Pseudomonas syringae* strains,[32] and *Pseudomonas entomophila*,[102] revealed that the *Pseudomonas* sp. core genome consists of about 2,100 genes. These analyses also provided significant new insights into the biology of KT2440 and the underlying genomic basis of its biosafety features, which have further increased the usefulness of this model laboratory organism and its potential biotechnological applications.

2. BASIC CHARACTERISTICS OF THE GENOME OF *P. PUTIDA* KT2440

The global features of the genome of KT2440 were described in the original report by Nelson *et al.*[66] and its subsequent reanalysis in this series of books by Martin dos Santos *et al.*[53,55] In this chapter, we review some of the most relevant characteristics that are useful to consider for the construction of a collection of mutants.

The genome of strain KT2440 consists of a single circular chromosomes of 6,181,863 basepairs (bp), and has a mean G + C value of 61.6%. The initial annotation suggested 5,420 open reading frames (ORFs) ranging in size from 90 bp (the arbitrary size cut-off the authors applied to the algorithm to find genes) to almost 30,000 bp (see below). This set of genes specifies the proteome of KT2440. In addition, there are seven ribosomal RNA operons,[66,78] one of which occurs as a tandem (171,000–182,000) with a spacer of just a few hundred bp. Also present are 74 tRNA genes and two structural RNAs. The intergenic sequences were estimated to comprise 12.5% of the genome.[66]

Nelson *et al.*[66] proposed that *P. putida* strain KT2440 has independently evolved its own repertoire of transcription factors, indicating that an

important factor in the adaptation of an organism to a new environment is the emergence of a distinct set of these proteins. This is similar to what Madan Babu *et al*.[52] proposed for *Escherichia coli*. These authors observed that organisms with similar lifestyles have similar regulatory networks and have incorporated orthologous genes with similar patterns of network interconnection. At the global level, analysis of the dataset revealed that conservation of transcription factors is independent of the number of their target genes, and depends on the lifestyle of the organism rather than phylogenetic relatedness.

One characteristic we reported in the intergenic space of KT2440 is that the genome contains more than 800 copies of a species-specific 35-bp Repetitive Extragenic Palindromic (REP) element.[3] The REP sequence consists of a central palindromic motif and conserved nucleotides that define the head and the tail of the REP sequence. The consensus sequence is 5′-ccggcctcTTC*GCGGG*t*aa*a*CCCGC*tcctacaggg-3′ (small letters: 50–89% conserved residue; capital letter: 90–100% conserved residue). In contrast to *P. aeruginosa* and *E. coli*, in which the REP elements are typically organized in complex "bacterial interspersed mosaic elements," most REP elements in *P. putida* occur as single units or pairs: 225 REP sequences occur singly, 372 are located in tandem arrangements on opposite strands, and clusters of three, four, and five REP sequences are found in 36, 12 and 1 case, respectively. The role of these repeats in *P. putida* is still unknown, but they do not seem to be involved in gene regulation. Recently, Ramos-González *et al*.[79] reported that the REP sequence is the target of the so-called ISPpu*10* insertion sequence.

As noted above, based on BLAST comparisons of the genomes from five representative species of *Pseudomonas*, Vodovar *et al*.[102] identified a set of almost 2,100 genes that constitute the core genome of *Pseudomonas*. This indicates that the number of noncore genes of KT2440 is larger than the number of core genes. In this context it should be noted that the genome of KT2440 contains more than 100 regions of atypical oligonucleotide composition, including a number of gene islands.[66] Nine of these islands are larger than 20 kb, and apart from mobilization functions, they also encode functions such as amino acid and opine uptake and metabolism, arsenate resistance, resistance to heavy metals (e.g., copper and cadmium), oxidative stress response (peroxidase), biosynthesis of secondary metabolites, and a set of proteins that exhibit typical motifs of enzymes of a restriction-modification system.[35] These noncore functions are considered to contribute to the fitness and versatility of *P. putida* in its natural habitat. Therefore, the analysis of mutant libraries of a given pseudomonad can provide clues on their lifestyle in certain ecological niches and reveal specific properties of each microbe. In addition, analysis

of core mutants can reveal information of general interest for the genus *Pseudomonas*.

Table 1 lists the assignment of the identified coding sequences in the TIGR annotation of metabolic categories and the number of ORFs in each group that have been knocked out (July, 2006).

Among the 5,420 genes predicted according to Table 1 to code for proteins, around 2,143 have no functional annotation and were annotated as genes encoding "hypothetical proteins" (600 ORFs), which indicated that in most cases, no significant similarity to any other gene could be found. Other genes have been annotated as conserved hypothetical proteins (1,039 ORFs) and proteins of unknown function (504 ORFs); as of this writing no clues are available on the function of the proteins in these two groups.

Of the remaining proteins, some were annotated erroneously since the annotation was based mainly on BLAST analysis without functional tests. Some of these annotations have been corrected on the basis of the isolation of mutants and/or enzyme characterization (Table 2). For instance, PP3591 was originally annotated as a potential malate synthase; subsequent genetic and biochemical analyses revealed that it was in fact the second enzyme in the catabolism of D-lysine in the so-called AMA pathway.[64,82] The current version of TIGR (July, 2006) incorporates the

Table 1. Metabolic categories of annotated genes in *P. putida* KT2440.

Metabolic function	Number of genes	Number of knockout genes
Amino acid biosynthesis	126	36
Biosynthesis of cofactors, prosthetic groups, and carriers	149	27
Fatty acid and phospholipid metabolism	112	30
Central intermediary metabolism	79	15
Energy metabolism	459	124
Purines, pyrimidines, nucleosides, and nucleotides	65	6
DNA metabolism	118	29
Transcription	66	19
Protein synthesis	132	14
Protein fate	180	47
Cellular processes	361	97
Regulatory functions	535	143
Signal transduction	140	50
Transport and binding proteins	656	190
Cell envelope	327	74
Related to mobile elements	183	31
Conserved hypothetical proteins, hypothetical proteins, and proteins of unknown function	2,143	321

Table 2. Re-annotation of some open reading frames in *P. putida* based on functional analyses.

PP number	Gene symbol	Current annotation	Previous annotation	Reference
0166	*lapC*	Membrane fusion protein, ABC transporter. Secretion of LapA	HlyD family secretion protein	[29]
0167	*lapB*	ATPase, ABC transporter. Secretion of LapA	Secretion ATP-binding protein	[29]
0168	*lapA*	Large adhesion, surface associated. Biofilm formation	Surface adhesion protein	[29]
0213	*davD*	Glutaric semialdehyde dehydrogenase	Succinate-semialdehyde dehydrogenase	[82]
0214	*davT*	δ-Aminovalerate aminotransferase	4-Aminobutyrate aminotransferase	[82]
0382	*davA*	δ-Aminovaleramide aminohydrolase	Carbon-nitrogen hydrolase family protein	[82]
0383	*davB*	Lysine monooxygenase	Tryptophan 2-monooxygenase, putative	[82]
0691	*proB*	Glutamate-β-semialdehyde dehydrogenase	Glutamate 5-kinase	[78]
0806	*lapF*	Adhesion protein. Involved in seed colonization	Surface adhesion protein, putative	[29]
1002	*lysP*	Lysine permease	Arginine/ornithine antiporter	Revelles *et al.*, unpublished
1206	*acrD*	Arginine/ornithine, lysine/ ornithine antiporter	Porin D	Revelles *et al.*, unpublished
1449	*hlpA*	Hemolysin-like protein. Seed colonization and iron acquisition	Surface colonization protein, putative	Molina-Henares *et al.*, unpublished
1450	*hlpB*	HplB transporter/activator protein	Activation/secretion protein, TPS family, putative	Molina-Henares *et al.*, unpublished
1530	*dapD*	Tetrahydrodipicolinate succinylase	2,3,4,5-tetrahydropyridine-2-carboxylate *N*-succinyltransferase, putative	Molina-Henares *et al.*, unpublished
1588	*dapC*	*N*-succinyl diaminopimelate aminotransferase	Aminotransferase, class I	Molina-Henares *et al.*, unpublished
3590	*amaC*	D-Lysine 6-aminotransferase	Amino transferase	Revelles *et al.*, unpublished

Table 2. Re-annotation of some open reading frames in *P. putida* based on functional analyses—cont'd.

PP number	Gene symbol	Current annotation	Previous annotation	Reference
3591	*dkpA*	Piperidine-2-carboxylate dehydrogenase	Malate dehydrogenase, putative	64, 82
3596	*amaD*	D-Lysine dehydrogenase	D-Amino acid dehydrogenase	Revelles *et al.*, unpublished
4140	*cadA*	Lysine decarboxylase	Decarboxylase, Orn /Lys/Arg family	[82]
4473	*lysC*	Aspartate kinase	Aspartate kinase, monofunctional class	Molina-Henares *et al.*, unpublished
4486	*ltpA*	Basic amino acid ABC transporter, periplasmic protein	Basic amino acid ABC transporter, periplasmic basic amino acid-binding protein	Duque *et al.*, unpublished
4519	*lapE*	Outer membrane protein, ABC transporter. Secretion of LapA	Agglutination protein	29
4615	*ddcA*	Membrane protein involved in seed colonization	Conserved hypothetical protein	29
4695	*cbrA*	Two-component system, sensory box histidine kinase; amino acid utilization regulator	Sensory box histidine kinase	Vílchez, S., and Ramos, J. L., unpublished
4696	*cbrB*	Two-component system, response regulator	Nitrogen regulation protein NR(1)	Vílchez, S., and Ramos, J. L., unpublished
5257	*amaB*	L-Pipecolate oxidase	Oxidoreductase, FAD-binding	Revelles *et al.*, unpublished
5258	*amaA*	Δ^1-Piperidine-6-carboxylate dehydrogenase	Aldehyde dehydrogenase family protein	Revelles *et al.*, unpublished

corrected information. However, we feel that in contrast with the *P. aeruginosa* community, no real community efforts have been devoted to updating the annotation of *P. putida* KT2440. We wish to call our colleagues' attention to this issue since continuous updating of the annotations will make the mutant collection more useful to researchers and will help disseminate better information about KT2440.

3. CONSTRUCTION OF THE TRANSPOSON MUTANT COLLECTION

A mini-Tn*5* derivative carrying a Km^R gene was used to generate the mutant library (Figure 1).[3,12] The mini-Tn*5* for mutagenesis was chosen because it was previously shown to integrate at relatively random positions in the chromosome of KT2440.[92]

Transposon insertions were generated in *P. putida* by triparental mating of the strain with *E. coli* donor CC118λ*pir* bearing pUT-Km and *E. coli* HB101 bearing the helper plasmid pRK600, which carries conjugation-proficient functions.[19,40] The mutagenized cultures were plated on large bioassay-scale M9 minimal medium agar plates containing kanamycin (to select for transposon insertions), rifampicin (the *P. putida* strain used is a Rif^R strain), and benzoate or citrate as the carbon source (to select against donor cells). We found that the best way to obtain randomness in the insertion was by using different cultures of KT2440 and by carrying out separate mutagenesis assays for short periods of time, that is, 6 h. In the early steps of construction of the library, when we began sequencing we found a higher rate of siblings than expected because the conditions included a single mutagenesis mating and a long (24 h) incubation period.

In addition to these steps to ensure random selection of mutants, at certain stages clones were used to screen directly for a number of phenotypes. Since mini-Tn*5* transconjugants were selected on M9 minimal medium with citrate, a search was set up to identify clones deficient in the use of different carbon sources, for example, glucose, L-lysine, vanillate, quinate, etc. In this series of assays we kept specific clones deficient in the use of a given carbon source. In another series of assays we set up a specific program to identify the genes necessary for the biosynthesis of several amino acids, or genes necessary to use inorganic nitrogen sources (M. A. Molina-Henares, unpublished data).

We performed several statistical tests to ensure that the distribution of transposon insertions was random and that the mini-Tn*5* transposons had no hot spots in the *P. putida* genome. An important parameter that attests to the randomness of transposon insertions is the number of genes that carry a transposon insertion. A low number of genes hit by the transposon indicates bias in the pattern of transposition. To determine the theoretical number of genes that should be hit by at least one transposon, we used the neutral-base-pair model.[45] This model makes it possible to estimate the number of gene hits based on genome length, the number of transposon insertions, and the gene sizes. After applying this model to the library of 2,200 *P. putida* transposon mutants sequenced so far, we predicted that 2,000 genes would be mutated. The actual number of genes that were hit by at least one transposon was 1,680 (84% of the predicted

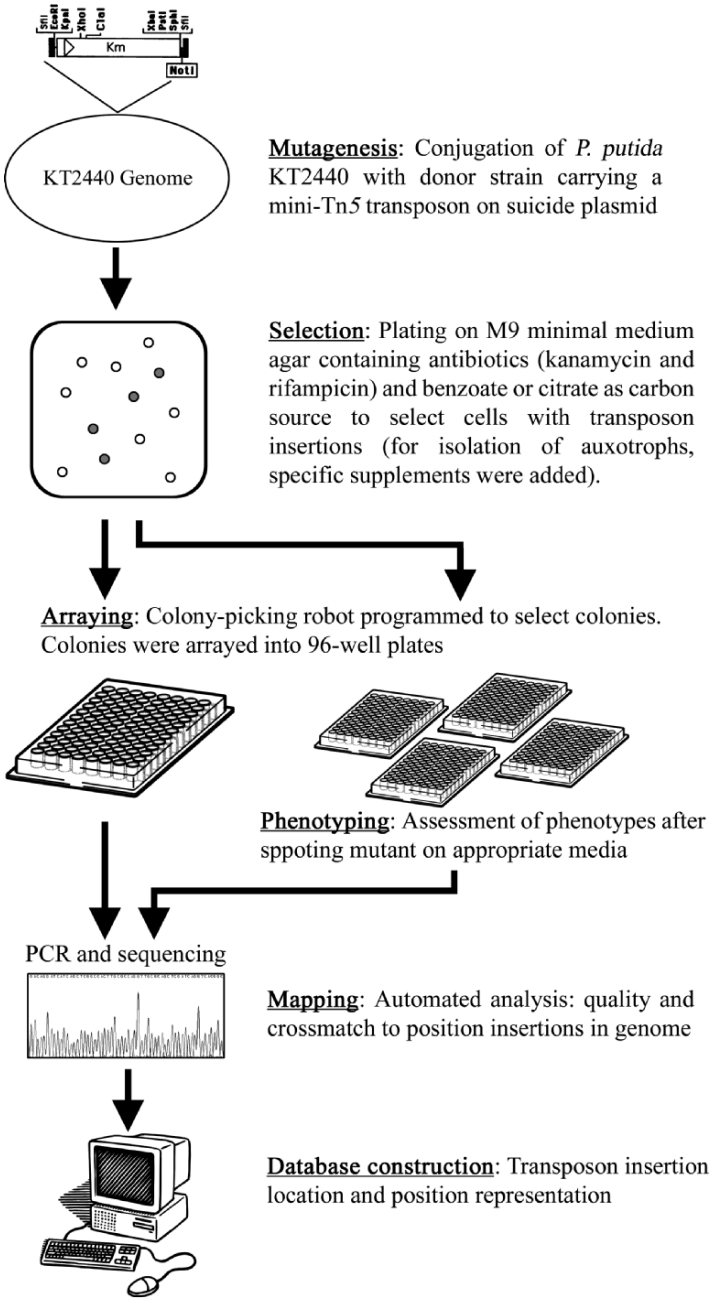

Figure 1. Generation analysis and maintenance of the *P. putida* KT2440 mutant library. (Details are given in the text.)

number in *P. putida*), which was consistent with expectations based on the neutral-base-pair model.

We also performed a genome-wide analysis of all transposon insertion sites in relation to G + C (A + T) content. Using a 100-bp window centered on the transposon insertion position, we calculated the mean G + C content. The differences between the mean G + C and A + T contents in all windows and the mean G + C and A + T contents of the whole genome were 0.4% (G + C) and 0.3% (A + T).

Once randomness had been ensured, we determined the best way to screen the mutant collection. We found that to avoid cross-contamination, 96-well plates were best, and in addition offered a format that allowed for efficient storage of all strains. The mutant collection consists of plates (96 strains per plate) that are stored frozen at –80°C. In our experience *P. putida* mutant strains stored in glycerol at –80°C are stable for several years, although certain clones lose viability rapidly. This is the case for mutants in a set of outer membrane proteins such as *oprL*, *tolB*, and *tolC*.[47,48] To avoid loss of viability, we set up a program for the long-term storage of specific mutant strains through lyophilization.[63] To prevent accidental losses, several copies of the collection are kept in different locations.

The methods used for sequence mapping and storage of mutants were originally developed for sequencing genomes in which no long reads are necessary. To identify the insertion site we have found it useful to be able to read the mini-Tn*5* border sequence because it unequivocally guarantees that the rest of the sequence is adjacent to it. DNA fragments which included transposon insertion junctions were amplified and sequenced using a semi-degenerate PCR scheme (Figure 1). The procedure consists of two PCRs. In the first, a series of mixed random primers are used together with a primer based on the sequence of the mini-transposon. After the first amplification, a second amplification is carried out with an internal primer based on the mini-Tn*5* sequence. This guarantees that DNA amplifications are based on previously amplified DNA.

An automatic search program written in HPP and available upon request was used to crossmatch the junction sequences against the *P. putida* genome, and then to determine the position of the mini-Tn*5* insertion relative to annotated ORFs. Data from the collection can be accessed at www.eez.csic.es/mutants.

3.1. Composition of the Mutant Collection

The collection is made up of more than 25,000 independent clones, and as of this writing we have identified the transposon insertion site in nearly 2,200 strains (see Table 1). Since the kanamycin resistant marker functions very well in counter selection, 100% of the Km^R

clones had an insertion at a location in the *P. putida* genome. Approximately 90% of the insertions were within ORFs, corresponding well to the fraction of the genome predicted to have coding functions.[15] Some of these insertions are in the same genes, but we kept only those that were located at different sites within the ORF. The ORFs that we hit most often were PP0168 and PP0806, which are the two longest ORFs in the genome and are the target of 9 and 11 independent insertions, respectively. The ORF encoding PP5076 (large subunit of glutamate synthase) was selected five times in our searches for specific types of mutants deficient in the use of inorganic nitrogen sources (nitrate or ammonia), or unable to use proline as a nitrogen source. Curiously, genes encoding peptidases, that is, PP0098, PP0435, and PP5320, were hit three times. Mutants in *catB*, *vanA* were found four times due to selective pressure. Only about 60 ORFs were the target of the mini-Tn*5* in two cases. In all, based on the limited number of clones analyzed to date, we can report that in almost 95% of the cases the mini-Tn*5* is located in a different gene. This is in accordance with the randomness of insertions by mini-Tn*5*.

3.2. Mutant Distribution

The assembly of the *P. putida* mutant collection, limited as it is for the time being, provides a source of mutants for researchers worldwide. Researchers can identify genes of interest in *P. putida* using bioinformatic searches, microarray or proteomic data. Once a list of interesting genes is generated, it can be compared with the mutant collection and the corresponding mutant strains can be ordered, saving time and resources that would otherwise be required to construct the mutants. To facilitate the distribution of mutant strains, we created a publicly accessible website at http://www.artemisa.eez.csic.es.

Researchers may search for strains by ORF number, gene name or gene abbreviation. If the requested mutant strain has been lyophilized we send an ampoule; if not we prepare mutants for distribution by streaking the appropriate strain onto an LB agar plate with kanamycin, and preparing a stab once the strain has grown. We recommend that immediately after receipt, each strain should be streaked out and a representative sample frozen and stored. We also strongly recommend that the identity of all strains be confirmed prior to use. Several tests can be run to confirm the nature of the mutation. One is to use individual colonies, PCR and appropriate primers based on the intact gene corresponding to the insertion, to verify that the target gene has been inactivated. Depending on the PCR conditions, either no fragment or a fragment

corresponding to a very large product will result from a correctly assigned mutant strain. To corroborate the exact position of a transposon insertion, the easiest approach is to carry out a PCR with a transposon-specific primer (5′-CGACCTGCAGGCATGCAAGCTTCGGC-3′) and a primer based on the sequence of the target gene. Sequencing of the PCR product identifies the precise insertion site.

4. METABOLIC FUNCTIONS

4.1. Brief Analysis of Transposon Mutants in Basic Carbon and Nitrogen Utilization Sources

The availability of the whole genomic sequence of *P. putida* KT2440 makes it possible to combine bioinformatics and experimental data on the metabolism of different nutrients[46,51,66,80,82,89,90,105] and allows us to reconstruct catabolic and anabolic pathways. Velázquez *et al*.[97] reconstructed the metabolic pathways available for the use of glucose, fructose, and gluconate in *P. putida* KT2440. Every protein involved in each of the steps was assigned a PP number as specified in the TIGR database (www.tigr.org). Figure 2 summarizes the steps in these pathways. It should be mentioned that the network of transformations that results from projecting genomic data is consistent with all observations made in various laboratories since the early 1970s on glucose, fructose, and gluconate metabolism in this bacterium.[46,89,98,99] Since *P. putida* KT2440 lacks fructose-6-phosphate kinase, glucose, and gluconate appear to be metabolized exclusively by the Entner–Doudoroff pathway,[28] while fructose is channeled through the Embden–Meyerhof (EM) route. An analysis of glucose metabolism in KT2440 provided a novel set of data (del Castillo *et al*., unpublished results) indicating that glucokinase and glucose dehydrogenase function simultaneously in the assimilation of glucose in strain KT2440, which contrasts with findings in other pseudomonads. The prediction that mutants in *edd* (PP1010 or phosphogluconate dehydratase) and in *eda* (PP1024 or keto-deoxy-phosphogluconate aldolase) would fail to grow on glucose was confirmed by del Castillo *et al*. (unpublished results), since these mutants appeared in screening assays intended to find mutants unable to use glucose (our unpublished results). Mutants in each of the steps confirm the proposed pathway based on bioinformatics analyses.

Regarding the use of inorganic nitrogen sources, mutants unable to use nitrate, nitrite, and ammonium were isolated as well as mutants that cannot derive nitrogen from certain sources of organic nitrogen (amino acids such as glycine, leucine, glutamate, phenylalanine, lysine, and proline). Mutants in assimilatory nitrate reductase (PP1703) and nitrite reductase

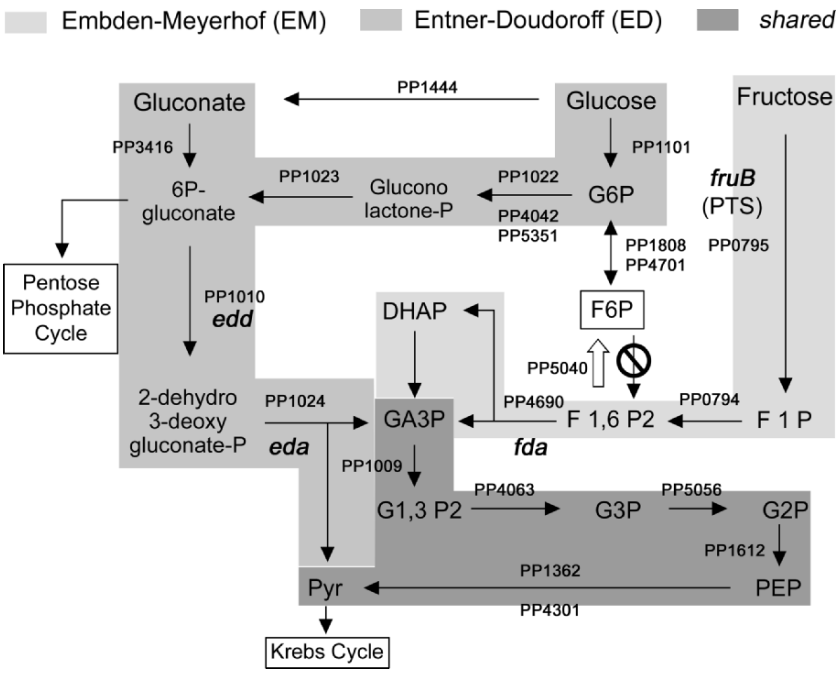

Figure 2. The metabolic network for glucose, gluconate, and fructose metabolism in *P. putida* KT2440. The Figure is based on the bioinformatics and experimental work of Velázquez *et al*. (2004), reproduced in part with permission from the authors and from the American Society for Microbiology, and on the work by del Castillo *et al*. (in preparation).

(PP1705) are available. We also identified clones unable to use NH_4^+ as a nitrogen source. A number of these mutants exhibited a knockout in one of the subunits of glutamate synthase,[9] which suggests that the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway is preferred for the assimilation of ammonium in spite of the fact that the strain encodes a glutamate dehydrogenase. An oddity of KT2440 is that it exhibits three GSs, and although mutants in each of them are available, each mutant grew well on inorganic and organic nitrogen sources, indicating that the three copies of GS can replace each other in functional terms.

Very few auxotrophs have been described for *P. putida* in the literature,[36,72,87] so we set up a specific program to isolate auxotrophs for Trp, Leu, and Ser. These mutants were selected to confirm the biosynthetic pathways for these essential metabolites (see Figure 3 for leucine and threonine biosynthesis). Alaminos and Ramos[1] showed that like other eukaryotic and prokaryotic microorganisms, *P. putida* KT2440 uses homoserine as the starting point for the biosynthesis of methionine. This first step

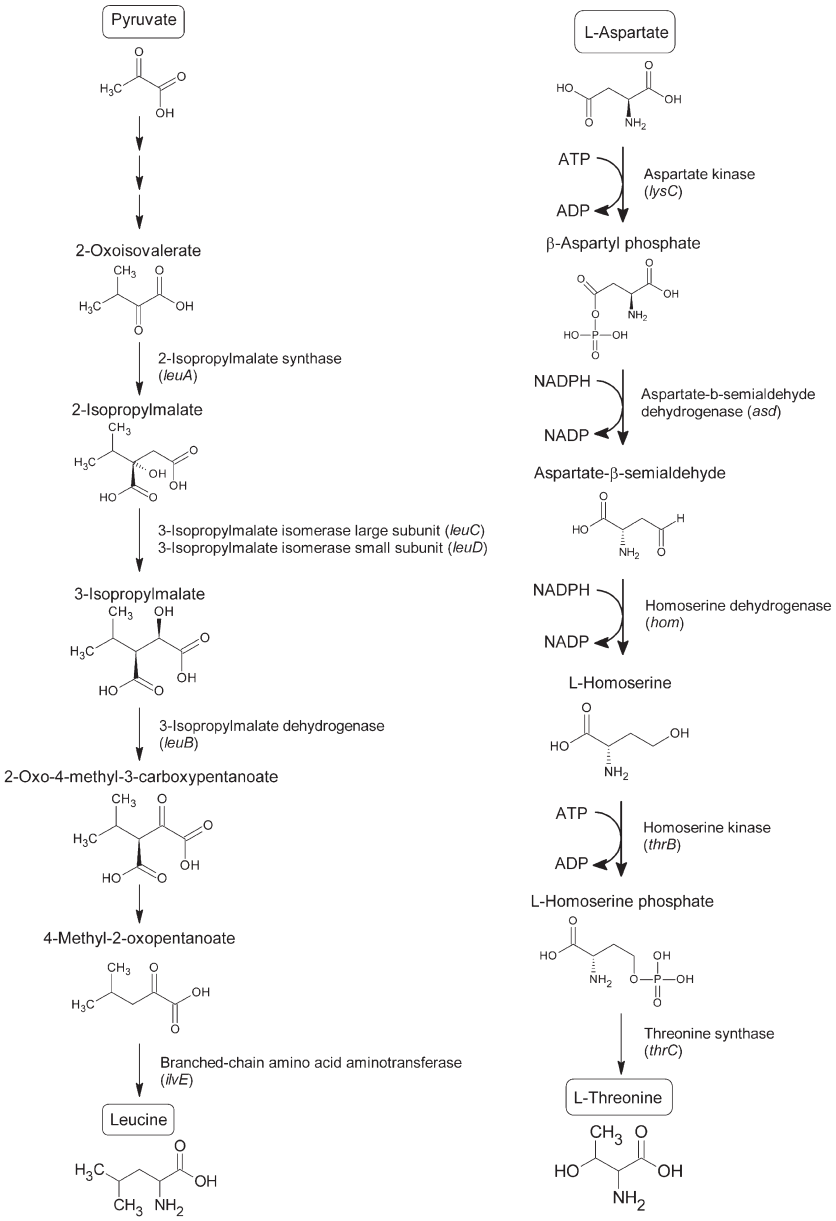

Figure 3. Biosynthesis of threonine and leucine in *P. putida*.

consisted on the acylation of homoserine to yield *O*-acetyl-homoserine in a reaction catalyzed by the *metW*, *metX* gene products, as is also the case in *P. syringae*.[2] The second step, as in Gram-positive bacteria and certain fungi, consisted on the direct sulfhydrylation of *O*-acetyl-homoserine into homocysteine, a reaction catalyzed by MetZ. This reaction also occurs in *P. aeruginosa*.[31] Eventually, homocysteine was converted into methionine in a reaction catalyzed by one of the two methionine synthase enzymes.

The use of rich LB medium facilitated the isolation of mutants in which metabolic steps related with the biosynthesis of an extensive range of amino acids and cofactors were affected, for example, amino acid auxotrophs or mutants deficient in the biosynthesis of biotin, folic acid, ubiquinone, pantothenate, coenzyme A, and thiamine.

A general strategy exploited by pseudomonads to degrade diverse aromatic compounds is to oxidize them to catechols, which can be funneled into a limited number of central pathways.[17,38] In KT2440, for example, Jiménez *et al*.[43] proposed that the initial steps in ferulate, *p*-coumarate, and *p*-hydroxybenzoate metabolism are mediated by different enzymes (upper pathways), but all routes ultimately converge via protocatechuate (ferulate and *p*-coumarate) or catechol (benzoate) on the 3-oxoadipate/β-ketoadipate pathway. Interestingly, this pathway is found almost exclusively in soil- and plant-associated microorganisms,[37,70,77,100] and presumably evolved in response to the large number of phenolic compounds synthesized by plants. Other relevant central pathways are the phenylacetate and homogentisate pathways. Some mutants unable to use these chemicals have been isolated, although despite several attempts, mutants unable to use quinate were not obtained, probably because several different pathways are available for the initial metabolism of this compound.

4.2. Nutrient Uptake Systems

P. putida KT2440 has very broad transport capabilities, with approximately 370 cytoplasmic membrane transport systems – 15% more than *P. aeruginosa* – that constitute about 12% of the whole genome. The largest family corresponds to the ATP-Binding Cassette (ABC) transporter (94 paralog members), a significant proportion of which is predicted to be devoted to amino acid uptake (Duque *et al*., unpublished). This is consistent with the ability of KT2440 to colonize plant roots, since root exudates are rich in amino acids, and reflects the physiological emphasis on the metabolism of amino acids and their derivatives to successfully compete in the rhizosphere.[44,49,50] In addition, *in vivo* expression technology (IVET) studies confirmed the induction of this type of transporter in *P. putida* when colonizing plant roots.[79] In the case of amino acids, multiple uptake systems can exist for a single compound. For

instance, we have reported that at least two complex uptake systems in KT2440 are involved in the uptake of L-lysine, and recently a third uptake system was identified as part of a cluster in which D-lysine dehydrogenase is present.[82]

P. putida KT2440 encodes various uptake systems for osmoprotectants such as glycine betaine (PP0871-PP0868) and proline (PP0294-PP0296 and PP2774-PP2775). Mutants in these systems are available and can shed light on the process. It has been proposed that up to five transporters are available for gamma-aminobutyric acid (GABA) uptake, namely PP4106, PP2911, PP4756, PP2543, and PP0284; however, no functional proof is available. We have found that one of these proteins, PP4106, is involved in the uptake of tyrosine, since PP4106 was isolated specifically as a mutant that failed to use this amino acid.

Consistent with its exceptional metabolic versatility, the KT2440 genome encodes more putative transporters for aromatic substrates than any currently sequenced microbial genome, including multiple homologs of the *Acinetobacter calcoaceticus* benzoate transporter BenK (PP3165), and of the *P. putida* 4-hydroxybenzoate transporter PcaK (PP1376).[67] In addition, in the KT2440 mutant collection we have identified porins such as PP3168 specifically involved in benzoate uptake. The genome of KT2440 also encodes the determinants for the import of some sugars; so far we have identified mutants in the fructose uptake system (PP0792-PP0793) as well as in gluconate uptake (PP3417).[95]

Martínez-Bueno *et al*.[54] reported that *P. putida* KT2440 exhibits up to 13 iron-siderophore uptake systems in spite the fact that the strain produces only one siderophore, pyoverdine, whose genes are clustered in three groups (PP4243-PP4246, PP4319-PP4327, and PP4219-PP4223) organized in a manner similar to that found in other pseudomonads.[14,34,101] Although the siderophore receptor for pyoverdine in each strain is highly specific for the siderophore the strain produces, fluorescent pseudomonads have been shown to use siderophores produced by other strains, uptake being mediated by other ferric-siderophore receptors. In the current collection, 11 mutants in different uptake systems are available, and their importance in niche colonization and biocontrol can now be tested.

Strain KT2440 has a large number of determinants for active efflux systems for metals such as arsenite (PP1929, PP2717), copper (PP5378-PP5379 and PP2204-PP2205), cadmium (PP0041-PP0045, PP2408-PP2411, PP5139), chromate (PP2556) and other toxic chemicals such as cyanate (PP0970, PP3751). Other efflux systems identified to date extrude paraquat (two paralogous sets: PP2576-PP2577 and PP0598-PP0599) and, interestingly, fusaric acid (PP1266-PP1263), a common fungal toxin produced by phytopathogens such as *Fusarium oxysporum*.[91] Mutants in the efflux systems that extrude arsenate and cadmium are available.

4.3. Regulation and Signal Transduction

As in other soil-dwellers, the genome of *P. putida* is large, and this is thought to reflect the fact that soil is relatively nutrient-poor, such that nutrients are varied and patchily distributed. To survive in soils, microorganisms need to use a variety of scarce nutrients, and therefore need several sets of metabolic genes and gene regulators.

Almost 10% of the genes in the KT2440 genome encode products involved in signal transduction and gene regulation, which reflects the evolutionary emphasis in this bacterium on monitoring and responding to a large number of environmental signals. Common transcription factors include the sigma factors RpoD (sigma-70), RpoN (sigma-54), RpoS (sigma-38), RpoH (sigma-32), FliA (sigma-27) and AlgT (sigma-22, homologous to RpoE in *E. coli*). The genome also contains a large number (19) of genes for extracytoplasmic function (ECF) sigma-70 factors, many of which are clustered with sensing or transport genes[54] (see Chapters 11 and 12 of Volume II in this series). The function of these ECFs is unknown. Site-directed mutagenesis mutants have been obtained for ECF-PP12, but only in *P. putida* DOT-T1E, a solvent-tolerant strain. A reduced number of genes making up less than 1% of the genome have been shown to be under the direct or indirect influence of this ECF.[26] Very few phenotypes have been associated to this mutant, the clearest example of it being increased solvent sensitivity.

4.4. Swarming and Chemotaxis

P. putida has previously been shown to attach as single cells to solid matrices, and can form biofilms on various surfaces. Intriguingly, proteomic analysis revealed that proteins belonging to the *P. putida* motility complex, including the flagellins, the basal body proteins and the chemotactic proteins, are expressed at higher levels in biofilm-grown cells than in planktonic cells.[4,13,88] Additionally, in liquid culture an aflagellate mutant was unable to form a pellicle at the air–water interface, and insertional inactivation of flagellar filament genes delayed pellicle formation, indicating that the motility complex is important for cell–cell interactions in mature biofilms.[10,41]

Bacteria respond to chemical stimuli by moving towards attractants or retreating from repellents. This phenomenon, called chemotaxis, enables bacteria to position themselves in their environments. The signals (stimuli) are perceived by membrane-spanning methyl-accepting chemotaxis proteins (MCP), which monitor environmental composition and transduce the signal via a number of receptor and transducer proteins to the flagellar apparatus, and thus influence the mode of rotation and the swimming

direction of the cell. *P. putida* KT2440 encodes 27 MCPs, suggesting that it can respond to a large variety of environmental signals. Adaptation is mediated by the level of methylation of MCP, which is controlled by methyltransferase CheR and methylesterase CheB. The activity of transducer CheY is regulated by the phosphatase CheZ.

It has been proposed that an MCP is involved in inorganic phosphate sensing (PP0562), that three MCPs (PP0320, PP1371, PP2249) are involved in aerotaxis, and that PP2257, PP2111, and PP4521 are able to sense amino acids. Mutants in PP1371 and PP4521 are available, and their role in energy sensing and movement towards oxygen can be tested. It is interesting to note that the uncharacterized chemotaxis cluster (PP1494-PP1488) is shared with *P. aeruginosa and P. syringae*, and a mutant is currently available for analysis.

Recently, Matilla *et al*. (unpublished) have shown that *P. putida* can swarm on solid plates. The process is dependent on lipopolysaccharides (LPS) and the presence of type IV pili, which are involved in the efficient colonization of the plant root.[18,23,24] The availability of the mutant collection has been of great importance in analyses of swarming in KT2440 (Matilla *et al*., unpublished results).

4.5. Adherence to Surfaces

The genome of *P. putida* encodes two unusually large proteins, the 6,310-amino acid PP0806 and the 8,628-amino acid PP0168, the second largest bacterial protein described so far. On the basis of work by Espinosa-Urgel *et al*.,[29] who found that transposon insertion mutants of both proteins fail to adhere to the surface of plant seeds, both proteins, as well as another large protein (PP1449, 1,508 amino acids), have been annotated as surface adhesion proteins. All three proteins exhibit a complex repetitive structure and atypical amino acid composition, and are threonine-rich and free of cysteine.[16,53] These clusters have often been knocked out through mini-Tn*5* insertions due to their large size rather than through sequence composition, since insertions occurred in different sites within the long ORFs. The availability of mutants in ORFs adjacent to these genes has been instrumental to elucidate their role in adhesion to surfaces.

Protein PP0168 contains three types of peptide sequence repeats: nine highly conserved N-terminal repeats of 100 amino acids, 29 highly conserved repeats consisting of 219 amino acids that form two subgroups (1–7, 8–29), and five hemolysin-type calcium-binding repeats at the C-terminus. Moreover, a C-terminal domain shows similarity to a von Willebrand domain involved in adhesion and signal transduction.[8] The second largest protein, PP0806, consists of about 65 repeats with an

average size of 85 amino acids. The first 35 repeats (with the exception of repeats 1 and 2) form seven clusters of five repeats. Towards the C-terminal end, the repeats exhibit an increasing degree of sequence variation. In-depth characterization at the molecular level of these proteins will help us to understand how *P. putida* KT2440 interacts with surfaces playing a role in the social behavior of this microorganism in different habitats.

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GENE REGULATION

9

THE USE OF MICROCALORIMETRY TO STUDY REGULATORY MECHANISMS IN *PSEUDOMONAS*

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Abbreviations: EMSA, electrophoretic mobility shift assay JTC, isothermal titration calorimetry

1. INTRODUCTION

Calorimetry or the measure of heat changes has been used for more than two centuries to study processes that release substantial amounts of heat. However, recent advances in instrumentation have made it possible to detect much smaller heat changes generated upon interaction of relatively small quantities of biomolecules. Over the last 30 years, the emergence of the new discipline of microcalorimetry has had increasing impact on life science by contributing to a better understanding of the thermodynamic mechanisms of interaction between biomolecules.[37,22]

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There are two microcalorimetric modes. Differential scanning calorimetry (DSC) detects heat changes that occur when a temperature gradient is applied to a sample. This technique is primarily used to study the thermodynamics of temperature-induced protein unfolding, which is an endothermic process.[3] Isothermal titration calorimetry (ITC) involves the titration of two biomolecules at constant temperature and monitoring heat changes caused by their molecular interaction.[9] Typically, an ITC instrument has a sample cell containing one ligand into which aliquots of a second ligand are injected from a syringe in a fully automated manner. Heat changes in the sample cell are measured with respect to a reference cell containing only water or buffer. ITC experiments are designed so that the biomolecule present in the sample cell becomes saturated by the ligand added via the syringe.

Figure 1 illustrates typical data from a simple ITC-binding experiment. The upper panel shows the raw data in which each peak corresponds to the heat changes caused by the injection of an aliquot of a ligand into buffer (A) or a solution of a protein capable of binding the introduced ligand (B). In both cases, peaks are directionally negative, indicative of exothermic events. Positive directional peaks represent endothermic events. Heat changes seen in trace B are the sum of heats caused by the binding and the

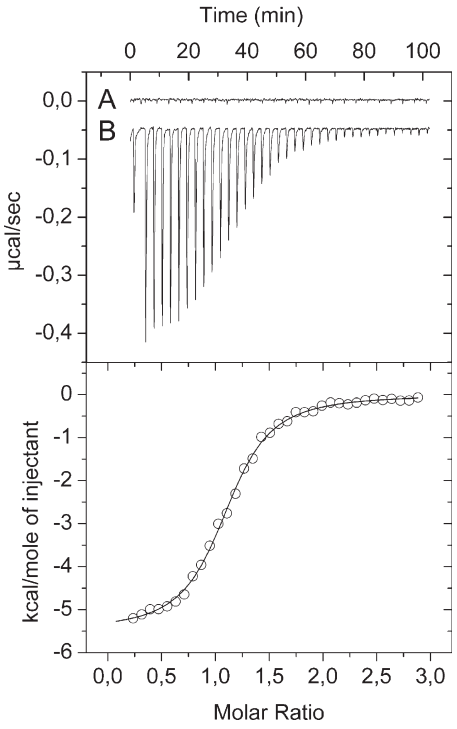

Figure 1. Isothermal titration calorimetry data for the binding of toluene to TodS. Heat changes (upper panel) and integrated peak areas (lower panel) for the injection of 1.6 µl aliquots of buffer (A) or 1 mM toluene (B) into 15.24 µM TodS. For clarity reasons, traces have been off-set on the *y*-axis. Derived thermodynamic parameters are given in Table 1.

dilution of the molecules, while trace A corresponds to heats of dilution only, which in this example are small. Since the aim of this experiment is to measure heats of binding, the integrated peak areas of trace B have to be corrected using the corresponding data from trace A and normalized for the concentration of each ligand. Such corrected and normalized data are shown as empty circles in the lower panel. Mathematical algorithms can then be applied to fit these data. In Figure 1, a model for the binding of a ligand to *n* identical independent sites[38] was applied using a global non-linear least-square method from Origin (Microcal Software, Inc.). A continuous line in the lower panel of Figure 1 shows the resulting curve fit.

Two parameters can be directly determined from the curve fit; the corrected heat changes represent a direct measure of the enthalpy change (ΔH) of the molecular interaction. For high-affinity binding, such as in Figure 1, this value corresponds roughly to the point where the curve meets the *y*-axis. The slope of the curve is related to the binding constant K_A. Generally, the steeper the slope, the higher is the affinity. Since the dissociation constant K_D is the inverse of K_A, the K_D value can be calculated from K_A. Having obtained K_A and ΔH, the changes in Gibbs free energy (ΔG) and entropy (ΔS) can be calculated using the equation:

$$\Delta G = - RT \ln K_A = \Delta H - T\Delta S,$$

where R is the universal molar gas constant, and T the absolute temperature. Binding stoichiometry can also be obtained from sigmoidal-binding curves such as that in Figure 1. This value corresponds to the molar ratio (lower *x*-axis in Figure 1) at the point of inflection of the curve. For the example shown, this molar ratio is close to 1:1, indicating that one molecule of the ligand from the syringe interacts with one molecule of protein in the sample cell. However, care needs to be taken since interpretation assumes that ligand concentrations are exact (a parameter that is sometimes difficult to determine for volatile compounds) and protein preparations are fully active (which is not always the case due to varying proportions of denatured and/or inactive protein).

A major advantage of ITC over alternative techniques is that both biomolecules are unmodified and in solution. In addition, the signal registered (heat) is a direct signal of a molecular interaction. Leavitt and Freire[16] provide further reading on the use of ITC. The aim of this chapter is to illustrate the kind of information obtained from microcalorimetric analysis as applied to regulatory studies in *Pseudomonas*. The examples we use here to illustrate the power of this approach are primarily derived from studies directed towards understanding the tolerance of *Pseudomonas putida* DOT-T1E towards environmental insults, but are generally applicable to regulatory studies in any organism.

2. THE EXPERIMENTAL SYSTEM

P. putida DOT-T1E is a paradigm of solvent-tolerant microorganisms since it can grow in the presence of high concentrations of extremely toxic and harmful compounds such as aromatic hydrocarbons.[25,26] This strain is not only able to survive in a growth medium saturated with toluene, but also to use this extremely toxic compound as a sole carbon and energy source. Amongst the molecular events which occur when this strain encounters toluene are the expression of three RND efflux pumps, termed TtgABC, TtgDEF and TtgGHI,[28] and the induction of the enzymes of the toluene dioxygenase (TOD) catabolic pathway[40] for conversion of toluene into Krebs cycle intermediates.

Each of the efflux pumps contains three types of subunits that share high sequence identity (60%) with those of the AcrAB-TolC multidrug efflux system,[21,36] and are thus likely to have a similar structure and subunit arrangement. Despite the high sequence similarity, the substrate profiles of the three toluene efflux pumps appear to be different. The TtgABC pump recognizes a wide range of hydrocarbons, antibiotics and secondary plant metabolites,[5,28,34] whereas the TtgDEF and TtgGHI pumps mainly efflux hydrocarbons.[28] The transcriptional repressor TtgR,[33] which belongs to the TetR family, controls the expression of the operon *ttgABC*,[5] whereas the IclR type repressor TtgV regulates the expression of operons for the other two pumps.[27,32] Both regulatory proteins function by the same mechanism; in the absence of effector molecules they bind to the –10/–35 region of their respective promoter thereby preventing transcriptional initiation by RNA polymerase. In both cases, binding of small effector molecules results in the release of the DNA-bound repressor to open the path for transcription.[8,33]

As mentioned above, a second event that occurs upon exposure of the *P. putida* DOT-T1E to toluene is the induction of the TOD pathway that enables the bacterium to use toluene as the sole carbon and energy source.[40] The initial enzyme of this pathway is a multi-component toluene dioxygenase that attacks the aromatic ring to yield toluene-*cis*-dihydrodiol. This compound is subsequently reduced to 3-methylcatechol, which is further metabolized to Krebs cycle intermediates. The substrate range of this pathway is narrow and restricted to toluene, benzene and ethylbenzene. The gene products of *todS* and *todT*[20] which form a two-component regulator system[15] control transcription of the *todXFC1C2BADEGIH* operon that encodes the pathway. In contrast to a typical sensor kinase, the so-called "double histidine kinase" TodS contains two input and two histidine kinase domains. A second unusual feature of TodS is that it appears to be a cytosolic sensor. *In silico* analyses showed no transmembrane domains and, unlike membrane-bound sensors,

purified TodS is soluble at high concentration in the absence of detergents. Toluene binding to TodS increases its autophosphorylation rate, which leads to an increase in the phosphorylation of its cognate response regulator TodT.[14] Phosphorylated TodT, bound to the *todX* promoter is thus the transcriptional activator of the catabolic operon.

The regulator proteins TtgR, TtgV and the two-component system TodS/T were all identified due to their role in toluene resistance and degradation. This however raises the question of where and how toluene binds to these proteins, if other related molecules also bind, and if so, how their affinities and *in vivo* activities compare to those for toluene. Such information is required to shed light on the nature of the compounds that have conditioned these proteins during evolution and which compounds can be considered as physiological ligands.

A traditional way of characterizing the effector profiles of ligand-responsive regulatory proteins is *in vivo* gene expression studies using a reporter gene such as β-galactosidase. However, the *in vivo* efficiency of an effector is a function of both its affinity for the protein and its intracellular concentration. The intracellular concentration of a compound in turn depends on the net efficiency of its uptake and expulsion from the cell, which will differ for structurally distinct effectors. Hence, the ratio of intracellular and extracellular concentrations will be different. This is even more pertinent in the case of regulators such as TtgV and TtgR whose effector molecules are substrates for their respective efflux pumps. In the following sections, we illustrate the use of ITC to measure the affinity of effector molecules to purified regulator proteins.

3. INTERACTION BETWEEN TRANSCRIPTIONAL REGULATORY PROTEINS AND EFFECTORS

3.1. Defining the Effector Specificity Profile

3.1.1. TodS: a Histidine Sensor Kinase with a Broad Effector Profile

The data summarized in Figure 1 and Table 1 provides the first experimental evidence for interaction between TodS and toluene and confirms the proposition by Lau *et al.*[15] that TodS is the sensor for toluene. The binding is driven by favourable enthalpy (−5.50 kcal/mol) and entropy changes (2.92 kcal/mol). In general, favourable (negative) enthalpy changes are attributable to bond formation whereas favourable (positive) entropy changes are primarily the result of ligand-induced expulsion of bound water into the bulk water, which represents an increase in the disorder (entropy) of the system. Jelesarov and Bosshard[10] provide additional information on the interpretation of enthalpy and entropy changes.

Table 1. Thermodynamic parameters obtained from the titration of TodS with effector molecules.

Effector	K_A (M^{-1})	K_D (μM)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)	Fold increase in induction (ref [14]) of P_{todX}[a]
1-Hexanol	No binding					0
Cyclohexane	No binding					0
Benzene	(1.31 ± 0.07) × 10^6	0.76 ± 0.04	−10.96 ± 0.2	−2.62 ± 0.18	−8.34 ± 0.03	41
Toluene	(1.54 ± 0.08) × 10^6	0.65 ± 0.03	−5.74 ± 0.07	2.56 ± 0.09	−8.30 ± 0.03	173
Ethylbenzene	(3.57 ± 0.2) × 10^5	2.8 ± 0.1	−3.81 ± 0.1	3.63 ± 0.12	−7.44 ± 0.04	11
Propylbenzene	(5.5 ± 0.5) × 10^4	18 ± 2	−2.7 ± 0.4	3.7 ± 0.5	−6.46 ± 0.06	0
Butylbenzene	(1.2 ± 0.1) × 10^4	81 ± 4	−5.4 ± 0.1	0.2 ± 0.1	−5.58 ± 0.03	0
Styrene	(1.74 ± 0.13) × 10^6	0.58 ± 0.13	−12.4 ± 0.3	−3.90 ± 0.60	−8.51 ± 0.04	67
Nitrobenzene	(1.52 ± 0.1) × 10^5	6.60 ± 0.4	−7.31 ± 0.85	−0.24 ± 0.86	−7.07 ± 0.04	74
Flourobenzene	(8.01 ± 0.4) × 10^5	1.25 ± 0.06	−5.28 ± 0.06	2.77 ± 0.06	−8.05 ± 0.03	58
Chlorobenzene	(8.5 ± 1.0) × 10^5	1.2 ± 0.1	−9.9 ± 0.4	−1.8 ± 0.5	−8.09 ± 0.07	92
Benzoate	No binding					0
Benzamide	No binding					0
o-Xylene	(1.72 ± 0.1) × 10^6	0.58 ± 0.03	−9.36 ± 0.14	−0.86 ± 0.15	−8.50 ± 0.03	0
m-Xylene	(8.24 ± 0.3) × 10^5	1.21 ± 0.04	−9.02 ± 0.12	−0.95 ± 0.12	−8.07 ± 0.02	18
p-Xylene	(1.32 ± 0.1) × 10^6	0.76 ± 0.06	−10.1 ± 0.1	−1.70 ± 0.09	−8.35 ± 0.02	46

[a]The last column shows the increase in the *in vivo* expression of a P_{todX}::*lacZ* fusion by the presence of 1 mM effector as detailed in ref [14].

As previously alluded to, the substrate range of the TOD pathway is restricted to the metabolization of toluene, benzene and ethylbenzene. However, there is *in vivo* evidence that differently substituted mono- and bicyclic aromatic compounds can induce the expression of pathway enzymes.[14,30] These observations motivated us to use ITC to study the binding of different effectors to purified TodS. Initial experiments involving an aliphatic alcohol (1-hexanol), cyclohexane and benzene showed that of these three compounds, only benzene has a dissociation constant similar to that of toluene (760 nM). The lack of binding activity towards cyclohexane provides suggestive evidence that TodS may have evolved to specifically recognize aromatic compounds. In the following, we will illustrate the use of ITC for structure–function studies of singly substituted benzene derivatives. These

compounds form only a selection of the complete TodS effector profile (Busch *et al*., Proc. Natl. Acad. Sci. USA, manuscript under revision).

Methyl- and vinyl-substitutions of benzene (toluene and styrene, respectively) slightly increase the affinity (Table 1). It is interesting to note that the binding mode of styrene and benzene (unfavourable entropy changes) is different from that of toluene (favourable entropy changes). Although the affinities for these three effectors are very similar, toluene has a three- to fourfold higher inducing activity *in vivo* (Table 1, last column). Since the three ligands are structurally very similar it is possible that the positioning of the toluene in the binding site is somewhat different and could potentially underlie the differences seen *in vivo*.

With respect of benzene, ethyl-, propyl- and butyl-substitutions cause a decrease in affinity by factors of 4, 23 and 106, respectively. As compared to styrene, ethylbenzene has a fivefold reduced affinity. Both ligands differ only in two protons and thus it becomes evident that very subtle changes to the effector structure significantly alter the binding parameters. Nitro-, fluoro- and chlorobenzene, which are efficient effectors *in vivo* have affinities in the lower micromolar range. There seems to be no apparent straightforward relationship between *in vitro* affinities and the *in vivo* capacity to induce gene expression as exemplified by nitrobenzene, which has only a relatively modest affinity of 6.6 μM, but which is one of the most potent effectors *in vivo* (Table 1, last column). Other singly substituted aromatic compounds such as benzoate and benzamide do not bind, which explains their lack of *in vivo* activity.

In summary, TodS provides an example of a sensory regulator with an effector range that far exceeds that of the substrate profile of the cognate pathway it controls. However, although TodS binds to a wide range of structurally different compounds, minor changes to the effector structure considerably alter the binding parameters. Obviously, compounds that do not bind *in vitro* do not induce gene expression *in vivo*. However, some compounds can be bound *in vitro* but fail to elicit *in vivo* activity (e.g. propyl- and butylbenzene). This observation will be discussed further in Section 3.2. Interestingly, the affinity of TodS for toluene is high (K_D of 649 ± 33 nM) as compared to corresponding values for other two-component sensor kinases (e.g. K_D of 5.5 μM for the binding of citrate to CitA,[6] 35 μM for nitrate binding to NarX[17] and 300 μM for Mg^{2+} ions to PhoQ[18]) However, further work is needed to establish if such high affinities are a general feature of two-component kinases involved in the sensing of toxic compounds or is related to the atypical structure of TodS with its two input and two histidine kinase domains.

3.1.2. TtgR and TtgV: Similar Function but Different Effectors

We also employed ITC analysis to define the effector profile for TtgV, the repressor regulating the expression of the TtgDEF and TtgGHI efflux pumps of aromatic hydrocarbons. ITC analysis revealed that this class of

compounds also binds tightly to the cognate regulator TtgV with mono- and bicyclic aromatic compounds such as 1-naphthol, 2,3-dihydroxynaphthalene or 4-nitrotoluene showing highest affinities. These effectors were also the most efficient effectors *in vivo* and in the case of TtgV, a correlation between *in vitro* affinity and *in vivo* activity was observed.[7]

Accordance between the effector/substrate spectra of the regulator and pumps is also found with TtgABC and its cognate regulator TtgR. TtgABC exports a wide range of hydrocarbons, antibiotics and secondary plant metabolites. Using ITC we have shown that hydrocarbons such as 1-naphthol or 1,3-dihydroxynaphthalene bind to TtgR with rather modest affinities, whereas plant secondary metabolites such as phloretin and quercetin had much higher affinities of around 1 μM.[34] Plant defence metabolites such as certain flavonoids possess antibacterial activity[31] which is due to the inhibition of DNA gyrases.[24] Based on the ITC-binding studies of effectors to TtgR combined with MIC (minimal inhibitory concentration) data of the TtgB deficient mutant, we were able to demonstrate that the physiological role of the TtgR/TtgABC system is the resistance to plant antimicrobials like phloretin, quercetin, naringenin or coumestrol that it confers. Therefore, similarly to other multidrug resistance (MDR) transporters found in soil- and plant-associated bacteria,[23] the TtgABC efflux pump may be a critical element in the competitive colonization of plant roots by *P. putida*.

3.2. Identification of Inhibitors – the Case of TodS and *o*-Xylene

In vivo gene expression studies of P_{todX}[14] via TodST show that *m*- and *p*-xylene induce gene expression, whereas *o*-xylene does not (Table 1). Since ITC experiments revealed that some compounds do not bind to TodS and consequently do not induce the expression of P_{todX} *in vivo*, we initially hypothesized that this might to be case for *o*-xylene. However, as illustrated in Figure 2, ITC analysis shows that all three isomers of xylene bind and that the *in vivo* inactive *o*-xylene does so with the highest affinity.

In addition to similar affinities, the thermodynamic mode of binding of these three compounds is very similar. Binding is driven by favourable enthalpy changes that are counterbalanced by weakly unfavourable entropy changes (Table 1).

To explain the lack of *in vivo* activity of *o*-xylene, we hypothesized that *m*- and *p*- xylene might bind to the effector-binding domain while *o*-xylene might bind to a secondary site unrelated to transcriptional activation. This appeared likely to us since TodS is a large protein of 108 kDa that has two PAS domains that potentially bind small ligands. We employed ITC to

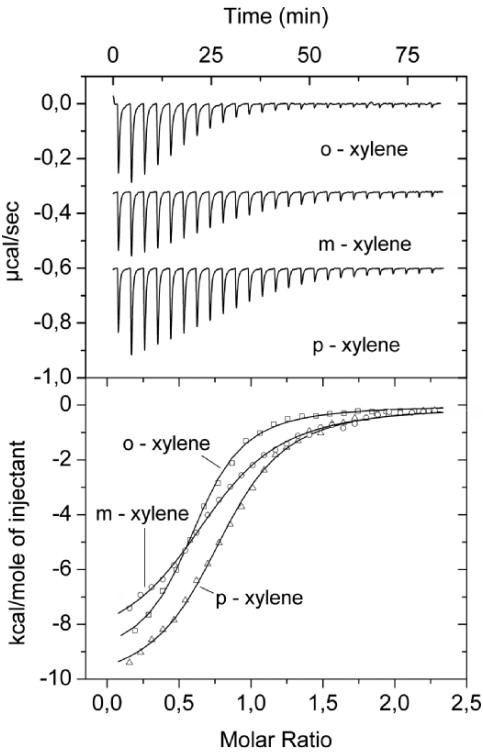

Figure 2. ITC data for the titration of TodS with *o*-, *m*- and *p*-xylene. Heat changes (upper panel) and integrated peak areas (lower panel) for the injection of 1.6 µl aliquots of effector (670–850 µM) into 10–12 µM TodS. Derived thermodynamic parameters are shown in Table 1.

determine whether toluene and *o*-xylene bind to the same or different sites. In these experiments, we first saturated TodS with one ligand, and then subject this complex to titration of the second ligand. If the second ligand binds, then it is likely that the binding sites of both ligands do not overlap. If the second ligand does not bind, it is likely that the binding sites are the same or overlap. Pre-saturation of TodS with either toluene or *o*-xylene blocked subsequent binding of the alternative compound, which makes it likely that binding sites overlap and that both effectors compete for binding at one site.

TodS is fully soluble in the absence of detergent and is thus likely to be located in the cytosol. In order to explain the lack of *in vivo* activity of *o*-xylene our next hypothesis was that this effector might not enter the cell or is expulsed efficiently leading to low intracellular concentrations. Thus, although the effector binds TodS *in vitro*, it might not be present at a sufficient intracellular concentration to achieve transcriptional activation. To test this hypothesis, *in vivo* expression studies in which β-galactosidase activity of a culture induced with toluene as compared to a culture to which *o*-xylene was added prior to the addition of toluene were performed. The expression level in the strain pre-exposed with *o*-xylene was four times lower than the control

culture containing only toluene, providing evidence for significant intracellular levels of *o*-xylene. Hence, the conclusions arising from both the ITC and *in vivo* studies is that toluene and *o*-xylene bind with almost identical affinities to the same site but only binding of toluene gives rise to an increase in gene expression *in vivo*. This conclusion was confirmed *in vitro* by demonstrating that toluene increases TodS autophosphorylation activity whereas *o*-xylene has no effect (Busch *et al*., Proc. Natl. Acad. Sci. USA, manuscript under revision). Therefore, *o*-xylene must be considered as a competitive inhibitor of the induction of the TOD pathway enzymes. As stated above the thermodynamic-binding parameters of the *o*-, *m*- and *p*-isomers of xylene are very similar, so there must be very subtle differences in the way these compounds bind to allow two of them to be active and the third one to be inactive. The most plausible way to explain these data is to suggest that the signal generated by the binding of *p*- and *m*- xylene can be efficiently transmitted to the kinase domain of TodS, whereas that upon binding of *o*-xylene cannot. It should be noted that *o*-xylene is not a singular case. As mentioned under Section 3.1, propyl- and butylbenzene, like *o*-xylene bind to TodS but do not elicit gene expression, and likely function in a similar matter (Table 1). Furthermore, we have identified several polysubstituted aromatic compounds that function as inhibitors (Busch *et al*., Proc. Natl. Acad. Sci. USA, manuscript under revision). Differences in the signal transmission efficiency for ligands belonging to different classes are also apparent with the TtgV transcriptional regulator (Guazzaroni *et al*., J. Biol. Chem., in press).

Toluene and xylene are frequently present as a mixture, for instance in petrol. The identification of inhibitors is a relevant finding that needs to be considered in potential biotechnological exploitation of toluene resistant strains for the biotransformation of toxic compounds into high added-value products or for decontamination purposes.

3.3. Overcoming the Limitations of ITC: Structure–Function Studies of Effectors with Reduced Solubility

As reported in Section 3.1.2, the effector profile of TtgR includes plant secondary metabolites that bind with high affinity. Compounds with the highest affinity were phloretin, a dihydrochalcone, and quercetin, which belongs to the class of flavonols. The obvious question was thus whether and how other structurally related flavonoids bind to TtgR.

In general, the concentration of the ligand placed in the ITC sample cell needs to be at least 10 μM to record data with reasonable signal-to-noise ratios. This implies that the ligand in the ITC syringe (in our case the flavonoid) needs to be at least at 200 μM to guarantee complete saturation of the protein. The problem we confronted was that a large number of the

flavonoids to be tested were insoluble in an aqueous buffer system at this concentration. We overcame this problem by the analyses of these compounds in a buffer system containing 5% (vol/vol) dimethylsulfoxide (DMSO) as has been used successfully by others[4] to overcome solubility problems.

Naringenin and phloretin were the only two effectors sufficiently soluble to allow analysis in the absence of DMSO. Initial experiments involved comparative analysis of the binding of these compounds to TtgR in a buffer system with and without DMSO. For both ligands, enthalpy changes as well as binding constants decreased proportionally following the inclusion of DMSO, which justified the use of this solvent in order to compare thermodynamic parameters of different effectors in the presence of DMSO (Figure 3).

Figure 3 compares naringenin, luteolin, phloretin and genistein with apigenin that represents a core structure. For clarity, only the dissociation constants for these compounds are given. The most dramatic change in

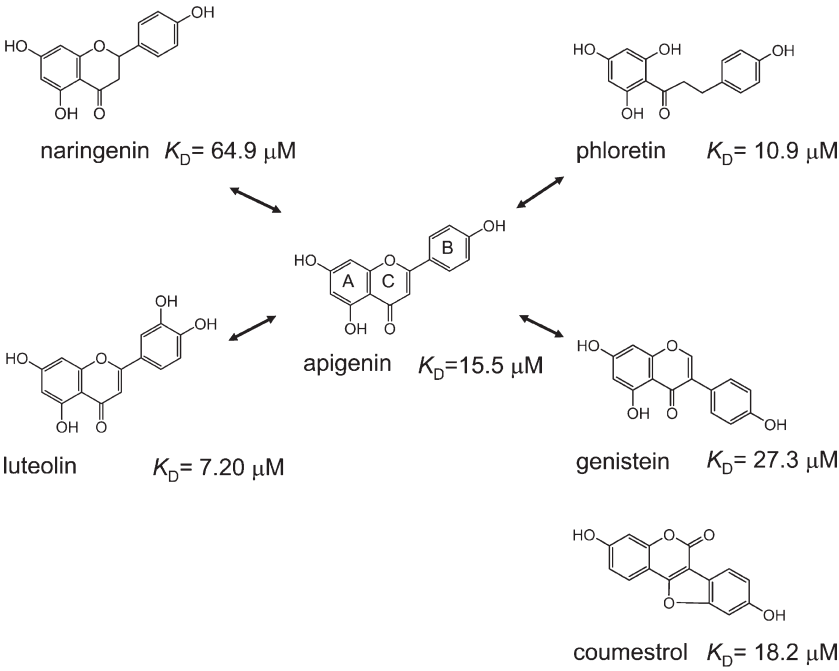

Figure 3. Structures of plant-derived effectors and their dissociation constants for the binding to TtgR. Data are from ITC experiments performed in a buffer system containing 5% (v/v) DMSO. Structures are positioned as pairs (*double arrows*) to illustrate the corresponding small changes in their structure with respect to apigenin.

binding parameters, a fourfold decrease in binding affinity, was observed for the transition of apigenin to naringenin that is characterized solely by the reduction of a double bond, which makes the molecule non-planar and allows free rotation of the B-ring. As a consequence of the ring opening, the increased flexibility of phloretin was accompanied by a slight increase in the binding affinity (K_D around 11 μM). The flavone apigenin has a twofold higher affinity as compared to its isomer genistein. Only modest changes in binding affinity were observed for coumestrol. However, it is important to note that the additional presence of a hydroxyl group of luteolin with respect to apigenin resulted in a twofold increase of the binding constant.

The conclusion from these studies is that TtgR recognizes a wide range of plant secondary metabolites with physiologically relevant affinities. The combined interpretation of all the effector-binding studies of TtgR has lead to the identification of two major structural determinants in the effector molecule for high affinity TtgR recognition. Firstly, the presence of at least one aromatic ring is essential for binding, and secondly, the number of hydroxyl groups in the effector structure directly correlates with affinity. The fact that the only two effectors (luteolin and phloretin) with four hydroxyl groups were those with the highest affinity confirmed the latter finding (Figure 3). Terán *et al.*[34] provides a comprehensive discussion of this data.

3.4. Gaining Mechanistic Insight – Titration of the TtgR-Operator Complex with Effector

Up to this point, we have illustrated the use of ITC titration of proteins with effectors in order to determine the binding parameters and the effector profile. We would like to illustrate here the use of ITC to obtain information on the binding stoichiometry that can give insight into the mechanism of a complex molecular interaction. For this purpose, we use the example of TtgR that belongs to the TetR family of transcriptional regulators. The stoichiometry of the binding of effector to unliganded protein of this family has been reported to differ; with one effector molecule binding to the QacR dimer,[29] whereas two effector molecules bind to the TetR dimer.[11] In general, a stoichiometry of two ligands to a protein-homodimer is much more common than the 1:2 stoichiometry observed for QacR, which is thus to be considered as unusual.

TetR, QacR and TtgR are all repressors that control gene expression through an effector-mediated de-repression mechanism in which effector binding causes the release of the repressor from the promoter thus allowing transcription. In understanding the mechanism of effector-mediated

release, the question of the binding stoichiometry appears to be essential. However, stoichiometries cited above are for the binding of effector to free protein. Since proteins undergo structural changes when they bind to DNA it cannot be excluded that the stoichiometry seen for the binding of effectors to free protein is different from the stoichiometry for the DNA-bound protein. In our attempt to elucidate the stoichiometry of effector-binding to DNA-bound protein we were facing the apparent dilemma that a ternary effector-protein-DNA complex is not stable, and therefore, stoichiometry cannot be obtained easily. However, the microcalorimetric analysis combined with gel electrophoresis has proved to be a useful approach to address this question.

Figure 4 A shows the titration of free TtgR with the effector phloretin. A satisfactory fit of these data with the "one binding site" model (Origin, Microcal) was achieved and the curve shape was sigmoidal (indicative of high affinity binding), from which information on the binding stoichiometry can be derived. In our experience, reliable information on binding stoichiometry can only be obtained from curves of this shape; in cases where hyperbolic curves are obtained (due to a lower affinity), information on the stoichiometry must be considered with caution. The point of inflection of the sigmoidal curve corresponds to the stoichiometry, given on the lower *x*-axis (Figure 4) as the molar ratio of the molecules injected over the molecules in the sample cell. In the case cited, the effector was in the syringe and TtgR in the sample cell (dimer concentration was used for analysis). The point of inflection is close to the molar ratio of 1, which implies that one effector molecule binds to each TtgR dimer, which is the same stoichiometry as seen for QacR.

In a subsequent experiment, we used TtgR pre-saturated with a 40-bp DNA fragment containing its operator sequence. To this end, DNA was added to TtgR in the sample cell until heat changes corresponded only to the heats of dilution (indicating complete saturation of TtgR with operator DNA). The TtgR-operator complex and excess free DNA was then titrated with phloretin as shown in Figure 4b. In this case, the heat changes measured correspond to the binding of the effector to the nucleoprotein complex and to the dissociation of protein from the DNA. Native polyacrylamide gel electrophoresis of aliquots of the ITC cell content taken before and after the experiment confirmed the absence of free protein at the beginning of the experiment and the absence of DNA-bound protein at the end of the experiment (data not shown), consistent with an immediate dissociation of the protein following effector binding. An additional control experiment confirmed that effectors have no affinity for free DNA (data not shown).

Figure 4c demonstrates unequivocally that phloretin binds with the same stoichiometry to free TtgR and to the TtgR-operator complex, that is with 1 effector molecule per TtgR dimer. This implies a mechanism in

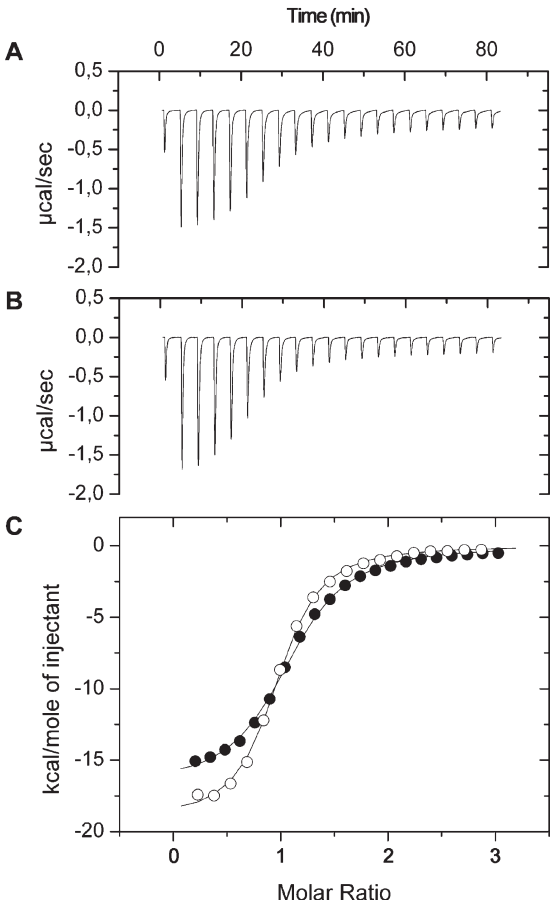

Figure 4. ITC of the binding of the effector phloretin to free and DNA-bound TtgR. Heat changes for the injection of a 2 µl and a series of 4 µl aliquots of 700 µM phloretin into 20 µM free TtgR (a) and TtgR-operator complex (TtgR at 20 µM in its DNA-bound form) (b). (c) Integrated peak areas and non-linear regression of the above data using the "One set of sites" algorithm of Origin. Closed symbols: free TtgR; open symbols: DNA-bound TtgR.

which binding of a single effector molecule releases a TtgR dimer from its promoter. Ongoing X-ray crystallographic studies should establish whether residues from the two monomers make a single effector-binding site, or whether the effector binds solely to one TtgR monomer, giving rise to a strong negative cooperativity and thus preventing binding of the second molecule.

3.5. Mutagenesis of the Multidrug-Binding Site of TtgV: Trade-off Between Binding of Effectors Belonging to Different Classes

The TtgV protein belongs to the IclR family of transcriptional regulators. The currently available five three-dimensional structures of IclR family proteins in the pdb database[39] (and unpublished) are all derived from proteins in the absence of physiologically relevant ligands and the effector-binding site for this class of protein remains to be identified. We generated a homology model of TtgV based on some of these structures, which shared only a modest 21–29% sequence identity with TtgV. The model revealed a cavity with a volume of 1200 Å as determined with the aid of the PASS software.[2] We hypothesized that this cavity corresponds to the effector-binding site, and generated alanine-substitution mutants of six amino acids located within this pocket to test our hypothesis. These purified mutant proteins were analysed by far-UV cd spectroscopy and no changes to the protein structure were detected.

As outlined previously, TtgV has multidrug-binding properties and the most potent effectors *in vivo* fall into two groups, namely monocyclic and bicyclic aromatic compounds.[7] Based on initial experiments involving the titration of the six mutants with a representative of each class, we chose 1-naphthol and 4-nitrotoluene which bind tightly to TtgV and which were shown to be amongst the most efficient effectors *in vivo*.[7] Figure 5 shows the titration of wild-type TtgV and the F134A mutant with both effectors. TtgV binding to 4-nitrotoluene is characterized by a K_D of 17.4 ± 0.6 μM and a ΔH of –9.7 ± 0.2 kcal/mol. The F134 to A mutation results in a dramatic drop in both the affinity (K_D = 221.2 ± 8.3 μM) and enthalpy change (ΔH = –3.0 ± 0.1 kcal/mol) (Figure 5a).

When the same proteins were titrated with 1-naphthol, the wild-type protein had a K_D of 40.1 ± 3.0 μM and a ΔH of –21.4 ± 3.3 kcal/mol (Figure 5b). Most interestingly, the tendency seen for F134A with 4-nitrotoluene was inversed that with 1-naphthol. The mutant protein had a sevenfold higher affinity for the latter effector as compared to wild-type protein (K_D = 5.7 ± 0.2 μM), which was accompanied by an increase in the enthalpy term (ΔH = –22.5 ± 0.6 kcal/mol). When the remaining five alanine-substitution mutants were analysed in the same fashion, an increase in affinity for 1-naphthol was observed for all cases. In direct contrast, a decrease in affinity towards 4-nitrotoluene, was observed for almost all mutants. When the logarithms of dissociation constants for both ligands are plotted (Figure 5c), a statistically relevant correlation is observed for the linear fit (r^2 = 0.82). The negative correlation between both affinities means that the more a mutation increases its affinity for 1-naphthol, the more it decreases it for 4-nitrotoluene, which can be regarded as a sort of trade-off.

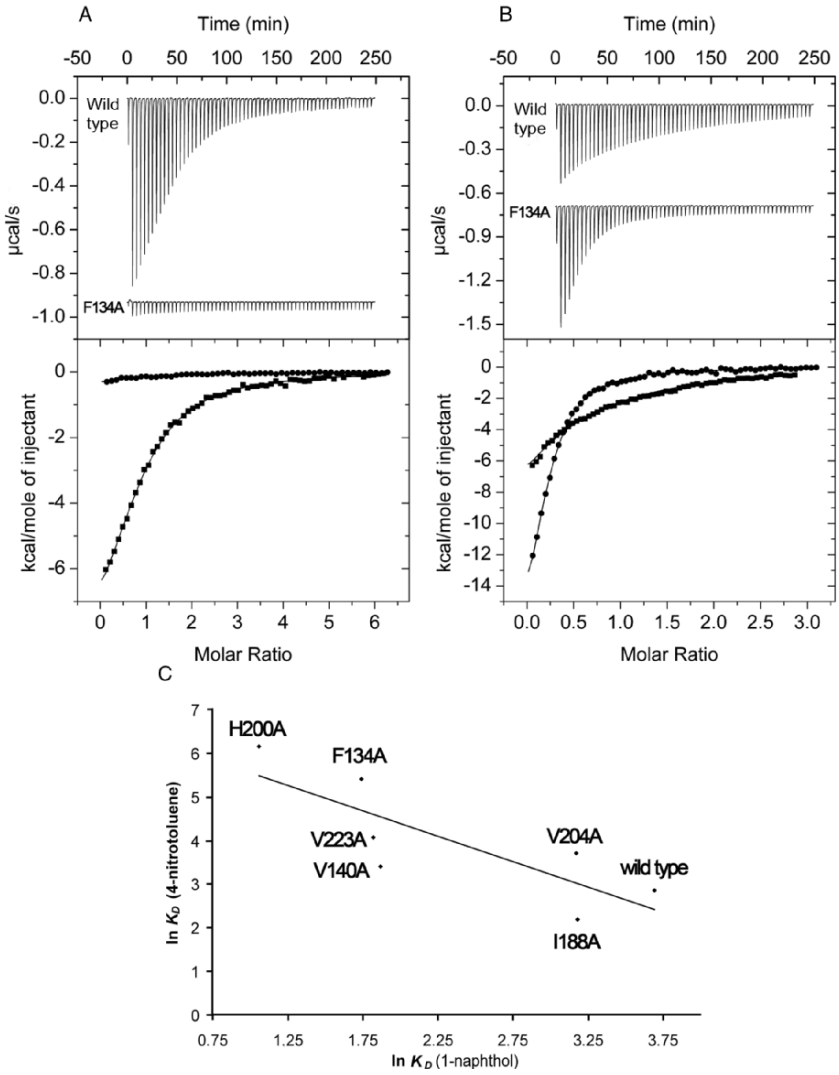

Figure 5. Study of the effector-binding site of TtgV. Titrations of wild-type and F134A mutant TtgV (35–40 µM) with 1 mM 4-nitrotoluene (a) and 0.5 mM 1-naphthol (b). Squares: wild-type TtgV, circles: F134A. (c) Plot of the logarithms of K_D for 1-naphthol and 4-nitrotoluene of the wild type and the six mutants of the effector-binding site. The linear regression has a correlation coefficient of 0.82.

A property common to all the mutations is that they increase the volume of the binding site. Since 1-naphthol is amongst the largest effectors of TtgV, we hypothesize that the size of the binding pocket of the wild-type protein partially restricts effector binding. This suggestion is sup-

ported by the fact that a slightly larger ligand, 2,6-dimethylnaphthalene is unable to bind (data not shown). The alanine-substitution mutations would partially relieve these size-restrictions, giving rise to tighter binding. This idea is further supported by the fact that the two mutations which most enlarge the binding site (F134A and H200A) cause the most pronounced increases in affinity for 1-naphthol (Figure 5c).

High precision determination of the affinities of four effector molecules (1-naphthol, 4-nitrotoluene, indole and benzonitrile) for native and mutant TtgV have been determined by ITC as indicated by an average error of only 5% for the K_D. Based on these data we conducted EMSA experiments with constant amounts of DNA and TtgV, but with effector concentrations that correspond to multiples of the K_D (data not shown). Under these conditions, the percentage of occupancy of protein with effector is the same. Differences in the effector-mediated protein release are thus not a consequence of differences in affinity but rather are due to differences in the efficiency of signal transduction. This approach thus allows dissection of the phenomenon of effector-mediated protein release into the two components, namely affinity and efficiency of signal transduction. Using this experimental approach, we noted that transmission of the signal caused by the binding of the four effectors to the wild-type proteins occurs with different efficiencies, and most importantly, that five of the six mutations alter the efficiency. This apparent ease in the modulation of the signal transmission efficiency is thus a parameter with relevance in terms of protein evolution (Guazzaroni *et al*., J. Biol. Chem., in press).

4. STUDY OF MORE COMPLEX INTERACTIONS – THE COOPERATIVE BINDING OF TTGR TO ITS PROMOTER

The analyses presented so far concern the binding of a molecule to one type of binding site. Thus, the mathematical model to fit these monophasic data is based on the existence of identical- and independent binding sites. However, biomolecules frequently bind ligands through multiple binding sites. The bacterial transferrin receptor is a good illustrative example of a protein that binds a ligand at different independent binding sites. This receptor is a heterotrimer, consisting of one TbpA and two TbpB subunits. Transferrin binds to both of these subunits in an independent fashion. Since the binding parameters for each subunit is different, binding gives a typical biphasic ITC curve.[12] However, biphasic ITC data does not necessarily imply different types of independent binding sites; biphasic ITC curves are also frequently obtained when the ligand in the sample cell has multiple sites (for instance a homodimer) and the binding of the first ligand increases (positive cooperativity) or decreases

(negative cooperativity) the affinity for the following ligands. Here we illustrate a case of cooperative binding of TtgR to its DNA-binding sites.

TtgR forms stable dimers, as determined by analytical ultracentrifugation, and two dimers bind to a 40-bp DNA fragment (referred to as WT) containing the operator site. This sequence is protected by TtgR in DNAse I footprint assays and covers the –10/–35 *ttgA* and *ttgR* promoter regions.[33] This fragment has a pseudopalindromic sequence, containing a 28-bp sequence in which only 65% of the nucleotides are related to each other in a palindromic manner. In addition to analysing the thermodynamics by which TtgR binds to its operator sequence, we also wanted to address a question of more general interest; namely, why some operator sequences are strictly palindromic whereas others, such as the TtgR-binding sequence, are rather irregular. Therefore, a 40-bp DNA fragment was designed which has an optimized, almost perfect palindrome, referred to as OP (for optimized palindrome). The sequences of the wild-type and OP DNA fragments are given in the header of Table 2. The microcalorimetric titration of both operators with TtgR is shown in Figure 6 and some of the derived thermodynamic parameters are listed in Table 2.

In contrast to effector-binding studies, the peaks go upwards indicating that the binding of TtgR to DNA is an endothermic process. The binding is thus entirely entropy driven since enthalpy changes are unfavourable. The binding of proteins to DNA is frequently endothermic,

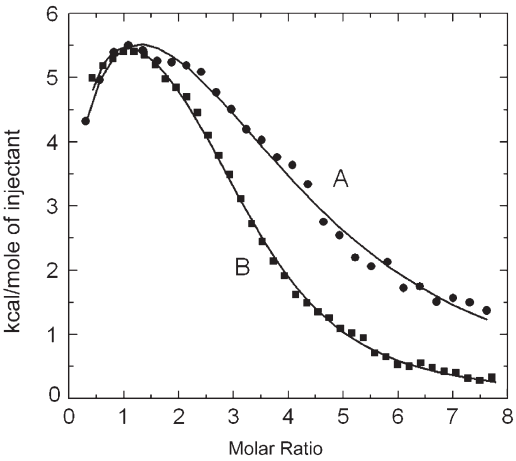

Figure 6. Binding of TtgR to 40-bp DNA fragments containing the operator. (a) Integrated heat changes upon injection of 64 μM TtgR-dimer into 1.85 μM WT DNA-fragment, and (b) of 78 μM TtgR-dimer into 1.81 μM OP DNA. Table 2 lists the derived thermodynamic parameters and the DNA sequences of the DNA fragments.

Table 2. The thermodynamic parameters for the binding of TtgR to different operator sequences. WT: 40 bp fragment containing the wild-type operator sequence containing a pseudopalindromic sequence (CAGCAG*TA*TT TACAAACAACCATGAATGTAAGTATATTCC, bases forming the palindrome are underlined). OP: 40 bp fragment derived from the WT, but in which the palindrome has been optimized to be almost perfect (CAGCAGTAT**A**TACA**T**ACA**TG**CATG**T**ATGTA**T**GTA TATTCC bases forming the palindrome are underlined, changed bases are in bold).

Operator	K_{A1} (M^{-1})	K_{D1} (μM)	ΔH_1 (kcal/mol)	K_{A2} (M^{-1})	K_{D2} (μM)	ΔH_2 (kcal/mol)	n_H[a]
WT	(5.5 ± 1.9) × 10^4	18.2 ± 6.3	21.0 ± 2.6	(1.1 ± 0.6) × 10^6	0.91 ± 0.49	9.0 ± 3.8	1.63 ± 0.13
OP	(6.3 ± 2.0) × 10^4	15.8 ± 5.0	10.5 ± 1.5	(5.5 ± 1.4) × 10^6	0.18 ± 0.05	8.6 ± 1.6	1.81 ± 0.05

[a]The Hill coefficient is a measure of cooperativity, it varies between limiting values of 0–2 for extremely negatively and positively cooperative systems, respectively. A value of 1 is obtained for non-cooperative systems. The Hill coefficient was calculated using: $n_H = 2 / [1 + (K_{D2} / K_{D1})^{1/2}]$.

thus entropy driven,[1,13] which is thought to be due to the positive entropic effect of the displacement of DNA-bound water.

The binding curve is biphasic suggesting multiple binding events. Since we know that two dimers of TtgR bind to the DNA fragment, curve fitting using a model based on two independent binding sites was attempted, which however failed. This led us to conclude that there is cooperativity between both TtgR-binding sites. A Hill plot of EMSA experiments using this DNA fragment and increasing concentration of TtgR, which showed positive cooperativity, further supports this conclusion. To analyse these data, a mathematical model was generated[35] which aims at determining four parameters, namely K_{A1}, K_{A2}, ΔH_1 and ΔH_2, which correspond to the binding constants and enthalpy changes for the initial binding event ($_1$) and the second binding event ($_2$). Other parameters such as K_D, ΔS and ΔG can be calculated from these parameters (see Section 1).

The dissociation constant for the binding of the first TtgR dimer to the WT sequence was 18.2 ± 6.3 μM whereas the binding of the second dimer was characterized by a K_{D2} of 0.91 ± 0.49 μM (Table 2). This represents a 20-fold increase in affinity as compared to the initial binding and is evidence for a positively cooperative binding of TtgR to the WT DNA, which is quantified by a Hill coefficient of 1.63 ± 0.13. The binding of the first TtgR dimer to the OP DNA fragment was comparable to the WT (K_{D1} of 15.8 and 18.2 μM, respectively). However, more pronounced positive cooperativity was observed with OP since a K_{D2} of 0.18 ± 0.05 μM

was obtained. This corresponds to an 88-fold increase to the first event (Table 2), giving a corresponding increase in the Hill coefficient to 1.81 ± 0.05. Given the similar K_{D1} for the two DNA fragments, and considering that the second TtgR dimer binds five times tighter to the OP fragment, the overall affinity of TtgR to the optimized operator sequence is also around five times higher as compared to the wild type.

So what is the physiological significance of these findings? In other words, why has the TtgR promoter evolved as it did? As mentioned previously, the action of three extrusion pumps primary underlies the toluene resistance of *P. putida* DOT-T1E, in which TtgABC provides an innate tolerance to antibiotics and plant antimicrobials, whereas TtgDEF and TtgGHI are primarily involved in the induced toluene tolerance.[19,28] This TtgABC-mediated innate toluene resistance is due to a leaky expression of the pump, which in turn is due to an only limited repression by TtgR. Thus, if a perfect palindrome were present at the *ttgABC* promoter, TtgR would bind with higher affinity, likely leading to reduced innate tolerance to antibiotics of *P. putida* DOT-T1E, which might represent an evolutionary disadvantage. The imperfect palindrome of the WT DNA sequence might thus represent a trade-off between guaranteeing innate resistance and tight binding of the repressor that would offer the possibility of more dramatic increases in gene expression in response to acute toxic stress situations.

5. CONCLUSIONS

In addition to the cellular concentration of a given regulator protein, their affinity for DNA and effector molecules are the primary parameters that determine their biological function. Microcalorimetry has proven to be an elegant tool to assess both these parameters. Here we demonstrate that one limitation of ITC, the solubility problem of ligands in the syringe, can partly be overcome by the inclusion of DMSO in the buffer. Although significant technological advances have increased the sensitivity of microcalorimeters over the last decade, a persistent limitation of this technique is the sample amount necessary for analysis. Typically, at least a 10 μM solution, usually a protein, needs to be present in the sample cell. Therefore, an efficient heterologous protein expression system is desirable prior to the commencement of ITC studies.

ITC is a rigorous approach since the heat changes measured are the sum of binding heats and other "unwanted events," such as protein aggregation. As such, in cases where "unwanted events" contribute significantly to the total signal, the data can simply not be analysed, which greatly reduces the risk of interpreting non-binding events as a binding event. When working with hydrophobic ligands such as toluene, there is the

constant worry that the compound binds non-specifically or partially denatures the protein leading to a high affinity for any hydrophobic molecule. However, if heat changes following saturation of the protein, such as in Figure 1, correspond to heats of dilution, no non-specific binding has occurred. Therefore, ITC is a suitable technique to differentiate well between specific (saturable) and non-specific binding events.

High-resolution protein structures in complex with physiological relevant ligands have contributed tremendously to advances in life sciences. However, these structures represent only a photo-image and information on the binding thermodynamics are frequently lacking. ITC appears thus to be a convenient tool to close this gap.

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10

CELL–CELL COMMUNICATION: QUORUM SENSING AND REGULATORY CIRCUITS IN *PSEUDOMONAS AERUGINOSA*

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1. INTRODUCTION

It is increasingly apparent that bacteria function in nature less as individuals and more as microbial communities, able to establish social interactions and to inhabit multiple ecological niches. Increased awareness of the role of cell–cell communication in the ecology of bacteria is matched by increasing knowledge of both the physiology and molecular biology that underlie this process.

Bacterial intercellular communication is based on the production and detection of diffusible signal molecules. Bacteria use a wide variety of signalling molecules, signal detection systems and signal transduction mechanisms. In Gram-negative bacteria, the signalling molecules are typically acylated homoserine lactones (acyl-HSLs or AHLs). In Gram-positive bacteria, peptides are the signalling molecules. Signal molecules are used for specific intraspecies communication and while interspecies communication also takes place, it is less well understood.

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The general denomination of Quorum Sensing (QS) was proposed by Fuqua *et al.*[24] because it appeared that the cell–cell communication process allows populations of bacteria to collectively control gene expression, and synchronizes group behaviour generally when a high population density is reached.

The *Vibrio fischeri* LuxR/LuxI bioluminescence system was the earliest-described bacterial cell–cell communication system and was rapidly considered as a paradigm. QS research has progressed rapidly over the past decade, since it is possible to study this phenomenon at a molecular level. QS is now regarded as a general system with numerous examples of this type of communication in a range of different bacterial species.[25,54,67,89,104,107,110] In Gram-negative bacteria two essential proteins are involved in the QS process: first, a LuxR-type protein, a transcriptional regulator homologous to the LuxR protein of *V. fischeri*; second, a LuxI-type protein, homologous to LuxI of *V. fischeri*, the autoinducer synthase which synthesizes the signalling molecule, an AHL.[24–26] Recognition of the AHL ligand by the R-type transcriptional regulatory protein is specific. In general, each R-type protein activates transcription in response to an autoinducer signal. For example, Schaefer *et al.*[81] showed that LuxR of *V. fischeri* is able to fully activate transcription only when its autoinducer *N*-3-oxo-hexanoyl-L-homoserine lactone (3O-C6-HSL) is added in the culture medium. This explains why most signalling molecules enable intraspecies, but not interspecies communication. Specificity in the interaction between the R-type transcription factor and its cognate AHL is essential for bacteria to distinguish AHLs produced by their own species from AHLs produced by other species. Nevertheless, such molecular specificity is not absolute, and some non-cognate AHL elicit partial responses.

A general scheme of the regulatory process over the growth curve is as follows: at low cell density, AHLs are synthesized at a basal level by the low concentrations of the constitutively produced I-protein. Newly synthesized ASLs diffuse out of the cell down a concentration gradient. The AHL synthesis, and accumulation in the environment, continues during bacterial growth. The local environment is often a confined space or other area with restricted diffusion.[76] When the AHL concentration reaches a threshold value, generally at high bacterial cell densities, there is enough signal to interact with the specific R-transcriptional factor. Binding of the AHL ligand activates the R-protein which, in turn, triggers expression of target genes through DNA binding and activation of transcription. Generally, the I-gene itself is rapidly activated, which causes autoinduction and an amplification of the signal ensuring that subsequent R-target genes are induced.[86] Consequently, there is a synchronization of intracellular responses, and perhaps whole communities of cells are synchronized. These

mechanisms ensure maximal production of specific proteins during the late-logarithmic and stationary phases of growth, when the density of the population is high.[86] The physiological processes controlled by QS are diverse, but are often related to virulence in pathogenic organisms.

This global regulatory process is not always strictly dependent on high cell densities and it is more and more recognized that the critical threshold concentration of signalling molecule required vary according to the prevailing growth environment. Indeed, QS regulatory systems do not function in isolation and the population density is only one parameter among others that cells within a population must integrate in order to adapt and survive within a given ecological niche. Consequently, a more general definition of QS can be given: it is a process of bacterial cell–cell communication by means of small signalling molecules which allows bacteria to respond to environmental changes and to synchronize global gene expression in a collective manner.

Since the discovery of the *V. fischeri* LuxR/LuxI bioluminescence system, many QS circuits have been described in different species of Gram-negative bacteria.[67,104] Only a few of them have been studied at the molecular and mechanistic levels. These systems illustrate an increasing complexity underlying QS in specific bacterial models and *Pseudomonas aeruginosa* is a particularly good model system of a complex regulatory circuitry with the existence of three LuxR-type proteins, LasR, RhlR and QscR.

2. QS REGULATORS: STRUCTURE, BIOCHEMISTRY AND FUNCTION

To date, many LuxR-type proteins have been identified in various bacteria but only a few of them have been extensively studied at the molecular and mechanistic levels, among them are LuxR (*V. fischeri*), TraR (*Agrobacterium tumefaciens*), ExpR (*Erwinia carotovora*), LasR, RhlR and QscR (*P. aeruginosa*). Based on sequence homologies and biochemical analysis, it appeared rapidly that LuxR-type regulatory proteins have a modular structure consisting of a DNA-binding domain typical of many transcriptional regulatory proteins and a separate and characteristic AHL-binding domain.[26]

Recently, the three dimensional structure of dimeric TraR bound to both its cognate AHL (3O-C8-HSL) and a DNA promoter fragment containing the TraR-binding site was reported.[95,115] These structures have considerably advanced our understanding of signalling in QS systems and have highlighted several fascinating questions about R-protein function.

2.1. Structural Features of the Activator TraR

TraR is composed of two structurally and functionally distinct protein domains, separated by a linker (residues 163–174). The amino-terminal portion of the protein is involved in ligand recognition and binding, while the carboxy-terminal domain is responsible for DNA sequence recognition and binding. Interestingly, the crystal structures showed that both these domains independently form symmetric dimers, but the overall protein structure of the dimer is asymmetric.

The structure of the N-terminal ligand-binding domain (residues 1–162) is a helix-sheet-helix sandwich. In this sandwich, the ligand is completely buried between the concave surface of the five stranded β-sheet and the superposed helices, while the three helices underneath the β-sheet are involved in dimerization of the domain. The N-terminal domain structurally resembles a PAS or GAF domain. PAS and GAF domains are two structural motifs which are found in many signalling proteins.[28]

The C-terminal domain (CTD: residues 175–234) has a four-helix bundle containing a DNA-binding helix-turn-helix (HTH) motif. The CTD is dimeric and the dyad symmetry axis corresponds to the dyad axis of the cognate DNA. This structure is particularly adapted to the recognition of the TraR inverted repeat recognition sequence in which the interaction between the two monomers ensures a fixed size for the promoter sequence.

2.2. Biochemical Function of R-type Proteins

The biochemical function of R-type regulatory proteins, such as TraR, is to bind their cognate ligands and as a result activate transcription. It seems clear that the binding of AHL is associated with large structural changes in the ligand-binding domain, as the binding pocket is completely buried in the hydrophobic core of the protein. It is reasonable to hypothesize that the structural changes modify the DNA binding ability of the protein, or the ability to interact with RNA polymerase. The nature of the ligand-binding site also offers an explanation for the exceptionally high affinity of the LuxR-family receptors[93,117] which enable the bacteria to detect the AHL at concentrations approaching one molecule per cell. The large structural changes proposed to occur after ligand binding are coherent with the altered protease resistance of TraR in the presence of ligand.[118] The reduced DNA-binding affinity of TraR in the absence of AHL[59,117] might indicate that in the unliganded state the N-terminal domain interferes with DNA binding.

2.3. Physiological Function of R-type Regulators

2.3.1. Transcriptional Activators

Many LuxR-type proteins are known to have a role as transcriptional activators (as a review, see ref [26]). However, there are only a few examples of demonstrated DNA binding by a R-protein, and then only *in vitro* in an autoinducer-dependent manner. TraR, CarR, LuxR, LasR, RhlR and QscR have all been shown to bind to DNA *in vitro*.[22,57,64,102,115,117] Palindromic "*lux* box" DNA motifs were identified in *V. fischeri* located upstream of LuxR-regulated genes which have been proposed to have a role as binding sites for the regulatory protein-autoinducer complex.[21] In *P. aeruginosa*, "*las* boxes", for which a consensus sequence was defined by alignment of 8 *las* sequences were only detected upstream of 7% of the QS-regulated genes identified in a global study.[99] This low proportion of genes that seem to represent *bona fide* targets for LasR indicates either that regulation is indirect, or that the bioinformatic method used was not sufficiently sensitive.

2.3.2. Transcriptional Repressors

Transcriptional activators and transcriptional repressors often have similar modes of interaction with DNA through a HTH motif.[79] An important clue to the function of a regulator is the position of the DNA-binding site relative to the transcription start site in the target gene promoter region, because this determines the interaction of the regulatory protein with RNA polymerase. The regulator is consequently an activator or repressor of transcription. In engineered repressor systems, in which a *lux* or a *tra* box has been inserted between the 0 and the −35 of a promoter sequence, it was shown that LuxR and TraR can function as AHL-dependent repressors that bind to these artificial promoters[22,59] which indicates that AHL-dependent repression might occur in natural systems. Likewise, it has been shown that RhlR, bound to a specific sequence in the *rhlAB* regulatory region in the absence of its autoinducer, acts as a transcriptional repressor of this promoter.[64]

Recently, Wagner *et al.*[99] and Schuster *et al.*[82] identified, using microarray analysis, numerous QS-repressed genes in *P. aeruginosa*. Only a few of these genes had a *las* box sequence upstream of the coding region, which probably indicate that indirect control mediates a global negative effect on gene expression. Obviously, further experiments are needed to evaluate whether LasR and RhlR protein repressor activity is physiologically important.

2.3.3. Layering Controls and Modulating R-Protein Function: Anti-Activators

Accumulating evidences indicate that heterologous interactions between different proteins can modulate regulatory protein functions. The existence of anti-activators is well demonstrated only in the case of *A. tumefaciens*. TraM, an anti-activator of the TraR protein binds TraR at a site close to the linker region and is able to inhibit transcription activation by TraR.[43] In *P. aeruginosa*, QscR could exert, in some defined conditions, such a function but this has to be further demonstrated as discussed in Section 3.2.

3. THE CENTRAL QS CIRCUITRY IN *P. AERUGINOSA*: MULTIPLE R/I SYSTEMS, MULTIPLE SIGNALLING MOLECULES, A COMPLEX REGULATION

An important feature of the QS circuitry in *P. aeruginosa* is its complexity, with the presence of two complete R/I systems (LasR/LasI and RhlR/RhlI) and one uncomplete system (QscR only) to control the expression of a wide range of genes. Moreover, these different QS systems are linked through intricate regulatory connections, involving other regulatory proteins (RsaL and VqsR) and another signalling system (PQS).

3.1. The Las and the Rhl Systems

The first system to be identified was the LasR–LasI system, originally observed in regulation of elastase.[29] A few years later was identified a second complete QS system, RhlR–RhlI, involved in regulation of rhamnolipids.[52,68] LasR and RhlR induce the transcription of their cognate synthase genes, creating a positive feedback loop, or autoinduction, that allows a rapid increase in signal production. The two LuxI-type proteins, LasI and RhlI, direct the synthesis of *N*-3-oxo-dodecanoyl-L-homoserine lactone (3O-C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL), respectively.[69,108] LuxI synthases are not under the scope of this review and for detailed informations, see refs [25] and [26]. The active conformation of transcriptional factors before they bind to DNA in most cases is a dimer or a homo-multimer oligomer.[112] The activation of LasR has been shown to require 3O-C12-HSL-dependent multimerization of LasR.[50] In contrast, although RhlR requires C4-HSL for transcription activation, it does not require C4-HSL for dimerization.[96]

These systems have been studied extensively. Nevertheless, two major features have to be emphasized in this review. The first point is the existence of a regulatory cascade between the Las and Rhl systems. Indeed, they are not independent and form a regulatory cascade in which LasR/3O-C12-HSL activates the expression of *rhlR* and *rhlI* (Figure 1).[53] Therefore, these two QS systems are arranged in a hierarchical fashion as the LasR–LasI system controls the RhlR–RhlI system. The second important feature of this network of regulators is that each system regulates a regulon that comprises an overlapping set of genes. LasR, in response to 3O-C12-HSL, controls many target functions. RhlR is responsive to C4-HSL and has also numerous genomic targets, many of which overlap to various degrees with the LasR regulon but others of which are clearly discrete.[82,99]

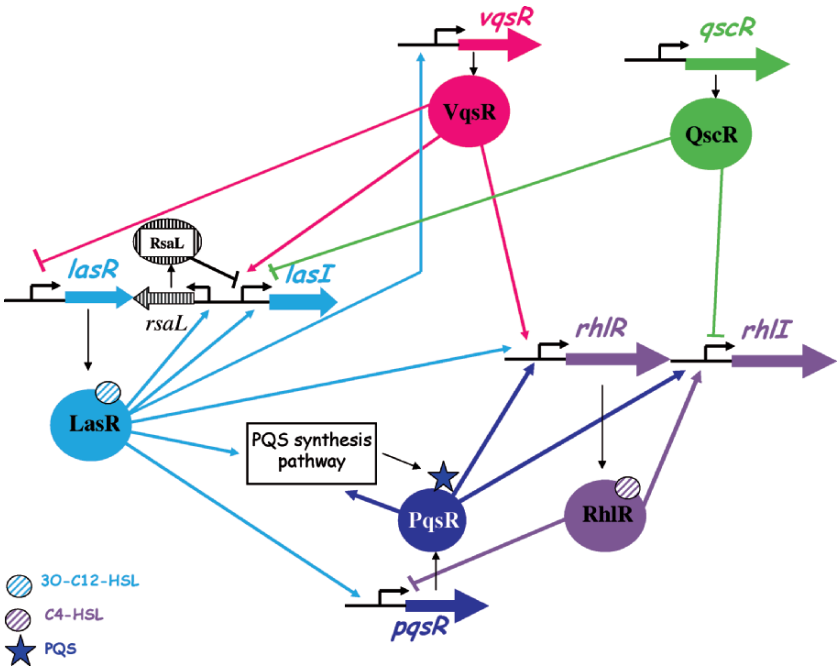

Figure 1. Interconnection between regulatory genes in the QS central regulatory circuitry. Genes and proteins are, respectively, indicated by *thick arrows* and *circles*. For clarity, the *lasI* and *rhlI* gene products have been omitted and only the two AHLs, synthesized by LasI and RhlI, respectively, are shown. Arrows and bars indicate positive and negative regulation of the gene(s), respectively. The same colour is used for corresponding gene, protein, arrows and bars. See the text for detailed explanations.

3.2. QscR, the Third Regulatory Protein Belonging to the LuxR Family

QscR (32% identical to RhlR and 29% identical to LasR) has both domains characteristics of a LuxR-type regulator, that is an AHL-binding domain and a DNA-binding domain and exhibits full conservation with AHL-responsive LuxR homologs.[10] Nevertheless, the *qscR* gene is not adjacent to an AHL synthase gene and has to be considered as an orphan gene.

Initial genetic studies suggested that this protein functions to modulate the activity of the Las and Rhl regulons, exerting a transient negative control on some QS-regulated genes (QscR, for *q*uorum-*s*ensing *c*ontrol *r*epressor).[10] In the *qscR* mutant, several QS-controlled genes were expressed early and more strongly than in the wild-type strain.[10,56] Consistent with this finding, there was early expression of *lasI* and *rhlI* and consequently, elevated levels of the 3O-C12-HSL and C4-HSL signals in early stage cultures (Figure 1). It seemed that QscR was limiting the activity of the LasR and RhlR regulators by a mechanism which remained unclear. The hypothesis that QscR could function as an anti-activator was strongly suggested.[10]

Based on fluorescence anisotropy measurements and *in vivo* cross-linking, Ledgham *et al.*[56] showed that QscR can form various complexes depending on the conditions: multimers in the absence of any acyl-HSL, lower order oligomers complexed either to C4-HSL or to 3O-C12-HSL and heterodimers with LasR or with RhlR in the absence of acyl-HSLs. Furthermore, the same approach suggested that in *Escherichia coli*, QscR exists as an oligomer that is destabilized by addition of either 3O-C12-HSL or C4-HSL. Taken together, these data suggested a potent mechanism for QscR-dependent repression through sequestration of LasR and RhlR monomers and perhaps through binding to each of their inducing ligands. What is the physiological significance of the various complexes observed? When do they form during the different phases of the growth of *P. aeruginosa*? It is important to consider that the formation of a complex is a dynamic process and is dependent on the relative amounts of regulatory proteins present at the same time inside the cell, and on the relative concentrations of the various acyl-HSLs. Additional studies will be required for a full understanding of the biological meaning of these phenomena.

Moreover, a recent study[58] using *P. aeruginosa* PAO1 DNA microarrays evidenced that QscR is a specific transcriptional regulatory protein that controls its own specific regulon (over 400 genes). Among these genes, many of them were repressed by QscR and others were activated which is a very important new data, since QscR was not suspected to have a positive function. Even though QscR seemed to bind both AHL[56] QscR uses the LasI-generated signal 3O-C12-HSL to activate transcription of numerous genes. The QscR regulon overlaps with the already overlapping

LasR and RhlR regulons. It was also shown that among the regulated genes, a few of them were probably directly controlled by QscR. These last results were confirmed by another study from the same laboratory[57] providing evidence that purified QscR is clearly a DNA-binding protein and that this binding to DNA requires 3O-C12 HSL.

To conclude, the regulator QscR appears to play an important role in QS modulation and in the interplay between the Las and Rhl systems. Its action could be either direct as specific transcriptional factor and/or indirect through sequestration of LasR and RhlR monomers or binding to each of their inducing ligands, depending on the cellular physiological conditions.

3.3. Two Other Regulatory Proteins, VqsR and RsaL, are Intimely Intricated in the Circuitry Defined by the Las and Rhl Systems

These two proteins play a major role in the hierarchy of QS regulators since their synthesis is dependent on *lasR* and, in turn, they regulate other QS regulatory genes. For this reason, they are described with the central QS circuitry even though they do not belong strictly to it.

3.3.1. The VqsR Protein

Recently, a fourth "LuxR-type" regulator termed VqsR (*v*irulence and *q*uorum-*s*ensing *r*egulator) was described.[44,45] The *P. aeruginosa* PAO1 sequence revealed the existence of multiple "LuxR-type" proteins, exhibiting more or less the same domain organization as LasR, RhlR and QscR. This point has to be clearly precised. A search for the DNA-binding HTH present in transcription factors of the LuxR/FixJ family of response regulators via Pfam (http://www. sanger.ac.uk/cgi-bin/Pfam/) results in the characterization of 36 proteins in the genome of *P. aeruginosa*. This domain, named "LuxR-type HTH domain" (PF00196) is located in the C-terminal part of the protein. Among these 36 proteins exhibiting such domain, only four of them also contain in their N-terminal part an autoinducer-binding domain (PF03472) characteristic of typical QS regulators. These four proteins are LasR, RhlR, QscR and a fourth possible transcriptional factor (PA1136) not yet characterized (S. Bleves, personal communication). Along these lines, VqsR possesses a HTH LuxR-type DNA-binding domain but no autoinducer-binding domain and therefore, cannot be considered as a true QS regulator. Nevertheless, it is integrated into the central QS circuitry.

VqsR synthesis depends on LasR/3O-C12-HSL.[99] The presence of the *las* box located in the promoter upstream of *vqsR* places this gene

under the direct control of the *las* QS system.[44] In turn, VqsR regulates positively the expression of *lasI* and *rhlR* and negatively the expression of *lasR* (Figure 1 and Table 1). An autoregulatory feedback loop could exist between *vqsR* and the *las* and *rhl* systems.[45] Moreover, VqsR is essential for the production of 3O-C12-HSL and C4-HSL since a *vqsR* mutant is impaired in the production of both signalling molecules.

A transcriptome analysis of the *vqsR* mutant provided evidence that VqsR is involved in the expression of certain sets of virulence, QS and iron-regulated genes.[44,45] Among the genes belonging to the VqsR regulon, many of them are QS regulated.

3.3.2. The rsaL Gene

The *rsaL* gene is located between the *lasR* and *lasI* genes and its expression is directly activated by LasR/3O-C12-HSL (Figure 1). The

Table 1. Effects of global regulators on the expression of *lasR*/*lasI, rhlR*/*rhlI, qscR* genes and on the levels of the two cognate AHLs.

	lasR	lasI	3O-C12-HSL	rhlR	rhlI	3O-C4-HSL	qscR	References
RsaL		–	↗					[13,73]
VqsR	n.e.	+	↘	+		↘		[44,45]
Vfr	+	+	↘	+				[1,65]
AlgR2	–			–				[55]
Fe O_2[a]	+	+						[4,49]
RelA[b]	+		↗	+		↗		[94]
GacA[b]	+		n.e.	+		↗	+	[77,56]
PprB		+	↘	+	+	↘		[19]
RpoN	–	–	↗	–	–	↗		[36]
RpoS	+			+				[53,99,105,106]
RsmA[b,c]	m	–	↘	m	–	↘		[33,71]
Hfq[c]	m	m		m	+	↘	m	[88]
MvaT		–	↗		–	↗		[16]
VqsM	+	+	↘	+	+	↘		[20]
PtxR	n.e	+	↘	n.e.	–	↗		[7]
AmpR	–	–		+				[51]

The effect of mutations in global regulatory genes on the level of expression of the QS genes were determined with the use of β-galactosidase transcriptional fusions. Only when indicated, β-galactosidase translational fusions were used.

–: indicates a negative control.

+: indicates a positive control.

↗, ↘: variation in the levels of 3O-C12-HSL and C4-HSL produced by a mutant strain compared to the WT strain.n.e.: no observed effect.m: minor effect.

A blank indicates that no effect was related in the cited article.

[a]iron deficiency and oxygen limitation.

[b]overexpression of the regulatory gene.

[c]use of β-galactosidase translational fusions.

RsaL protein specifically represses transcription of *lasI*. Overexpression of *rsaL* resulted in a 20-fold reduction of 3O-C12-HSL production.[13] A mutation in the *rsaL* gene produces higher amounts of ASL with respect to the wild type[73] (Table 1). The fact that *rsaL* expression itself is dependent upon LasR adds further complexity to this regulatory mechanism. It was recently shown that RsaL binds to the *lasI* promoter and DNA-binding studies suggested that RsaL and LasR could be in competition for binding on the *lasI* promoter. Bioinformatical analysis of the RsaL protein sequence did not show significant homology with already characterized transcriptional factors.[73] All these results highlight the key role of this negative regulator in QS.

3.4. Another Complexity Comes from the Multiplicity of the Signalling Molecules

Considering the signalling molecules synthesized by a LuxI-type synthase, only the major AHL species are generally considered, but it is well established, in different bacteria, that a typical LuxI-type synthase can produce one predominant AHL, and one or more minor AHLs in different proportions. The reason for this is not well understood, but one possibility is that the relative concentrations of these AHLs could vary with growth phase and growth conditions allowing a fine-tuning of the system. The two LuxI-type proteins, LasI and RhlI, direct mainly the synthesis of 3O-C12-HSL and C4-HSL, respectively.[69,108]

A third signalling system based on 2-heptyl-3-hydroxy-4-quinoline, designated as "*Pseudomonas* quinolone signal" (PQS), was first identified as a further component playing an important role in the QS hierarchy of *P. aeruginosa*.[70] *P. aeruginosa* culture supernatants have been known to contain a series of 2-alkyl-4-quinilones.[18] These compounds possess a variety of biological properties and it was shown that PQS functions as a quorum-sensing signal molecule via a regulatory network which is closely integrated with the AHL regulon. Like AHLs, PQS is a diffusible signalling molecule that accumulates in the local environment and can modulate gene expression.[17,70]

Synthesis of PQS is intricately linked to the QS hierarchy of *P. aeruginosa* (Figure 1). It has been shown that LasR regulates PQS production, which, in turn, is necessary for expression of *rhlR* and *rhlI* systems, creating a regulatory link between the *las* and *rhl* QS systems.[18,63,70] PQS is synthesized from anthranilate[5] by the products of the *pqs* operon (*pqsABCDE*). The immediate precursor of the PQS signalling molecule is HHQ (4-hydroxy-2-heptylquinoline) which is itself released from, and taken up by bacterial cells.[15] The expression of *pqs*ABCDE is positively regulated by a LysR-type

regulator, PqsR (corresponding to MvfR in strain PA14 previously identified.[6] It was also found recently that production of PQS was not only induced by the Las system but also repressed by the Rhl system, suggesting a balance between QS systems.[62,98] Conversion of HHQ to PQS is dependent on the *pqsH* gene product which is positively regulated by LasR/3O-C12-HSL.[27] Besides, PqsR bound to PQS modulates the expression of the *rhlI* gene.[98] Therefore, PQS is responsible of another regulatory link between the Las and the Rhl QS systems.

PQS is produced maximally during the late stationary phase of growth.[63] Nevertheless, under certain growth conditions, PQS is detectable at the onset of stationary phase and is produced in the absence of LasR.[17] Under these conditions there is a LasR-independent activation of the *rhl* QS genes, which is accompanied by *lasR*-independent production of PQS.

3.5. Complexity and Dynamics of the Regulation of the Central QS Circuitry

All these studies provide interesting new insights into the regulatory interactions possibly existing between each regulatory element of the central QS circuitry. Nevertheless, they do not elucidate the dynamics of the system. How is the timing of QS established during growth and colonization of bacterial communities? To ensure an adequate behaviour of the bacteria in variable environmental conditions, it is likely that the interactions previously described (Figure 1) do not exist all in the same time and, depending on physiological conditions, these interactions may be highly variable, leading to differential regulations of target genes. Regulation of the central QS circuitry can be realized at two different levels: either at the level of the R-proteins or at the level of AHL concentrations present in the cell.

3.5.1. Regulation of AHLs Concentrations

For years, the canonical Lux model postulated that, when a threshold AHL concentration was reached, the R-protein could activate its targets. It was more or less accepted that QS regulation and the perception of cell density was a result of variations in the AHL concentration both inside and outside the cells. The trigger for induction of the target genes was signal accumulation. Indeed, there are considerable evidences that fluctuations in AHLs levels could be attributed in part to alterations in the expression of the *luxI* gene.[25]

A second level of control could be the transient nature of AHLs, allowing a cascade of differently timed responses to different molecules. Two *N*-acyl-homoserine lactone acylases have recently been evidenced in *P. aeruginosa* PAO1.[41,42,85] The PA2385 protein is a member of the N-terminal

nucleophile hydrolase superfamily and several results suggest that this protein has *in situ* quorum-quenching activity.[85] Of the two main AHL signal molecules, only 3O-C12-HSL is degraded by the enzyme. This quorum-quenching AHL acylase may enable *P. aeruginosa* PAO1 to modulate its own QS-dependent pathogenic potential.[85]

The importance of the AHLs concentration is also well emphasized by Fagerlind *et al.*,[23] who developed a mathematical model of the AHLs regulatory network system in *P. aeruginosa*. At either low or high levels of extracellular 3O-C12-HSL, the bacterium remains in an uninduced or induced state, respectively. At moderate levels, the behaviour is characterized by several states. The bacteria can switch suddenly from an uninduced to an induced phenotype in response to small changes in the concentration of extracellular 3O-C12-HSL.

3.5.2. Regulation of R-protein Synthesis

Nevertheless, this regulatory scheme, based only on AHL concentration, was too simple to explain many experimental results. Firstly, most of the studies on the regulation of synthesis of the LuxR proteins were performed with transcriptional fusions which showed the transcription capacities of the promoters, but ignored any post-transcriptional regulation of these proteins which could be degraded by proteases in the absence of autoinducers. Secondly, several diffusible antagonists have been identified, which are produced by the same bacteria that produce AHLs. For example, cyclic dipeptides (diketopiperazines, DKPs) have been isolated from *P. aeruginosa* and other Gram-negative bacteria.[39,111] These diffusible antagonists could modulate the activity of the R-type regulators through interactions with the proteins and functioning as competitive inhibitors.[111] Thirdly, it is often difficult to prematurely induce the expression of QS-controlled genes in an exponentially growing culture by adding AHLs to the growth medium, particularly with *P. aeruginosa*.[16,105] Possible explanations are that the R-protein is limiting during the exponential phase growth, or that as-yet-uncharacterized regulatory proteins superimpose control on the expression of some QS-regulated genes.

3.6. Concluding Remarks

It is important to stress the high complexity of the central QS network which is still incompletely understood. In this regulatory circuitry, the chromosomally encoded LasR/I-proteins appear to be at the top of the regulatory hierarchy. But all the regulatory factors are linked through intricate regulatory loops and delineate a very complex network.

It is likely that, depending on physiological conditions, these interactions are highly variable, leading to differential expression of target

genes. Indeed, the formation of an active regulatory complex depends on the relative amounts of regulatory proteins present at the same time inside the cell, and is also dependent on the relative concentrations of the various acyl-HSLs. Therefore, studies of the status of the R-type proteins on the one hand and of the production of the different AHLs on the other hand, both in a range of growth conditions, would be helpful to understand more extensively the molecular mechanism of integration of QS networks in bacteria (see Section 4).

4. QS: AN INTEGRAL COMPONENT OF GLOBAL REGULATORY NETWORKS

Over last years, research has begun into the integration of QS control into bacterial global regulatory networks. Such integration links the central QS circuitry to other aspects of cell physiology and expands the range of signals – environmental and/or physiological – that influence target gene expression in addition to cell density. A very complex scheme of regulatory processes is emerging (Figure 2). Numerous transcriptional regulators were shown to have complicated interplays with the central QS circuitry and in most cases, the molecular mechanisms underlying these interactions are not yet understood.

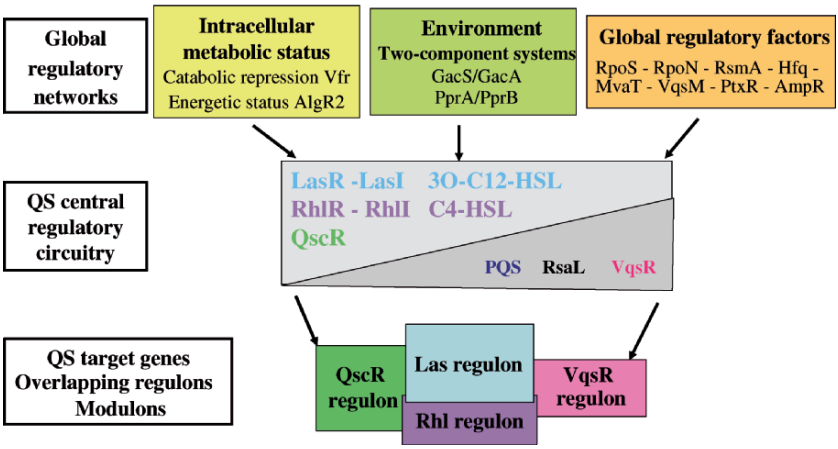

Figure 2. Integration of the QS systems into other global regulatory networks. See the text for detailed explanations.

4.1. Importance of the Intracellular Metabolic Status

4.1.1. Vfr

In *P. aeruginosa*, two adenylate cyclases CyaB and CyaA, their product cAMP, and the cAMP-dependent transcriptional regulator Vfr constitute an important signalling network that acts as a master regulator of the expression of a 100 genes.[113] Vfr, a functional homolog of the catabolite repressor protein (CRP), responds to the metabolic and energetic status of the cell.[103] Several years ago, it was shown that *lasR* gene expression is under the control of Vfr and that Vfr specifically binds to the *lasR* promoter and positively regulates *lasR* expression (Table 1).[1] Probably as a consequence, *lasI* expression is also decreased in a *vfr* mutant. More recently, the expression of *rhlR* has also been shown to be partly dependent on Vfr (Table 1).[65]

Interestingly, this dependence is variable: Vfr is required for signal production in the early phase of growth, but in the latter stages of growth, the *vfr* mutant is able to synthesize wild-type levels of signal.[23]

4.1.2. AlgR2 (AlgQ)

Originally identified as a regulator of alginate biosynthesis,[14,46] AlgR2 seems to regulate several functions that are related to the metabolic and energetic status of the cell, for example the synthesis of polyphosphate.[48] AlgR2 is also a more global regulator since it regulates the synthesis of a variety of secretable virulence factors, such as neuraminidase, pyoverdine and proteases. In some conditions, AlgR2 was shown to negatively modulate the expression of *lasR* and *rhlR* (Table 1)[55] and results obtained from DNA retardation assays provided evidence that AlgR2 can bind specifically to the *lasR* and *rhlR* promoters.[55]

4.1.3. Iron and Oxygen Limitations

The nutritional status of the cell must be taken into account when one is evaluating QS-based gene expression. Reporter gene experiments showed increased *lasI-lacZ* transcription in response to iron limitation (Table 1). The overall iron effect on *lasI* expression was small but highly reproducible and thus directly linked the iron stress response with QS circuitry.[4] It was later shown that expression of *lasR* was strongly affected by iron and oxygen concentrations in cultures of *P. aeruginosa* irrespective of cell density (Table 1).[49]

4.1.4. Amino acid Starvation

Amino acid starvation induces the cellular stringent response, characterized by high concentrations of guanosine tetraphosphate (ppGpp) which, in association with RNA polymerase, regulates transcription of many genes.[8] When the level of ppGpp is increased by overexpression of

the gene *relA* encoding the starvation response regulator, premature expression of *lasR* and *rhlR* genes was observed as well as high AHL levels in early phases of growth (Table 1). Therefore, the stringent response plays a positive role in activating QS regulatory genes.[94]

4.2. Response to Environmental Factors via Two-Component Regulators

Besides modulation of the frequency of transcription initiation, the second function of transcriptional factors is to sense changes in environmental conditions or other internal signals encoding changes. Bacteria constantly monitor extracellular physicochemical conditions, so they can respond by modifying their gene expression patterns to adjust growth. In general, the nature of environmental signals influencing the QS regulators is yet unkown.

4.2.1. GacS/GacA

The GacS/GacA system in *P. aeruginosa* is a two-component (TC) global regulatory system essential for virulence in this organism. This system is widespread in *Pseudomonas* spp. and other Gram-negative bacteria.[32]

In *P. aeruginosa*, transcription of the *lasR* and *rhlR* genes is positively controlled by the GacA TC response regulator. Moreover, *gacA* mutants are affected in the production of C4-HSL.[77] The transcription of *qscR* is also positively regulated by GacA (Table 1).[56] Therefore, in response to still unknown environmental signals, the expression of three R genes can be modulated.

Besides this regulation exerted at the transcriptional level, another level of regulation exists between GacA and the QS circuitry, depending on the RsmA/RsmZ /RsmY system[33] (see subsection 4.4.1).

4.2.2. PprA/PprB

The TC system PprA/PprB has been reported as an essential element controlling membrane permeability and antibiotic sensitivity of *P. aeruginosa*.[101] Later on, a transcriptome analysis revealed that among the 175 genes regulated per PprB, 85% were activated by QS. Particularly, the expression of *lasI*, *rhlR* and *rhlI* were significantly decreased in the *pprB* mutant (Table 1).[19] Measurement of 3O-C12-HSL and C4-HSL corroborated these results. It was also shown that mutation in *pprB* resulted in a large decrease in the sensitivity of *P. aeruginosa* to exogenous 3O-C12-HSL. Therefore, PprB appears to be a novel QS modulator that positively regulates AHLs synthesis probably by affecting the 3O-C12-HSL signal influx and thereby influencing global expression of QS-dependent genes.[19]

4.3. Sigma Factors

4.3.1. RpoN

RpoN was shown to exert global negative control on the QS machinery of *P. aeruginosa*.[36] In *rpoN* mutants, the expression of the *lasR* and *lasI* genes was elevated at low cell densities, whereas expression of the *rhlR* and *rhlI* genes was markedly enhanced throughout growth (Table 1). Considering the pleiotropic effects of *rpoN* mutations, the observed effects on QS machinery are quite complex to understand. Nevertheless, at least part of this effect appears to be mediated by GacA.[36]

4.3.2. RpoS

Synthesis of LasR and RhlR proteins is regulated by growth phase and growth conditions.[99] The *rpoS* gene encodes the specific stationary phase sigma factor, RpoS. The connection between QS and RpoS is complex and the two regulatory systems have several mild effects on each other. There were some controversies between several published data. But recent studies[83] confirmed that all available data are consistent with the fact that QS stimulates *rpoS* expression to a small degree, about twofold.[56,82,99,106] Moreover, a small induction of *lasR* and *rhlR* by RpoS was also found.[83]

4.4. Integration with Other Global Regulators

4.4.1. The RsmA/RsmZ/RsmY System

RsmA is a global post-transcriptional regulatory protein that, in *P. aeruginosa*, works in tandem with two small non-coding regulatory RNA molecule, RmsZ and RsmY, to regulate the synthesis of several virulence genes.[33] RsmA is homologous to CsrA (in *E. coli*) and RsmA (in *E. carotovora*) proteins involved in the post-transcriptional control of secondary metabolite production.[12,78] These proteins are considered to function as RNA-binding proteins that control access to the ribosome-binding site of target mRNA and by altering mRNA stability. The regulatory activity of the protein is modulated by binding to numerous molecules of small untranslated RNAs (sRNA), thus titrating the available concentration of free protein.[61,78]

In *P. aeruginosa*, RsmA functions as a pleiotropic post-transcriptional regulator of secondary metabolites directly and also indirectly by modulating the QS circuitry.[71,33] RsmZ and RsmY are two sRNAs, 117 and 124 nucleotides long, respectively, that share 72% identity. It was shown that RsmA exerted a negative effect on the synthesis of both 3O-C12-HSL and C4-HSL, which was confirmed by using *lasI* and *rhlI* translational fusions. The temporal expression of the *lasI* gene was induced

much earlier and to a higher level during the exponential growth phase in an *rsmA* mutant. The effects of RsmA on the QS regulators *lasR* and *rhlR* appear to be minor (Table 1).[33,71]

Another level of complexity resides in the fact that the two systems GacS/GacA and RsmA/RsmZ/RsmY are linked: GacA is essential for *rsmZ* and *rsmY* expression but expression of *rsmA* is independent of the GacS/GacA system.[71,37] Therefore, the GacS/GacA TC system may serve to coordinate bacterial responses controlled by the post-transcriptional regulatory system RsmA/RsmY/RsmZ to various different environmental conditions by sensing signal molecules.

4.4.2. Hfq

It was recently shown that Hfq alterations had effect on the QS circuitry.[88] The Hfq protein belongs to a large family of Sm-like proteins involved in RNA processing in eukaryotic cells. Major functions of Hfq is to control the stability of small regulatory RNAs and mRNAs as well as positive or negative translational regulation of target mRNAs by sRNAs.[31] A *P. aeruginosa* PAO1 *hfq*-mutant is attenuated in virulence.

The Hfq protein affects approximately 5% of the *P. aeruginosa* transcripts. Among these transcripts, 72 were identified to be QS regulated. Expression studies of translational fusions revealed that Hfq exerts only slight effects on the Las system, LasR and LasI, and on translation of the *rhlR* and *qscR* genes. However, Hfq considerably stimulated translation of the *rhlI* gene during late-logarithmic growth. Consequently, the C4-HSL levels were reduced in a *hfq* mutant strain (Table 1). In fact, Hfq stabilizes the sRNA RsmY. RsmY has been shown to bind both, the translational regulator RsmA and Hfq. All these data suggest that Hfq indirectly affects QS via regulation of the RsmA/RsmY system.[88]

4.4.3. MvaT

MvaT is a putative transcription regulator recently evidenced in *P. aeruginosa*.[16] Proteins similar to MvaT represent a new family of transcriptional regulator that are unique to *Pseudomonas*. Despite limited sequence similarity, MvaT may belong to a new class of H-NS-like proteins.[2,92] H-NS proteins are DNA-binding proteins that regulate gene transcription by modulating DNA topology. They also play a role in the expression of genes involved in adaptation to environmental challenges.[2,40] In *P. aeruginosa*, MvaT is a global regulatory protein that influences the expression of a large number of genes and which is also involved in the growth-phase-dependent control of several QS-regulated genes.[16] Deletion of *mvaT* led to premature expression of several QS-regulated genes including *rhlI* and *lasI* and to increased levels of both C4-HSL and 30-C12-HSL suggesting that MvaT may act as a repressor of quorum sensing (Table 1).[16]

4.4.4. VqsM

VqsM is a novel AraC-type global regulator and a QS modulator that positively regulates the QS systems in *P. aeruginosa*.[20] A mutation in *vqsM* resulted in a much reduced production of AHLs. Global gene expression profile analysis showed at least a total of 302 genes to be influenced, directly or indirectly, by VqsM. Among these VqsM-promoted genes, 52% were known to be QS upregulated. Several genes encoding the key regulators implicated in QS, such as *rhlR, rsaL, vqsR, pqsR(mvfR), pprB, rpoS* and the AHL synthases genes, *lasI and rhlI*, were suppressed in the *vqsM* mutant (Table 1). Moreover, overexpression of *vqsR* in *vqsM* mutant more or less restored the production of both AHLs. These results demonstrated that VqsM, largely through modulation of *vqsR* expression, plays a vital role in regulation of QS signalling in *P. aeruginosa*.[20]

4.4.5. PtxR

PtxR, originally described as a transcriptional activator of the exotoxin A gene *toxA*, also modulates the expression of some virulence factor genes through QS.[7] The expression of the *rhlI* gene and the production of the C4-HSL were increased in a *ptx* mutant while the expression of the *lasI* gene and the production of 3O-C12-HSL were reduced (Table 1). PtxR also reduced the expression of the PQS synthesis operon *pqsABCDE* and, in turn, reduces PQS production. *lasR* and *rhlR* expressions were not affected and it was also shown that Vfr positively regulates *ptxR* expression.

The example given by PtxR is a very good example of the complexity of regulation exerted on the QS regulatory genes. PtxR appears to regulate the three QS systems differently: it regulates the *las* system positively but regulates the *rhl* and PQS systems negatively.[7] The mode of action of this regulator is still unkown.

4.4.6. AmpR

AmpR is a DNA-binding protein that belongs to the LysR family of regulatory proteins. In *P. aeruginosa*, AmpR acts as a global transcriptional activator of β-lactamase production (both AmpC and PoxB) and also as a global regulator of other virulence factors through QS.[51]

Reporter fusion studies indicated that AmpR plays a dual role, positively regulating the *rhlR* expression levels and negatively regulating the *lasI* and *lasR* expression levels. The observed factors of regulation were not very important, but suggested clearly an additional level of complexity in the regulation of these genes.[51] This study provides new insights into the role of AmpR as a global regulator involved in antibiotic resistance and the expression of many virulence factors.

4.5. Concluding Remarks

Over past years, many global regulatory proteins have been uncovered that affect expression of the three QS regulatory systems, *lasR/I, rhlR/I* and *qscR* (Figure 2). This has very much complicated studies on QS signalling. The convergence (direct or indirect) of multiple-signalling pathways at this level and the resulting integration of extra- and intracellular signalling information result in appropriate responses of the bacteria.

Transcription factors amplify their range of control through the regulation of other transcription factors and encompass a set of indirectly regulated genes. This strategy, adopted by many bacteria[60] allows the integration of diverse environmental signals. Therefore, the additional regulation of QS by the mentioned regulatory proteins most likely affects the timing of the response and increases the range of environmental and metabolic signals, in addition to cell density, to which QS responds. The mode of action of these regulators in most cases is still unknown. However, as transcription factors regulate other transcription factors, including themselves in feedback loops and complex relationships, we cannot assume a strict hierarchical organization of the network. The cell is unlikely to have a single hierarchical perspective of regulatory networks.

The *P. aeruginosa* QS system is finely tuned and integrated within the global cell regulatory networks. Knowledge of the number of regulatory factors involved in *P. aeruginosa* QS regulation is increasing with ongoing genetic and microarray studies.[84,97] However, the real impact of these regulators in determining the amount of AHL produced and the molecular mechanisms underlying their interaction with *las* and *rhl* genes in most cases remains poorly understood. Much experimental work lies ahead to provide understanding of these dynamic regulatory networks.

5. THE ROLE OF QUORUM SENSING IN CELL PHYSIOLOGY: A GLOBAL ANALYSIS

The globality of the QS regulation in cellular physiology was a field of interest over recent years. With the availability of microarray technology, a more comprehensive evaluation of *P. aeruginosa* QS regulation was recently made. High-density DNA microarrays has provided tools to decipher the underlying transcription networks. Three different research groups have used microarrays to analyse the LasR- and RhlR-regulated transcriptome of *P. aeruginosa* enabling comparisons to be made between independent studies.[35,82,99] Besides identification of regulated genes, these studies provide interesting glimpses into QS regulons and a wealth of new informations. More recently, microarray studies and subsequent reporter

gene experiments have been performed to define the QscR and VqsR transcriptomes.[44,45,58]

5.1. A Large Number of Genes: 6–10% of the Total Genome, are QS-Regulated

In all three studies[35,82,99] an overwhelmingly large number of genes were shown to be LasR- and RhlR-regulated, with 6–10% of the total genome affected depending on the strains used and the conditions of growth. Estimates of the number of QS-regulated genes included 315 activated genes and 38 repressed genes,[82] 394 activated genes and 222 repressed genes[99] or 163 QS-regulated genes.[35] Ninety-seven induced genes were identified in all three studies. In experimental conditions comparable to those used by Schuster *et al*.,[82] QscR was found to control its own regulon (more than 400 genes. The QscR regulon overlaps with the already overlapping LasR- and RhlR-dependent regulons (150 genes).[58] This large percentage of genes identified as QS-regulated reflects the importance of the global level at which QS influences cellular behaviour.

5.2. Dependence on Environmental Conditions

Only 97 induced genes were identified in all three studies. What is the real meaning of such fluctuations? Besides limitations inherent to the technology used, it became obvious and important to conclude that under different experimental conditions, additional genes may be differently regulated by QS. Changes in media composition and oxygen concentration have shown significant effects on the microarray data produced[99]giving new insights into the links between environmental conditions, QS regulation and cell physiology.

5.3. Modalities of the Regulation

An important finding of these global studies is that a significant number of genes seem to be LasR and RhlR repressed. In *P. aeruginosa*, LasR and RhlR are generally described as transcriptional activators. As an exception, a negative control of the type III secretion (T3S) regulon expression in *P. aeruginosa* PAO1 was evidenced.[3] Detailed studies clearly identified the T3S regulon, except for the regulatory operon *exsCBEA*, as a negative target for QS, control depending on the RhlR/C4-HSL system. Nevertheless, it was not established if this control was direct or indirect.

Wagner *et al*.[99] and Schuster *et al*.[82] identified, using microarray analysis, numerous LasR- and RhlR-repressed genes in *P. aeruginosa*.

Only a few of these genes had a *las* box sequence upstream of the coding region, which may indicate that indirect control mediates a global negative effect on gene expression. Among the QscR-regulated genes, most of them were repressed (329) and 76 genes appeared to be induced by QscR. As in the case of LasR and RhlR, it is likely that QscR indirectly influences gene expression in many cases, but it was clearly shown that QscR is a DNA-binding protein that at least controls directly some genes.[58] As discussed before, further studies need to be performed in order to understand mechanisms underlying this apparent repression.

Temporal patterns of gene expression during growth show that most genes are activated during the transition from logarithmic phase to the stationary phase (at OD_{600}s between 0.8 and 2.0). Nevertheless, a small number of transcripts showed the highest induction early in growth (OD_{600}s from 0.2 to 0.4). Finally, induction is not a permanent state because transcription of some genes is transient and ceases shortly after induction.[82]

Signal specificity was observed. Some genes were transcriptionally activated or repressed in response to 3O-C12-HSL, other genes responded to C4-HSL, and a subset of genes responded differentially to the two AHLs, indicating signal specificity.

Moreover, in agreement with previous studies,[16,105] induction of most genes could not be prematurely induced by the addition of autoinducers. So, microarray analyses have confirmed and extended earlier observations that adding exogenous AHL has little effect on the timing of gene expression to a large number of genes.[82]

5.4. Besides Virulence Factors, Numerous Basic Cellular Processes are Regulated

It was not a surprise that many virulence factors are regulated by QS but a new concept emerged from those studies. Many QS-regulated genes are involved in basic cellular processes, such as DNA replication, RNA transcription and translation, cell division and amino acid synthesis.[82,99] QS regulation of so many diverse functions underline particularly the large physiological impact of QS.

5.5. Concluding Remarks: Overlapping Regulons and Modulons

Since the first studies on LasR and RhlR regulons, it was clear that these two transcription factors regulated some target genes in common and others independently. Microarrays studies amplified these studies and

clearly defined overlapping regulons, not only for LasR and RhlR, but also for QscR and VqsR, as schematically represented in Figure 2.

This complex regulatory network is even more complicated since a second level of signal integration occurs at the promoters of target genes, therefore defining various modulons. Indeed, many QS-target genes are regulated by additional specific regulations at the promoter level, such as regulation by O2, iron deprivation, different sigma factors, etc. Regulation of gene expression by multiple transcription factors occurs quite frequently and in most cases, it seems that a global regulator works together with more specific local regulators.[60] The interplay of several regulatory proteins on the same promoter, as well as the stimulus to which the regulatory protein could respond, deserves further investigations. This genetic organization responds to the necessity of tight regulation and fine-tuning regulation.

6. QUORUM SENSING AND MICROBIOL ECOLOGY

In recent years, many studies have been devoted to decipher the molecular mechanisms of intercellular interactions between bacteria of a same community. Nevertheless, the role of AHLs signalling in intercellular interactions between bacteria and their biotic and abiotic environments is still poorly understood.

Commensal flora, biofilms, are good examples of complex and mixed communities. Intra- and interspecies chemical communications between bacteria have been explored but not completely elucidated.[89] In addition to bacteria– bacteria interactions, many bacteria are also evolved for interaction with eukaryotic hosts (plants or animals) and, in many instances, QS signals of the bacteria have been implicated in these interactions. The nature of interactions through QS signals does not only involve cooperative signals, but also other interactions such as cues, and chemical manipulations.[47]

In the case of *P. aeruginosa*, it has been shown that the 3O-C12-AHL can have varying effects on host cells, including the induction of apoptosis,[90] the inhibition of expression of the nucleotide P2Y receptors in cystic fibrosis tracheal gland cells,[80] and an important immune modulatory activity.[72] While it is clear that both PQS and the long chain AHL, 3O-C12-HSL, have well defined immune modulatory potential *in vitro*[38,91] and that AHLs molecules are detectable in body fluids,[9,66] the true impact of these molecules on host physiology *in vivo* has yet to be fully determined. Therefore it seems that *P. aeruginosa* is capable of manipulating its host through the small QS chemicals.

Moreover, a recent study suggested that *P. aeruginosa* can actively monitor the host immune system and respond by enhancing their

virulence phenotype. It was found that interferon-gamma binds to OprF, an outer membrane protein in *P. aeruginosa*, resulting in the synthesis of a QS-regulated virulence factor, the PA-1 lectin.[114] These data illustrate a new aspect of bacteria–host interactions in which the *P. aeruginosa* QS system both senses and modulates the host immune state.[100]

Interestingly, anti-QS strategies are present in both bacteria and eukaryotes. Indeed, it has been shown that *P. aeruginosa* QS signal 3O-C12-HSL is inactivated by human airway epithelia.[11] Besides inactivation, another example is the production by the host of QS analogues, that could disrupt QS regulatory systems, probably for protection. For example, the marine algae *Delisea pulchra* produces halogenated furanones, structurally similar to the AHL signals, that act as a competitive inhibitors of the AHL-signalling system of bacteria that colonize the surface of the algae.[30,34] This discovery was of prime importance since QS is strongly implicated in the virulence of the bacteria.[87,109] The absence of one or more components of the QS system results in a significant reduction in virulence. This feature has attracted a lot of attention because it could form an interesting target for the development of drugs that could antagonize the production of and/or the response to signal molecules, resulting in extensive studies during recent years.[35,74,75]

7. CONCLUDING REMARKS

The past ten years have seen the publication of a large number of studies on *P. aeruginosa* QS. The mechanisms involved are much more complex than the simple models originally presented.

The QS circuit plays a central role in regulating many gene expression. It is a major global regulatory system that has been estimated to control approximately 5% of the genes, including the most important virulence genes, exoenzymes, motility, nutrient acquisition and biofilm formation.

The central QS circuitry is complex, involving multiple regulatory proteins and multiple-signalling molecules. Many other cellular regulators have been uncovered that affect expression of the three QS regulators. This has complicated studies of QS signalling and has made it particularly challenging. Classical paradigms of prokaryotic transcriptional regulation are simple. However, as the control of individual genes is examined in greater detail, the situation often becomes more complex. Transcription of individual genes may reflect input from multiple DNA-binding proteins, regulatory cascades, RNA-binding proteins, small RNA or metabolites, as well as factors influencing genome structure and local superhelicity. This large panorama corresponds well to *P. aeruginosa* QS.

Microarray technology has revolutionized the ability to comprehensively examine bacterial transcriptional responses to environmental changes and to examine the contribution of specific regulatory loci. Such analyses have revealed unanticipated complexity of regulatory phenomena, and to date, it clearly appears that regulation by "quorum sensing" is more than just quorum sensing.

Finally, the concept of the integration of QS factors into global regulatory networks of the cell is particularly important. The challenge for the future will be to integrate different signals and regulators in order to get a better picture of the extraordinary adaptability of *P. aeruginosa* to changing environmental conditions. There is clearly a need for systematic, global and temporal methodologies for understanding quorum sensing, which could have implications for interruption of biofilm formation, and design of new antibacterial molecules.

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11

MODULATION OF BACTERIAL LIFESTYLES VIA TWO-COMPONENT REGULATORY NETWORKS

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1. INTRODUCTION

The ability of bacteria to survive in specific habitats requires the coordination of the expression of thousands of environmentally regulated genes. The complexity of these regulatory networks increases with the breadth of environments a bacterial species occupies. Species that are able to survive in a broad range of environments generally possess a large coding capacity and

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devote a significant portion of their genome to signal transduction and gene regulation. *Myxococcus xanthus*, a free-living bacterium known for its complex multicellular development and differentiation, uses 8% of its genome for regulation.[67,99] In contrast, the habitat-restricted human pathogen *Helicobacter pylori* dedicates less than 2% of its genome toward this aim.

Transcriptional and posttranscriptional regulators allow the bacterium to selectively activate specific genetic programs in response to a specific environment, and are closely tied to several major signal transduction mechanisms. These include components of the RNA polymerase holoenzyme (the alternative sigma factors), accessory transcriptional regulators, posttranscriptional regulators and small regulatory RNAs,[30,73] together with intra- and extracellular small molecules that act as second messengers.[10] In this chapter, we will give a strong emphasis on an important class of proteins, the two-component systems (TCSs). TCSs are widespread signal transduction devices in prokaryotes, which act both as signal transducers and regulators to directly translate environmental signals into regulatory responses. Currently, more than 4,000 TCSs have been identified in 145 sequenced bacterial genomes[4] demonstrating the enormous importance of these systems for environmental adaptation in bacteria.

The opportunistic pathogen *Pseudomonas aeruginosa* is found in numerous environments including natural reservoirs (fresh and sea water as well as sewage and most soils) and tissues of animal or human hosts. In this organism, almost 10% of the genome is dedicated to the regulation of the remainder.[84,99] One of the striking features of *P. aeruginosa* is that its genome encodes 129 TCS proteins.[72] While the functions of many of these proteins remains unknown, we will discuss the increasing evidence that *P. aeruginosa* uses TCS signaling to modulate global adaptive changes required to transition between different lifestyles (Figure 1).

2. TWO-COMPONENT SYSTEMS, PHOSPHORELAYS, AND VIRULENCE

2.1. Global Definition of Two-Component Systems

TCSs share a conserved core architecture and general signaling mechanism. The primary components include a histidine kinase protein (the "sensor" protein) and a cognate response regulator. These two proteins are often encoded by adjacent genes. The sensor kinase consists of a signal recognition domain (also known as the "input domain") coupled to an autokinase transmitter domain. In many cases, the input domain contains membrane-spanning segments and a periplasmic region that imparts a unique signal specificity by binding a ligand. Cytoplasmic ligand binding

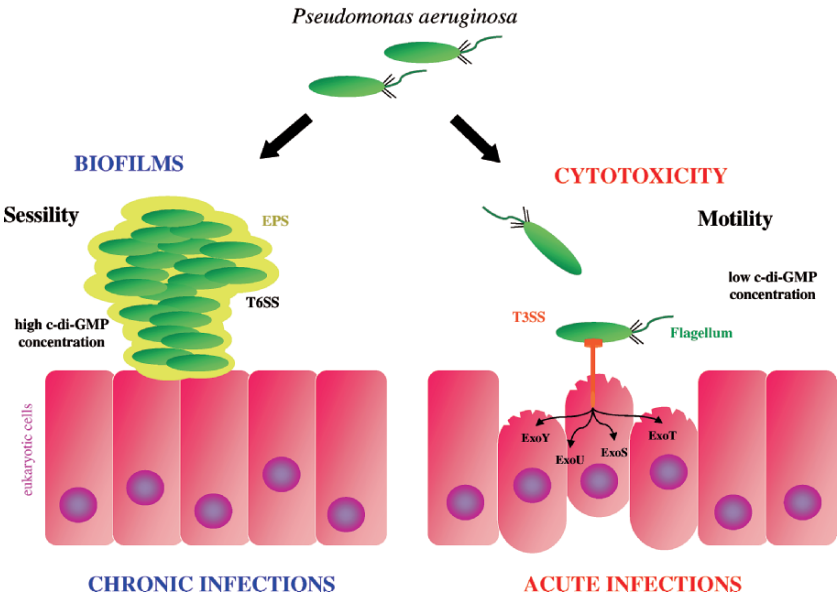

Figure 1. Transition between chronic and acute infections. Free swimming *P. aeruginosa* (in green) may develop a biofilm on host tissues (eukaryotic cells in red). The bacterial community is then embedded in a matrix rich in exopolysaccharide (EPS in yellow) and biofilm formation is favored by high levels of the intracellular messenger molecule c-di-GMP. Expression of several other molecular determinants may be induced in strains developing a hyper biofilm phenotype such as the Type Six Secretion System (T6SS). Biofilm is a favored lifestyle for persistence of bacteria, which results in chronic infections. In the course of an acute infection, cytotoxicity is the favored mode of action, and the Type Three Secretion System (T3SS) is the major molecular determinant involved. It allows the injection of effectors (ExoS, T, U, and Y) into the eukaryotic cells, which results in the subversion of host cell signaling, morphological changes and cell death.

domains, such as HAMP linker, PAS and GAF domains are also present in many sensor kinases.[94] Signal binding causes activation of the autokinase resulting in ATP hydrolysis and phosphorylation of a conserved histidine on the transmitter domain of the protein. The transmitter domain is specific to a receiver domain of a response regulator; transfer of the phosphoryl group to a conserved aspartic acid on the receiver domain of this response regulator activates the signaling pathway. Sensor kinases can also have phosphatase activity toward their cognate response regulator.[11,21] Receiver domains normally inhibit the effector domain (also known as "output domain") of the response regulator and phosphorylation relieves this inhibition, freeing the output domain to carry out its function. That function is usually transcription activation, although enzymes or domains of other types may be controlled in this manner. Response regulator output domains can also contain GGDEF and EAL domains, catalyzing the

production or degradation, respectively, of the second messenger bis-(3′,5′)-cyclic dimeric diguanosine monophosphate (c-di-GMP).[75] Some output domains function in anti-anti-σ factor dephosphorylation (PP2C family) or protein methylation (CheB family). Other output domains with no known function have also been identified.[94] Furthermore, certain response regulators, such as CheY, function without an output domain at all.[51]

2.2. The Phosphorelay Mechanism

Signal transduction by multistep phosphorelay differs from TCS signaling by having a more complicated signal transduction pathway using additional regulator and phosphotransferase domains. For example, in the sporulation phosphorelay of *Bacillus subtilis*, these domains are on individual proteins but in other systems, the domains are associated with the kinase in a multidomain protein. In either case, the phosphoryl group is transferred in the order His→Asp→His→Asp. The additional components give more targets for regulation of the signal transduction pathway, including the ability to sense multiple input signals or to regulate different targets from a shared signal. Regulation of the phosphate flow by dephosphorylation of the receiver domains at multiple stages in a phosphorelay system can also provide added regulatory checkpoints. When the phosphorelay signal transduction pathways were discovered, it was suggested that their increased complexity over the classical TCSs may reflect the need to integrate both positive and negative signals into the output of the pathway.[18] Many phosphorelay-based developmental pathways have been uncovered, including heterocyst formation in cyanobacteria,[32] sporulation timing in *M. xanthus*,[13] development and fruiting body formation in *Dictyostelium*,[86] and hypheal formation and properties in fungi including the human pathogen *Candida albicans*.[9] In most lower eukaryotes the phosphorelays are minor signaling pathways and they appear to be absent in mammals, whereas in plants they are used for several functions.

P. aeruginosa possesses 63 sensor kinases that can be subdivided in several classes, including in 42 classical sensors involved in TCSs, 4 CheA-like histidine kinases involved in chemotactic responses, and 17 sensors involved in phosphorelay pathways. Most of the known families of response regulators are encoded by the *P. aeruginosa* genome: 24 OmpR-like, 14 NarL-like, 8 NtrC-like, 4 CheB-like, 5 CheY-like, and 11 atypical response regulators.[94]

2.3. TCS and Bacterial Pathogenicity

Although the basic biochemistry of TCSs is well understood and some structural insights in the phosphorylation-dependent conformational changes of TCS domains and their interactions have been worked out, several important points remain to be elucidated. This is particularly true regarding the nature of the environmental clues sensed by the TCS, which has been verified experimentally only in very few cases. The presumptive physical and chemical signals that are believed to be detected by TCSs include different ions, temperature, pH, oxygen pressure, osmolarity, autoinducer compounds, the redox state of electron carriers, and contact with host cells. Moreover, in many cases the role of TCSs in the pathogenicity of bacteria is poorly understood, although an attenuation of virulence in animal models of infection has been observed for several TCS mutants.[4] In many cases, the attenuated phenotype of these mutants may be caused by interference with the cells' metabolic requirements rather than with changes in the expression of specific virulence factors. Investigation of the interplay between bacterial and host metabolism has so far been neglected, although it is key to further understanding the principles underlying the successful infection of a host by a pathogenic microorganism.

There are only a few examples in which the mechanisms of virulence regulation by TCSs are well understood. Two-component signaling in bacterial virulence gene regulation exhibits different levels of complexity when integrating various systems into regulatory networks. The regulation systems for *Salmonella* and *Staphylococcus aureus* virulence properties are well characterized and involve a sophisticated interaction of several TCSs and additional regulators to control expression of virulence factors at different stages during infection. For example, the regulation of *S. aureus* virulence involves the AgrA-AgrC TCS, which responds to cell density to control the transcription of the regulatory RNA III, as well as the three additional TCSs SaeR/SaeS, SsrA/SsrB, and ArlR/ArlS.[6,58] The BvgA/BvgS TCS is an intriguing example of a system that appears to be the master regulator of virulence controlling virtually all known virulence traits of *Bordetella pertussis*.[14]

Of the 129 proteins involved in two-component signal transduction pathways in *P. aeruginosa*, only 48 have been studied.[94] All the chemotactic proteins (13 in total) have been well characterized and control swimming, twitching motility and aggregation.[15,42,98] The other 35 TCSs studied, to date, regulate diverse processes such as ethanol and glycerol metabolism, and phosphate, iron and nitrogen starvation.[94] Moreover, more than two thirds of the already studied TCSs have been shown to modulate resistance to antibiotics and virulence mechanisms in *P. aeruginosa*.

3. *P. AERUGINOSA* CAN LIVE MULTIPLE LIFESTYLES

3.1. *P. aeruginosa* as a Human Pathogen

Although *P. aeruginosa* is a ubiquitous environmental bacterium, it is also one of the top three causes of opportunistic human infections. It is likely that the ability of *P. aeruginosa* to sense and respond to specific host factors enables this pathogen to establish infection in a range of sites, evade the host immune system, and activate virulence mechanisms in the appropriate context. Genome sequences revealed that, in *P. aeruginosa*, mechanisms for monitoring environmental stimuli are encoded in both conserved and accessory elements of the genome. The conserved (core) genome consists of approximately 5,000 genes found in all strains analyzed to date.[100] Included in this core genome are a surprisingly large number of virulence factors, which may provide an explanation for the broad pathogenic potential of this particular member of the genus *Pseudomonas*. The observation that these virulence factors are conserved in nonclinical isolates suggests that such factors may also have a role in the natural reservoir.

In most cases, human *P. aeruginosa* infections require some underlying breach of host defense mechanisms. The acute infections are characterized by a rapid progression, associated with the expression of multiple virulence factors. *P. aeruginosa* virulence factors validated in animal models of acute infections are correlated with a poor prognosis for human infections. In some cases, *P. aeruginosa* is capable of causing acute pneumonia, breaking down lung defenses and disseminating in the bloodstream, resulting in systemic infection, septic shock, and death of patients in hours or days. In this case, *P. aeruginosa* is invasive and cytotoxic. As an example, *P. aeruginosa* has a type III secretion system (TTSS) and produces a battery of extracellular toxins that are believed to play a role in acute infections such as pneumonia.[3,27,55] The TTSS of *P. aeruginosa* has also been shown to play a role in survival of this pathogen in the blood and in systemic dissemination.[92] Treatment of wounds, burns and corneal injury is commonly complicated by acute *P. aeruginosa* infection and this is considered the most serious cause of life threatening burn-related infections.[23,68,78] Similarly, organ transplant procedures and various malignancies are often accompanied by *P. aeruginosa* infection. In hospitals, urinary tract catheterization and the use of respiratory ventilators commonly result in *P. aeruginosa* colonization at the installation site.[2] Overall, *P. aeruginosa* is implicated in 10% of nosocomial infections.[77] Infection of otherwise healthy patients is less common and manifests as keratitis infections of contact lens wearers, hot tub folliculitis, and otitis externa and media. In most cases, innate

and acquired elements of the immune system are able to recognize and clear the invading bacteria. For seriously compromised patients, only prompt and intensive antibiotic treatment can prevent a rapid progression of *P. aeruginosa* bacteremia, sepsis, and death. Successful treatment of *P. aeruginosa* infections, however, is further complicated by the high level of antibiotic resistance of the majority of clinical isolates of this organism.

P. aeruginosa overcomes rigorous antibiotic treatment and an apparently intact immune system in patients with cystic fibrosis (CF) and remains in the lung for decades until chronic inflammation leads to death.[28] Natural reservoirs appear to be the major source of the infecting organism, although incidence of person-to-person transmission among patients in certain CF clinics has also been reported.[12,59] The colonization of the respiratory tract of CF patients by *P. aeruginosa* can be divided in two phases. Initially, *P. aeruginosa* remains restricted to the upper airway, a habitat shared with other pathogens such as *S. aureus* and *Haemophilus influenzae*. In many of its clinical features, this early phase of infection resembles an acute infection, limited by the functional innate immune defenses of the lung, and shows minimal symptoms of respiratory disease. Progression of infection into the chronic phase is accompanied by colonization of lower airways, displacement of other respiratory pathogens and a pronounced neutrophil-dominated inflammatory response, which is a major contributor to the decline in pulmonary function. Despite the heavy bacterial load within the CF airways ($>10^8$ cfu/g of sputum), *P. aeruginosa* remains essentially noninvasive, is not cytotoxic, and does not progress into a systemic infection. Several phenotypic changes accompany this persistent phase of infection, including overproduction of the exopolysaccharide alginate (mucoidy) and the acquisition of mutations that block toxin secretion and motility.[40,54] It is likely that *P. aeruginosa* persists for decades without these virulence factors by growing in a matrix-protected biofilm community in which the bacteria are more resistant to neutrophil-mediated clearing and antibiotic treatment.[19,79] Bacteria that detach and regain some of the phenotypes of planktonic cells can establish a new biofilm at a different location in the respiratory tract. This cycle very likely contributes to the large bacterial load in CF patients.[38,65] Therefore, production of secreted toxins and motility seem to play a larger role during initial colonization, while growth as a biofilm allows the bacteria to cause a chronic infection (Figure 1).

The observation that there is positive selection for the loss of toxin secretion and motility mechanisms during chronic phase of CF infection suggests that expression of these factors may compromise establishment of a persistent infection. It has frequently been reported that a significant fraction of *P. aeruginosa* isolates from patients suffering of chronic

infections are defective in motility and lack a functional TTSS.[17,54] Conversely, since the production of copious amounts of the exopolysaccharides required for biofilm formation could interfere with the effective function of factors necessary for acute infection, the genes for these biofilm-promoting factors may be actively repressed during initial stages of disease. This is particularly true of the TTSS, which requires direct contact between the surface of the bacterium and the host cell for proper function of the injection apparatus. It is therefore not surprising that bacteria employ some form of reciprocal regulation during acute and chronic infections.

3.2. Biofilms and Chronic Infections

In *P. aeruginosa* and other bacteria, genetic analysis indicates that biofilm formation is a complex process with discrete, temporal stages of development, and specific factors required for each stage.[60] The development of bacterial biofilms involves the regulated and coordinated transition from free-swimming planktonic bacteria to highly differentiated communities of surface attached organisms.[83] In *P. aeruginosa*, this developmental pathway proceeds from the initial attachment of bacteria to a surface, followed by the accumulation of cell clusters or microcolonies, and ultimately, a mature biofilm characterized by large aggregates of bacteria separated by fluid-filled channels.[44] Hallmarks of a mature biofilm include the production of an extracellular matrix (extracellular polymeric substance, EPS). The EPS is composed of nucleic acids, proteins, and exopolysaccharides. To date, three different exopolysaccharides have been identified in *P. aeruginosa*. Alginate, a polymer of mannuronic and guluronic acids, is thought to play a protective role in the relatively harsh environment of the CF lung, perhaps by enhancing the formation of biofilms. The other exopolysaccharides are less well characterized. Two gene clusters, *pel* and *psl*, are involved in the production and secretion of glucose- and mannose-rich extracellular matrix, respectively.[24,25,93,102]

Multiple lines of evidence suggest that *P. aeruginosa* biofilm growth may be responsible for some of the features of chronic human infections, particularly those in the CF lung. The profile of quorum sensing signaling molecules detected in CF sputum samples is similar to that produced *in vitro* by biofilm-grown but not planktonic bacteria.[80] Images of polysaccharide-encased bacteria in CF sputum are also consistent with the idea that *P. aeruginosa* exists in a biofilm or biofilm-like state in the CF lung. In CF infections, *P. aeruginosa* is characterized by its resistance to many antibiotics; this is one of the physiological adaptations observed in bacteria growing as a biofilm *in vitro*.

The transition from initial colonization to chronic infection could proceed in several ways. Environmental signals present in previously uninfected hosts could diminish as the disease progresses, preventing activation of acute infection-associated genes at later stages. Alternatively, signals present only during chronic infection could activate specific genetic programs in later stages of disease. Recent work by several laboratories provides some insight into signal assimilation that may be operating during the adaptation of *P. aeruginosa* to its host in different disease settings. The adjustment of the transcriptome to fit the requirements of a specific environment occurs by the utilization of sensors that act as signal distribution switches, often with reciprocal outcomes on the expression of groups of genes. Several common themes have emerged from these studies: (i) the assimilation of multiple inputs into a shared signaling pathway, (ii) the ability of a single pathway to disseminate positive and negative regulatory outputs, (iii) transcriptional as well as posttranscriptional mechanisms are used in regulating target genes, and (iv) the importance of activating certain pathways and repressing others to favor one phenotype and completely abolish another.

4. PHOSPHORELAYS CONTROL KEY TRANSITIONS DURING THE INFECTIOUS PROCESS OF *P. AERUGINOSA*

The TTSS could be considered a molecular weapon that plays a major role in *P. aeruginosa* cytotoxicity. It is therefore an excellent marker for the success of an acute infection. In contrast, exopolysaccharides and cell surface adhesions are key players in the formation of a bacterial biofilm. Biofilm formation is the favored developmental mode for the success of a chronic infection and the persistence of the bacteria within its host. Expression of genes involved in biofilm formation and cytotoxicity might thus be controlled by environmental conditions that are encountered during the infection process. In this respect TCSs may play an important role. For example, one of the most studied TCSs of *P. aeruginosa* is the PilS/PilR system. This system is dedicated to control the production of type IV pili that mediate adhesion to epithelial cells, surface colonization,[41] and biofilm formation.[62] Another TCS, CopS/CopR, has been shown to regulate the expression of the *ptrA* gene in response to high copper concentration.[31] PtrA neither shares homology with any known protein nor has any conserved domain or motif. Nevertheless, PtrA binds directly to the transcriptional activator ExsA, which is absolutely required for TTSS gene expression. In the presence of high copper concentration, the CopRS system shuts down the TTSS.

With these two examples one can see that one signaling pathway influences expression of determinants involved in biofilm formation whereas another pathway represses the production of determinant required for cytotoxicity. We would like to highlight the emerging concept that complex regulatory networks control, simultaneously but in an antagonistic manner, expression of target genes that are required for biofilm formation versus cytotoxicity. In an extension of previous reviews,[22,26,61,103] we would like here to give a particular emphasis to two signaling pathways involving phosphorelays. In fact, several recent publications have reported that *P. aeruginosa* integrates a range of environmental signals to determine whether the cell will be competent to activate the TTSS and other virulence factors required for acute infection or instead to upregulate production of exopolysaccharides and related factors associated with chronic infection (Figure 1).

4.1. The RetS/LadS/GacS System

4.1.1. The RetS Kinase Sensor

A recent report described a histidine kinase sensor (RetS, *r*egulator of *e*xopolysaccharide and *t*ype III *s*ecretion) as a molecular switch that reciprocally regulates genes associated with acute infection and chronic persistence.[29] The *retS* gene corresponds to the *orf* annotated PA4856 on the *P. aeruginosa* PAO1 genome and is conserved among all *P. aeruginosa* isolates analyzed to date. The structure of RetS consists of an input domain and a transmitter domain that includes a classical histidine kinase and an ATPase motif. RetS is a hybrid sensor that contains two predicted receiver domains at its C-terminus, which is a rather unusual feature. The *retS* gene was identified because a mutant of the *P. aeruginosa* strain PAK in this gene showed a hyperadhesive phenotype. The *retS* mutant strain had a strong propensity to clump when grown in liquid culture, and to avidly attach to the walls of the tubes or the culture flasks. The enhanced adherence of the *retS* mutant was further tested on biotic supports and it was observed that the *retS* mutant was hyperadhesive on cultured CHO cell monolayers. However, when the *retS* mutant was tested in a murine acute pneumonia model, it appeared to be unable to establish an infection. Another study by Zolfaghar *et al.*,[104] indicated that a *retS* mutant in the *P. aeruginosa* PA103 strain also displayed attenuated virulence in a murine model of acute corneal infection. A microarray analysis comparing wild-type *P. aeruginosa* versus the *retS* mutant indicated that each of the 40 structural and regulatory genes of the TTSS were downregulated in the mutant,[29] which might explain its attenuation in the infection model. Expression of TTSS genes and production of TTSS effectors is indeed strongly associated with *P. aeruginosa* virulence in animal models but also in studies of human diseases.[33,76,82] Furthermore, the microarray analysis showed that gene clusters required for exopolysaccharide production were

strongly upregulated in the *retS* mutant.[29] These gene clusters, namely *psl* and *pel*, contribute to the production of a mannose-rich or glucose-rich extracellular matrix, respectively.[24,25,93] The construction of a double mutant *retS*/*psl* confirmed that the hyperadhesive phenotype of the *retS* mutant was strongly *psl*-dependent. This is also supported by the observation that microarray analysis revealed that genes involved in type IV pili assembly are downregulated in the *retS* mutant, which nevertheless shows an increased level of attachment to epithelial cells. Interestingly, the *retS* gene was also identified in an alternative screen for strains defective in twitching motility.[104] In this study, the PA103 mutant with a Tn insertion in *retS* presented a small colony phenotype indicative for a poor surface motility.

Another study by Laskowski and collaborators[49] has also confirmed the role of RetS in expression of TTSS genes. The authors had initially called this protein RtsM for regulator of *t*ype III *s*ecretion. The gene was identified from a screen for noninvasive *P. aeruginosa* mutants toward epithelial cells. Using northern blot analysis, Laskowski and colleagues went on to demonstrate that mRNAs encoding the effectors ExoT and ExoU were not detectable in a *retS* mutant of the *P. aeruginosa* strain PA103. They also showed that expression of the operon containing *exsA*, the gene encoding the TTSS master regulator, was decreased in a *retS* background as compared to the wild-type strain. The *exsA* expression was monitored by using a *luxCDABE* reporter fused to the *exsCBA* promoter. The lack of TTSS function resulted in a lack of *in vitro* cytotoxicity on Hela cells, and for the attenuated virulence in a murine model of acute pneumonia. Moreover, overproducing the TTSS master regulator ExsA in a *retS* mutant fully restored TTSS effector production and secretion.[49] It should be noted that the *retS*-dependent attenuation phenotype is not exclusively linked to an ExsA-dependent mechanism and TTSS function. The microarray analysis of the *retS* mutant indicates that more genes are targeted by the RetS signaling pathway, including the type II secretion system and a subset of its substrates.[29] Moreover, Zolfaghar and collaborators compared the phenotypes of *retS* and *exsA* mutants and could identify significant differences.[104] For example, these authors showed that a *retS* mutant was less efficient, as compared to an isogenic *exsA* mutant, at corneal epithelial cell invasion, and that in contrast to the *exsA* mutant a *retS* mutant lost viability within epithelial cells during the first few hours following invasion.[104] Zolfaghar and collaborators performed another study in which they compared the fate of PA103 and isogenic *exsA* or *retS* mutants several days postinfection in the murine corneal infection model.[104] The data presented indicated that the attenuation of the *retS* mutant is only temporary and last the first few days postinfection. However between days 2 and 7, the *retS* mutant showed a significant increase both in colonization and disease severity. This

increased virulence was significant both when compared to the *exsA* mutant and the parental PA103 strains.[105] These findings suggest that whereas the TTSS is an important virulence factor during early stages infection, at later stages other molecular determinants could compensate for the loss of the TTSS. Since the production of exopolysaccharides by the enzymes encoded in the *pel* and *psl* genes is increased in a *retS* mutant, these are obvious candidates for promoting *retS* strain colonization, biofilm formation and inhibition of bacterial clearance by phagocytes at later stages of infection.

Laskowski and Kazmierczak further characterized the role of the RetS domains in signal transduction.[48] As mentioned earlier the presence of two receiver domains at the C-terminus of a hybrid sensor is unusual and such organization cannot be found in any of the other 64 kinase sensors encoded on the PAO1 genome. The PleD sensor from *Caulobacter crescentus* contains a tandem receiver domains. However, in this case only one domain has a phosphorylatable aspartate residue.[34] In the case of the 929 amino acid-long RetS protein, the phosphorylatable aspartate residues from the receiver domains called R1 and R2 were found at position 713 and 858, respectively.[48] In addition, residues required for the formation of an acidic pocket in the R1 and R2 domains are also found at position D664/D665 and E814/D815, respectively. Introduction of single mutations replacing aspartate residues for alanine residues revealed that R1 and R2 are not functionally equivalent. Indeed, mutation resulting in D713A substitution in R1 allows complementation of a *retS* mutant, whereas mutation resulting in D858A substitution in R2 did not. Complementation was determined by following transcription of the *exoT* gene in the PA103 strain. The presence of two phosphorylatable, but not functionally equivalent domains was also reported in the case of the FrzZ sensor in *M. xanthus*.[88] This observation does not necessarily indicate that the R1 domain has no functionality. Indeed, and as expected, a substitution at the site of autophosphorylation in RetS, H424Q, resulted in a severe lack of function, but the protein was not completely inactive. However, combining an additional mutation in the R1 domain, increased significantly the level of *exoT* mRNA in the double mutant strain, which suggests that the R1 domain might interfere with RetS-dependent induction of TTSS expression. These results were confirmed in a mouse model of acute pneumonia, in which only the bacteria expressing the R2 mutant allele RetSD858 were avirulent. Bacteria expressing RetSH424Q were recovered in numbers, less than the parental strain but 100 times more as compared to a *retS* mutant. Finally, bacteria producing RetS with the double substitution H424Q and D713A, were nearly as virulent as the parental strain and were able to disseminate to the liver and spleen.[48] Since the RetS kinase devoid of an autophosphorylation site (H424Q) is still functional, and only the R2 domain appeared required for RetS function in all

tested assays, it is conceivable that additional sensor kinases besides RetS might use the RetS D2 domain as a phosphorylation substrate.

4.1.2. The LadS Kinase Sensor

Shortly after the publications describing RetS, another paper reported the characterization of a sensor, unlinked to a response regulator, with antagonistic activity on exopolysaccharide production and TTSS activity.[95] This kinase was called LadS, for lost adherence sensor, since a *ladS* mutant was characterized by an altered biofilm development. Strikingly, the structure of the LadS histidine kinase hybrid sensor was very similar to RetS: the only difference was the presence of only one receiver domain at the C-terminus of LadS whereas RetS had two (Figures 2 and 3). Interestingly, microarray and transcriptional reporter fusion analysis revealed that the gene targets for the LadS signaling pathway included the *pel* and TTSS genes (Figures 2 and 3). It was shown that in the *ladS* mutant, *pel* gene expression was downregulated which may account for the defective biofilm phenotype. Furthermore, the overproduction of LadS resulted in a hyperbiofilm phenotype (as seen by thicker rings of attached bacteria on an abiotic surface) that was not observed in a *pel* mutant, indicating that the LadS effect was *pel*-dependent. Furthermore in a *ladS* mutant TTSS genes were upregulated, which resulted in an increased cytotoxicity on cultured Hela cells. These results indicated that RetS and LadS initiated two antagonistic signaling pathways (Figures 2 and 3). A more extensive comparison of the *retS* and *ladS* transcriptome also supported the idea that LadS and RetS have a global mirror effect on their gene targets. Indeed, when one considers only those targets whose expression varies both in *retS* and *ladS*, it is readily observed that in most cases the variation is reciprocal; that is to say, genes that are upregulated in the *ladS* mutant are downregulated in the *retS* mutant and vice-versa.

Most of the known genes that are reciprocally regulated by LadS and RetS encode virulence factors; it is therefore probable that some others genes, also reciprocally regulated by these two sensors, encode some yet uncharacterized virulence factors. In a recent report by Mougous and colleagues,[56] it was demonstrated that a gene cluster with previously no associated function, regulated in a antagonistic manner by both LadS and RetS, actually encodes a type VI secretion machinery and is involved in secretion of at least one exoprotein called Hcp1, required for *P. aeruginosa* virulence. This gene locus, named HSI-1 for Hcp1 *s*ecretion *i*sland is positively regulated by the LadS regulatory pathway and negatively regulated by the RetS pathway (Figures 2 and 3). It was further demonstrated that this locus actively secretes Hcp1 during chronic infection of CF patients.[56] All these data argue that the HSI-1 type VI secretion system has an important role during *P. aeruginosa* chronic infections. Moreover,

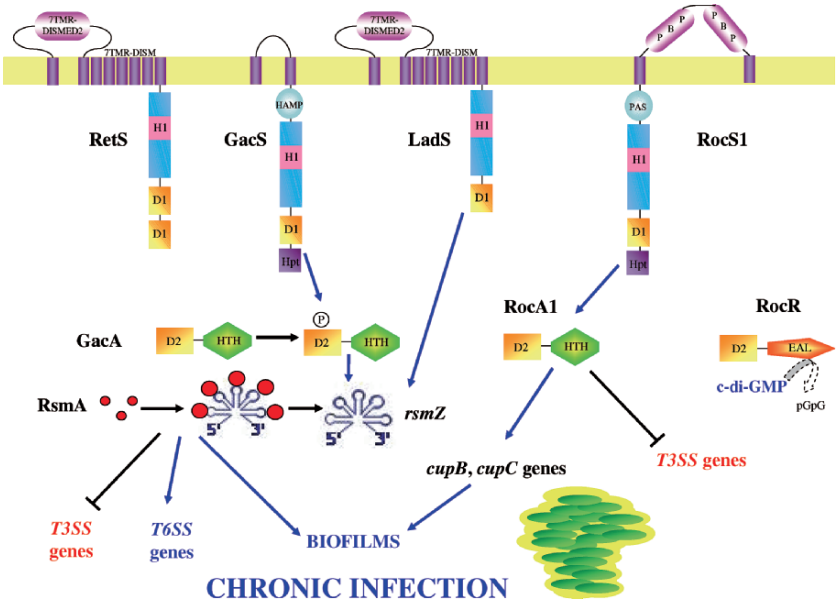

Figure 2. Signaling network and chronic infections. The four sensors, RetS, GacS, LadS, and RocS1 are represented inserted in the cytoplasmic membrane (yellow) with their transmitter domain (blue) on the cytoplasmic side. The detector domain of each sensor is variable although RetS and LadS have a similar 7TMR-DISMED2 domain. The receiver domain found on the sensors or the response regulators GacA, RocA1 or RocR are represented in yellow. GacS and RocS1 are nonorthodox sensors, which contain an additional Hpt domain (purple). LadS and GacS are positively controlling the high level of the small RNA *rsmZ*, allowing titration of the RNA binding protein RsmA (red circle). Whereas GacS influences gene expression via phosphorylation of GacA (P), it is not know which phosphorelay intermediates are involved in the LadS signaling pathway. If RsmA is titrated upon *rsmZ* binding, it will allow expression of T6SS genes or *psl* and *pel* genes involved in exopolysaccharide biogenesis and biofilm formation, whereas expression of T3SS genes is decreased. Moreover, RocA1 activation through the sensor RocS1 results in induction of the *cupB* and *cupC* genes, which favor biofilm formation, whereas T3SS expression is reduced. The combination of these two pathways thus promotes biofilm formation and chronic infections. Both RocA1 and GacA possess a HTH (helix turn helix) domain (green) for binding DNA and controlling gene expression.

the association between genes activated by RetS and chronic infection was strengthened by the observation that the HSI-1 locus was upregulated in a retS mutant, repressed in a ladS mutant, and required for persistence in a chronic rat lung infection model.[52] Therefore, targets of coordinately regulated genes can provide important and unexpected clues about the lifestyle of an organism in a specific pathological niche.

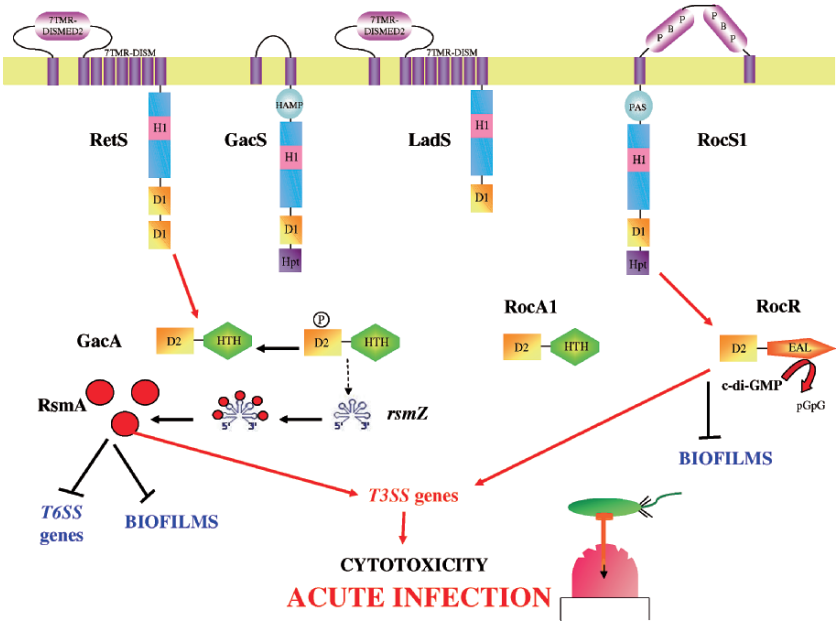

Figure 3. Signaling network and acute infections. The representation of the molecules is as in Figure 2. Here, the signaling through the RetS sensor resulted in low *rsmZ* level and thus high levels of free RsmA. As a consequence, T3SS genes will be activated and molecular determinants of biofilm formation repressed. Moreover, when RocR is activated by RocS1, c-di-GMP levels are low due to the activity of the output domain of RocR which is a phosphodiesterase containing the EAL motif (red). As a result, *cupB* and *cupC* gene expression is down regulated. The combination of these two pathways enhances bacterial cytotoxicity via the T3SS, prevents development of biofilm formation, and thus promotes acute infections.

4.1.3. The RetS and LadS Detector Domains

RetS and LadS are membrane hybrid sensors that share a common organization of their cytoplasmic domains, with a histidine kinase transmitter domain followed by a receiver domain. These sensors are also homologous in their N-terminal putative sensing or detector domain. Both sensors have an N-terminal transmembrane segment followed by a long periplasmic loop and a transmembrane domain composed by seven transmembrane segments (named 7TMR-DISM).[1] The periplasmic domain of RetS and LadS belongs to a class of bacterial periplasmic sensor modules called 7TMR-DISMED2. The secondary structure of the periplasmic loop, a series of α-helices bordered by two β-sheets, is reminiscent of carbohydrate binding motifs (CBM) and could be involved in carbohydrate binding,[1] suggesting that these two proteins may respond to carbohydrates of host or bacterial origins.

There is no information available about the function of the 7TMR-DISMED2 in LadS-mediated signaling, but Laskowski and Kazmierczac investigated the role of this detector domain of RetS.[48] They have generated two deletions, one resulted in the removal of the periplasmic loop (RetS Δ37–185) and the other included both the loop and six out of seven transmembrane segments of the 7TMR-DISM domain (RetS Δ37–360). By testing the complementation of *retS* mutation by those variants in the murine model of infection, two different roles for each domain were revealed. Deletion of both the periplasmic loop and the 7TMR-DISM domain led to a protein unable to complement a *retS* mutation. In contrast, the RetS variant lacking only the periplasmic loop (Δ7TMR-DISMED2) restored a wild-type phenotype of the *retS* mutant. These observations suggest that reception of the stimulus through the 7TMR-DISMED2 domain might result in inhibition of RetS function *in vivo*. Moreover, the transmembrane domain may have an independent sensing function or be involved in interaction and cross-talk with other proteins leading to activation of RetS.

Interestingly, *P. aeruginosa* possesses two other genes in its genome encoding proteins with both the 7TMR-DISM and the 7TMR-DISMED2 domains. One such protein is PA3452, another hybrid sensor. Preliminary results show that this sensor, in contrast to LadS and RetS, is not involved in the control of the attachment process or biofilm formation.[29] The second related protein, PA4929, belongs to the so-called GGDEF family of regulatory proteins.[75] A recent extensive study of *P. aeruginosa* proteins belonging to this family revealed that PA4929 controls neither biofilm formation nor cytotoxicity.[46] From these observations, one might conclude that the 7TMR-DISM and the 7TMR-DISMED2 domains, even if they are shared between LadS and RetS, are not dedicated to the specific regulation of biofilm formation or TTSS-mediated cytotoxicity.

4.1.4. Convergence of RetS and LadS Pathways into the GacA/GacS/RsmZ/RsmA Circuitry

A search for additional components in the RetS signaling pathway used a library of mariner transposon insertions in a *retS* mutant/*exoS* reporter background. This screen selected for suppression of hyperadhesion and TTSS repression caused by the absence of RetS. The majority of the strains that lost their capacity to adhere to the walls of the culture flask and presented an activation of the *exoS* promoter under low calcium conditions carried mutations in *gacS*, *gacA*, or *rsmZ*.

GacS is an unorthodox kinase sensor, which likely autophosphorylates in a signal-dependent manner and subsequently phosphorylates the response regulator GacA. GacA is required for transcription of the small

RNAs that control the posttranscriptional regulator RsmA.[36,66,69] RsmA positively and negatively regulates a broad range of downstream targets by binding to their mRNAs and stabilizing or destabilizing them.[8,35,36] Two small noncoding RNAs, termed RsmY and RsmZ (also called RsmB), bind RsmA and inhibit its translational regulatory function by sequestering it.[43] In *P. aeruginosa*, RsmA was shown to be a negative control element for pyocyanin, hydrogene cyanide, PA-IL lectin, elastase (LasB), and staphylolytic enzyme (LasA).[66] RsmA was also shown to have a positive control on swarming, rhamnolipid, and lipase production.[36] Supporting these data is the observation that a *retS* mutant presented both the pyocyanin overproduction and the swarming deficiency phenotypes reported for a *rsmA* mutant. More recently, it was shown that RsmA, in the absence of RsmY and RsmZ, induces the TTSS and other factors associated with acute infection in response to the appropriate signals such as host cell contact.[57] Finally, in a variety of bacteria including *P. aeruginosa*, RsmA also functions to reduce the levels of mRNAs encoding exopolysaccharide biosynthetic genes.[74,97]

The GacA/GacS two-component system is strictly required for expression of *rsmZ* in *P. aeruginosa*[36]. RsmZ RNA has a predicted secondary structure with four stem loops that contain GGA motifs, which may be used as RsmA binding sites. Because of the relation between RetS and GacA/GacS, the role of RetS in *rsmZ* expression was investigated. An *rsmZ–lacZ* fusion was shown to be drastically upregulated in a *retS* mutant,[95] and a *retS* mutant was shown to have 18 times more *rsmZ* transcript than the wild-type strains.[48] These data agree with the *retS* phenotypes. We have also seen in previous paragraphs that the LadS and RetS signaling pathways act antagonistically. It was observed that in contrast to the *retS* mutant, *rsmZ* expression was downregulated in a *ladS* mutant, similarly to what was observed with *gacA* or *gacS* mutants.[95] Thus, RetS functions in an opposite manner to GacS and LadS. Whereas GacS and LadS are required for *rsmZ* expression, RetS represses *rsmZ* transcription. However, one main difference between the GacS and LadS pathways is that whereas a *gacS* mutation suppresses the *retS* phenotype, *ladS* mutation does not.[95] The observation that GacS and GacA, but not LadS, are required for RetS-dependent *rsmZ* repression suggests a hierarchical organization of these signaling systems. RsmY, a second Gac-dependent small RNA,[43] may be controlled similarly.

As a result, by modulating the activity of GacS, LadS, and RetS, it is conceivable that *P. aeruginosa* transitions from a state in which it is competent to establish an acute infection to a state in which the bacterium is insensitive to cues such as host cell contact. In a coordinate manner, factors required for biofilm formation and other aspects of chronic infection are produced, potentially allowing the bacterium to

persist for decades. It is likely that multiple criteria must be met to mediate the transition between acute and chronic adaptation. These may include the disappearance of the RetS-activating signal as well as the presence of GacS- and LadS-activating ligands. These signals are integrated into a single transduction pathway, which ultimately determines the expression of small RNAs, such as RsmZ and RsmY, involved in posttranscriptional regulation via the RNA-binding protein RsmA. The pathway results in the reciprocal expression of specific downstream genes involved in cytotoxicity and biofilm formation. This complex regulatory network could thus allow *P. aeruginosa* to make the choice between developing a strategy which favors an acute or a chronic infection (Figures 2 and 3).

How RetS and LadS intersect with the GacS pathway at the molecular level is not yet understood. As we already discussed, RetS might be used as an intermediate phosphorelay, via its second receiver domain, which might be phosphorylated by another kinase. GacS and/or LadS are putative candidates. Moreover, it cannot be excluded that RetS preferentially acts as a phosphatase on the GacS/GacA pathway, which would then result in an antagonistic activity as compared to GacS. RetS could directly dephosphorylate GacA, but alternatively could act directly on GacS to counteract its activity. The absence of a null phenotype of the RetS H424Q derivative might support the idea of the RetS phosphatase activity being critical for its function.[48] Indeed, it is reported that histidine to glutamine mutations in the conserved histidine residue systematically disrupt the kinase activity, however these mutants can retain their phosphatase activity. This was shown, for example, with the EnvZ sensor from *Escherichia coli*.[39] It is also important to recall that both RetS and LadS are hybrid sensors, which means that they need an additional histidine phosphorelay to transduce the signal from the aspartate of their receiver domain onto the aspartate of the receiver domain of a cognate response regulator. Such a phosphorelay may function as an independent histidine phosphotransfer (Hpt) protein. It will thus be important to investigate the role in this regulatory cascade of the three putative Hpt proteins annotated on the PAO1 genome.[72] Moreover, it has also been shown that nonorthodox sensors could be used in phosphorelay pathways independent of their kinase/phosphatase activity.[87] Finally, it is known that YojN (RcsD) from *E. coli* is used as a Hpt protein in the RcsCB pathway, even though it has the structure of an atypical sensor.[85] At that stage, no direct evidence for the relation between LadS and GacA/GacS pathways has been shown. Obviously, biochemical approaches and protein–protein interaction studies will be required to characterize the direct partners, their mode of action, and the phosphotransfer route within this complex regulatory network.

4.2. The Three-Component System RocS1/RocR/RocA1

4.2.1. Role of RocS1/RocR/RocA1 in Adhesion

In *P. aeruginosa,* it appears that in addition to polysaccharide synthesis, biofilm development is promoted by the production of fimbrial structures encoded by the *cupA*, *cupB*, and *cupC* gene clusters.[90] The expression of *cup* genes is very low when strains are grown in standard laboratory conditions. In the case of the *cupA* gene cluster, a mutation in *mvaT*, which encodes an H-NS-like protein, is required for high level of *cupA* gene transcription.[89] Expression is further controlled by an MvaT-dependent phase variation mechanism.[91] In case of the *cupB* and *cupC* genes, expression was assayed by engineering *cupB–lacZ* and *cupC–lacZ* transcriptional fusions onto the chromosome of a *P. aeruginosa* strain. A subsequent screen of mutant libraries generated with a modified form of the mariner transposon resulted in the identification of mutants in which *cupB* and or *cupC* genes were upregulated. Most of the transposon insertions recovered were found to be located in a cluster of three genes that was named *roc* for regulation of *cup* genes.[47] The genes encode a phosphorelay system composed of three different partners. It consists of the multidomain sensor kinase RocS1 and two adjacent response regulators, the c-di-GMP phosphodiesterase RocR and the NarL-like response regulator protein RocA1. RocS1 is a nonorthodox sensor, which contains a typical kinase domain, a receiver domain, and an Hpt (or H2) domain containing a phosphorytable histidine residue. Overexpression studies suggest that the sensor RocS1 activates *cupB* and *cupC* expression in a RocA1-dependent manner, while RocR inhibits expression of these targets.[47] Therefore, RocS1 may activate RocA1 by a phosphorelay mechanism and RocR somehow interferes with the activities of the RocS1/RocA1 signal transduction system. It should be noted that the upregulation of the *cupB* and *cupC* gene clusters is concomitant with an increased ability to develop a biofilm.

In another study, Kuchma and collaborators characterized the very same gene cluster, which they called *sadARS*.[45] The surface attachment defective (*sad*) mutants were previously identified in a screen for biofilm deficient mutants in 96-well microtiter plate assay.[62] One such mutant, *sad160*::Tn5, had an insertion located within the intergenic region between *sadR* (*rocR*) and *sadA* (*rocA1*). The phenotype of the mutant versus the wild-type strain was rather subtle; after a 4-h incubation, the air/liquid interface biofilm ring was observed in the mutant but not in the wild type, whereas after 8 h incubation the phenotype was reverted and a biofilm ring was seen with wild type but not in the mutant.[45] However, while the biofilm was reduced at the air/liquid interface in the mutant, the amount of bacteria below the interface increased. When deletion mutants

were engineered in each of the three genes (*sadARS* = *rocA1RS1*), decreased biofilm formation at 8 h was observed, but after 24 h all strains attached to the surface of the microtiter wells to a degree comparable to that of the parental strain. The architecture of the biofilm was also analyzed by using a flow cell system and confocal microscopy. The *sadARS*/*rocA1RS1* deletion mutants all form biofilms lacking distinct macrocolonies and had a drastically reduced network of channels as compared to the parental strain. These observations strongly suggest that the mutants are defective for mature biofilm architecture and that the Roc signaling pathway plays an important role in the control of biofilm development in *P. aeruginosa*.

4.2.2. RocR is a c-di-GMP Phosphodiesterase

RocR is a truly novel component of this signal transduction pathway. RocR displays a modular arrangement with an amino-terminal receiver/ CheY-like domain, followed by an EAL domain found in phosphodiesterases that hydrolyze the second messenger c-di-GMP. Examination of the RocR sequence using the Simple Modular Architecture Research Tool (SMART) reveals that this modular arrangement, where response regulator domains are fused to c-di-GMP phosphodiesterases and diguanylate cyclases, occurs in almost 240 different proteins in a wide range of predominantly Gram-negative bacteria.[16] In contrast to phosphodiesterases, diguanylate cyclases are responsible for the synthesis of c-di-GMP from GTP, and possess a canonical GGDEF motif. In the annotated genome of *P. aeruginosa* PAO1, 17 proteins with a GGDEF motif, 5 with an EAL motif, and 16 that contain both domains have been identified.[46] The genome of *P. aeruginosa* PA14, compared to PAO1, lacks one PDE (PA2818, called *arr*)[37] and one DGC (PA2771), whereas it carries a PDE encoding gene, *pvrR* on a large pathogenicity island, which is absent from the genome of PAO1.[20] In a recent study,[46] systematic analysis of the DGC and PDE encoding genes from the PA14 strain was conducted, in which TTSS, cytotoxicity of *P. aeruginosa* on epithelial cells and biofilm phenotypes of the corresponding mutants were tested. In this case, neither a mutation in *rocR*, nor the overexpression of the gene resulted in a significant difference in biofilm formation as compared to the parental PA14 strain. Interestingly, overexpression of *rocR* abrogated PA14 TTSS cytotoxicity on CHO cells. According to these data, RocR negatively regulates both biofilm formation via the regulation of the *cup* genes and cytotoxicity mediated by the TTSS. However, global microarray analysis and quantitative RT-PCR performed by Kuchma and collaborators showed that expression of the TTSS genes is positively regulated by SadR (RocR) and negatively regulated by SadS (RocS1) and SadA (RocA1) in the *P. aeruginosa* strain PAK.[45] Overall, it is not easy to reconcile the data from these various studies. For example, why did the *sadR*/*rocR* mutant in

the Kuchma study downregulate the TTSS, whereas overexpression of RocR in Kulasekara study[46] downregulated cytotoxicity? However, it seems obvious that the Roc signaling pathway both acts on the biofilm and on TTSS cytotoxic phenotypes of *P. aeruginosa*. Therefore, this system also participates in reciprocal regulation of functions involved in acute and chronic infections. In support of this hypothesis, Kuchma and collaborators also reported that mutants affected in the TTSS genes had enhanced biofilm formation.[45]

To briefly summarize the data acquired from different studies in the *P. aeruginosa* strain PAK,[45–47] a model can be drawn for the Roc system controlling the transition between acute and chronic infections. In contrast to LadS, RetS, and GacS pathways where three different sensors converge on a single effector molecule, namely *rsmZ* (Figures 2 and 3), in the Roc system, the single sensor RocS1 transduced the information on two response regulators (Figures 2 and 3). The RocA1 regulator positively controls biofilm formation and negatively regulates TTSS, whereas RocR could antagonize this effect to favor TTSS-mediated cytotoxicity and acute infections against biofilm formation and chronic persistence.

In the study by Kulasakera and collaborators,[46] overproduction of several PDE domain-containing proteins resulted in reduced cytotoxicity. This phenotype may be a consequence of an overall reduction in c-di-GMP levels and not necessarily a direct effect. The increased PDE activity in a *rocR* overexpressing strain could be detected from a direct measurement of c-di-GMP content in a whole cell extract.[46] On the contrary, it was shown in this study that overproduction of several DGC domain-containing proteins resulted in hyperbiofilm formation. This is in agreement with several reports in other bacteria that suggest that the increased level of c-di-GMP promotes sessility and biofilm formation through increased production of exopolysaccharides and extracellular adhesive structures.[75]

In a previous study,[29] a transposon insertion that suppressed the *retS* phenotype was found to be located in PA4332 which encodes a protein with a GGDEF domain. In the study by Kulasakera and collaborators,[46] a mutation in PA4332 or overexpression of this gene resulted in a hyperbiofilm formation. Furthermore, whereas no obvious cytotoxic phenotype was observed, it was shown that mutation in PA4332 resulted in an attenuated phenotype in a murine model of acute infection, equivalent to the phenotype observed in the case of a TTSS mutant. If a mutation in PA4332 suppressed the *retS* phenotype, it might indicate that as with GacA/GacS, RetS interferes with the function of PA4332. A mutation in PA4332 will have thus been expected to behave in an opposite manner as compared to the *retS* mutant. This is not really the case since a PA4332 mutant is hyperadhesive as is a *retS* mutant.[46] Furthermore, the PA4332 mutant appeared to be attenuated in a murine model of acute infection as was the *retS* mutant.

4.2.3. How does RocR Integrate in the RocS1/RocA1 Pathway?

Two-hybrid analysis has demonstrated an interaction between the Hpt domain of RocS1 and both the response regulator domains of RocR and RocA1.[47] This could result in a regulatory effect by interfering with the phosphorylation of RocA1. It is possible that binding of RocR to the Hpt domain of RocS1 (and concomitant exclusion of RocA1) leads to phosphorylation of RocR and activation of its phosphodiesterase activity. The consequent reduction of c-di-GMP levels likely has multiple effects on the cells, including reduction of biofilm formation. Binding of RocR to RocS1 would compete with RocA1 for interaction with this sensor and would likely interfere the ability of RocA1 to be phosphorylated. RocA1 lacking phosphate would be unable to activate the expression of genes involved in biofilm formation (such as the *cupB* and *cupC* loci) and to repress the expression of TTSS genes. In this way, RocR could have dual functions: preventing phosphorylation of RocA1 and regulating the levels of a second messenger implicated in biofilm formation. At that stage, many questions remain to be addressed. For example, how does RocS1 distinguish the situation required to activate the RocA1 regulator or the RocR regulator? Is the phosphodiesterase activity of RocR required for the regulation of both TTSS and/or *cup* genes expression?

5. CONCLUDING REMARKS

Bacteria monitor complex environments by integrating multiple signals into signal transduction pathways that mediate a wide set of downstream responses. This strategy allows the bacteria to launch a coordinated response in which factors that are beneficial at the same time are expressed synchronously.

The decision process undertaken by *P. aeruginosa* when colonizing its host to develop a persistent and chronic infection, most probably as a biofilm, or to disseminate in a systemic infection that will result in quick death of the host is a very attractive model to explore at the molecular level. Why do *P. aeruginosa* infections last for decades in the CF lungs without disseminating to other organs? We have discussed the importance of TCSs, such as the RetS sensor, in this decision process. RetS is part of a cascade that could command this switch between chronic versus acute infection upon detection of specific stimuli in the environment. An interesting phenotypic illustration of this switch is the one reported by Zolfaghar and collaborators.[105] In this study, the authors revealed that the attenuated virulence of the *P. aeruginosa retS* mutant in the acute murine corneal infection model is temporary, and lasts only for the first few days of the disease. Indeed, after a week postinfection, the colonization rates by

the *retS* mutant exceeded those of wild-type bacteria. This supports the idea that if the infection shifts to a chronic state, the TTSS is no longer a major player in the success of the infection. Instead, the increased biofilm formation of the *retS* mutant may facilitate its ability to maintain infection in the corneal model.

It is conceivable that during chronic infections, the utility of secreted toxins is limited, and the TTSS plays a minimal role in promoting bacterial survival in the biofilm state because the bacteria are adherent to surfaces and to each other and are surrounded by a thick EPS. This provides a rationale for the evolution of regulatory networks that would coordinate the expression of these opposing survival mechanisms. However, analyses of coordinate regulation present a more complex picture. For example, three *P. aeruginosa* quorum sensing systems also combine multiple inputs to mediate reciprocal regulation of factors required for acute infection and chronic persistence.[50,53,81,96] Mutant *P. aeruginosa* strains that do not produce autoinducers form flat, fragile, or undifferentiated biofilms[64] and concurrently show increased TTSS expression, and this phenotype can be suppressed by the addition of purified autoinducer.[5] Overall, these results suggest that *P. aeruginosa* represses TTSS expression and activates biofilm formation in response to quorum conditions. The role of quorum sensing and RetS/GacS/LadS system in regulating the TTSS and biofilm formation may be connected or complementary. As described above, RetS functions to repress *rsmZ* transcription, which in turn leads to increased levels of free RsmA. RsmA exerts a negative effect on autoinducer production, so deletion of *retS* may lead to increased autoinducer levels.[66,69] However, at a genome-wide level there is almost no overlap between *retS*-dependent and quorum sensing-dependent genes, suggesting that most *retS*-dependent changes in mRNA levels are likely not due to overproduction of autoinducers.[29,63] Another level of complexity comes from the observation that in *cyaB* and *vfr* mutants, TTSS gene expression was downregulated.[101] The role of the proteins coded by these genes is linked; the adenylate cyclase CyaB is involved in the production of the cofactor (c-AMP), which is required for Vfr function. However, overproduction of the TTSS master regulator ExsA in these mutants bypassed this control and restored normal TTSS function. We have seen previously that in a *retS* mutant, the TTSS system was also downregulated. It could be shown that overexpressing *exsA* in a *retS* mutant restored TTSS, and so did overexpression of the *vfr* gene.[49] However, by no means could *retS* overexpression in an *exsA* mutant restore TTSS function, which suggests that RetS acts upstream from ExsA in controlling TTSS gene expression. That is rather expected when one considers that most TTSS genes do have ExsA binding sites. It should be important to note that RsmA has a positive effect on *vfr* transcription.[8] We have also seen that Vfr positively controls TTSS genes,

So the decreased free RsmA levels in a retS mutant is consistent with downregulation of Vfr and subsequently a down regulation of TTSS genes.

In each of these examples, regulation of various components of biofilm formation is inversely coupled with TTSS expression. Several mechanisms may account for these observations. It is possible that the physiological state of the cell is altered when exopolysaccharides are produced; metabolic cues have been shown to influence TTSS expression.[70,71] Alternatively, upstream regulatory pathways for various biofilm components may each be coupled with TTSS regulators. In the case of RetS signaling, it is unlikely that exopolysaccharide production causes repression of the TTSS. Second-site insertion mutations in *pel* or c-di-GMP genes block biofilm overproduction but not TTSS repression in *retS* mutant strains, suggesting that these two pathways are genetically separable. Further dissection of these regulatory networks will provide greater insight into the connections between genes required for acute and chronic infection.

Owing to their versatility in sensing diverse intracellular and extracellular signals and their variable modular architecture, TCSs are convenient devices for the regulation of the expression of virulence properties. Despite detailed knowledge about the phosphorylation-based signal transduction mechanism itself, surprisingly little information is available about the molecular basis for its contribution to bacterial virulence in most pathogens. What is not known is the nature of infection relevant signals; their mechanisms of perception, the targets of TCS-mediated regulation and the regulatory networks into which the TCSs are integrated to control the expression of such a multifactorial phenotype as bacterial virulence. The studies described here suggest that *P. aeruginosa* may be an ideal model system for understanding how such coordinated responses are mediated. Future research should be aimed at understanding these features because, owing to the absence of TCSs in mammals, these systems might be relevant targets for antimicrobial strategies.

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PATHOGENESIS

12

PSEUDOMONAS AERUGINOSA INTERNALIZATION BY NON-PHAGOCYTIC CELLS

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Abbreviations: type III secretion system, T3SS

1. INTRODUCTION

Pseudomonas aeruginosa is one of the most virulent opportunistic infectious agents of man. Despite constant exposure to this bacterium, ubiquitously found in water, plants, and soil, normal individuals rarely get *P. aeruginosa* infections. However, in the setting of epithelial barrier damage combined with some degree of immunocompromise, *P. aeruginosa* is able to effectively colonize and unleash its vast armamentarium of virulence factors.[28,82] This leads to further local epithelial damage, inhibition of wound repair, and dissemination to surrounding tissues and distant organs. *P. aeruginosa* infections manifest clinically as bacteremia and sepsis in patients receiving cytotoxic chemotherapeutic agents; nosocomial

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pneumonia and respiratory failure, particularly in mechanically ventilated patients; wound infections in post-surgical patients; skin infections and sepsis in burn patients; corneal ulcers in contact lens wearers; bladder infections in catheterized patients; and are a contributing factor to severe pulmonary damage and consequent death in patients afflicted with cystic fibrosis (CF).[97] More recently, bacterial infections due to *P. aeruginosa* are reported as a complication of HIV infection and are becoming increasingly frequent as patients with HIV live longer.[4,46,50]

Despite aggressive antibiotic therapy, the fatality rate in many *P. aeruginosa* infections is as high as 40%.[97] These figures have not improved in decades, and new approaches to treatment are even more critical, now that antibiotic resistance is increasingly widespread among *P. aeruginosa* isolates. Unraveling the complex interactions between *P. aeruginosa* and host epithelium will lead to novel insights into how bacteria attack host cells and outmaneuver the immune system. These studies will have a significant impact on the survival of *P. aeruginosa*-infected patients. They may help to identify host proteins that can be used as novel drug targets that are much less likely to engender resistance compared to conventional anti-microbial therapies.

This chapter will focus primarily on one aspect of *P. aeruginosa* interaction with the host, namely its ability to enter into (or be "internalized") by normally non-phagocytic cells, such as the epithelial cells that line the mucosal barrier and endothelial cells that form the vascular lumen. In many cases, these interactions are also relevant for uptake by so-called professional phagocytes, including tissue macrophages, monocytes, and neutrophils. Many medically important bacteria are facultative intracellular pathogens, including *Shigella, Salmonella*, and *Listeria*. More recently, it has been appreciated that bacteria that are usually thought to exert their pathogenic potential as extracellular pathogens also have the capacity to invade and perhaps reside or persist intracellularly during early phases of infection. It is clear from the studies reviewed below that *P. aeruginosa* deserves to be included in this latter group. Dissecting the pathways by which microorganisms enter into non-phagocytic cells, such as the epithelial cells that comprise the mucosal barrier, will reveal essential information into the mechanisms of pathogenesis as well as yielding new insights into basic mammalian cell biology.

2. OVERVIEW OF *P. AERUGINOSA* INTERNALIZATION

P. aeruginosa is primarily considered an extracellular pathogen; for example, in histologic sections from patients with pneumonia, the organism is usually extracellular. However, many clinical and laboratory isolates demonstrate measurable internalization by various assays. For example,

gentamycin protection assays or direct microscopic visualization of bacteria demonstrate intracellular *P. aeruginosa* in cultured epithelial, endothelial, and macrophage cell lines in vitro.[13,41,43,44,63,67] Intracellular bacteria have also been found in more physiologically relevant models of human infection, including in isolated mouse corneas,[12,43] isolated mouse trachea,[45] experimental murine lung infections,[45] and in alveolar and tracheal epithelial cells in vitro and in vivo.[52,53]

Over the years it has emerged that many important human pathogens are facultative or obligate intracellular pathogens, including *Salmonella, Shigella, Listeria, and Chlamydia*, indicating that invasion is an essential step in the development of disease. Similar to these pathogens, uptake of *P. aeruginosa* may permit intracellular replication in an immune-privileged environment. It also may allow transcytosis across epithelial cells, allowing access to deeper tissues. Indeed, transcytosis of *P. aeruginosa* across Madin–Darby Canine Kidney (MDCK) monolayers has been experimentally observed.[66] Uptake of *P. aeruginosa* may be more beneficial to the host, as a defense mechanism. For example, ingestion by macrophages may lead to bacterial death and presentation of bacterial antigens to the immune system. In addition, it has been suggested that internalization of *P. aeruginosa* by epithelial cells followed by desquamation of the epithelial layer may be a useful host defense mechanism in the setting of pulmonary infections in CF patients.[104,106] It is possible that under some environmental conditions, it is beneficial to *P. aeruginosa* to enter into eukaryotic cells (for transcytosis, for immune evasion, or during its life in water environments), whereas under other circumstances, the bacteria actively prevents its uptake, through the actions of the type III secreted effectors ExoS and/or ExoT.

Importantly, different strains and mutants vary in their internalization efficiency into cultured cells, but all strains are capable of entering into both phagocytic and non-phagocytic cells to some degree.[42] The observation that the ability to enter cells has been maintained during the evolution of *P. aeruginosa* indicates that it is likely to play a fundamental role in the pathogenesis of *P. aeruginosa* infections and/or in surviving in the environment.

The first step in entry is adhesion to the host cells. Not surprisingly, the bacteria may have multiple and even redundant adhesins it utilizes to bind to the plethora of host cell types that it may encounter in diverse environments and hosts. Likewise, multiple host cell receptors and pathways may be subverted for entry into non-phagocytic cells. Finally, it is clear that invasion is subject to complex regulation. Detailed analysis of the invasion phenotypes of various strains and their mutants has been instrumental in defining bacterial factors that are crucial for virulence, such as the type III secretion system (T3SS).[42,61] The potential adhesins, the host receptors and signaling pathways, and the regulation of *P. aeruginosa* internalization are explored in more detail in the following sections.

3. BACTERIAL ADHESINS

3.1. Pili and Flagella

Early studies revealed that a strain of PAK carrying a mutation in the pilin structural gene showed 80–90% reduced adherence, while a strain harboring a mutation in the pleiotropic regulatory gene *rpoN* showed no detectable adherence.[12,13] A direct comparison of the efficiency of binding of a pilin mutant of PAK, a flagellar mutant of PAK, and the double mutant (deficient in type IV pili and flagella) reveals that loss of one adhesin decreases binding and entry (anywhere from 50 to 90%) to HeLa cells, MDCK cells, or RAW macrophage cells but loss of both adhesins almost completely abolishes binding or entry[34] (Engel, unpublished results). These findings suggest that both pili and flagella can function as adhesins for entry.

Pili and flagella have been shown to function as bacterial adhesins to multiple cell types including human buccal cells,[26,130] lung pneumocytes,[13,33] exfoliating tracheal cells,[139] immortalized human airway cells,[34] HeLa and MDCK cells,[12] and endothelial cell lines.[13,108] Mutants deficient in these adhesins show decreased virulence relative to their parental strains in animal models of pulmonary,[34,126] intraperitoneal,[33] and burned skin[27,100,117] infections. Other components of the flagellar apparatus, including FlhA, have been shown to play a role in invasion of PA01 into corneal epithelial cells.[39] The FliC mutant, defective in production of flagellin, showed decreased binding and invasion. Interestingly, the FlhA mutant adhered better than wild-type bacteria, but exhibited an 80% decrease in invasion. Neither mutant exhibited a decrease in intracellular survival.

These findings are consistent with roles of PilA and FliC as adhesins in vitro, though they may have alternative or additional functions in vivo. For example, pili and flagella have also been shown to mediate early steps in biofilm formation,[101] which is thought to be important in catheter-associated infections as well as chronic lung infections seen in CF patients. As discussed in Section 7, intracellular *P. aeruginosa* may form biofilm like structures, similar to the pods as reported for uropathogenic *E. coli*.[48] In addition, these adhesins may be targets for the host immune response. Flagellum can activate the inflammatory response via TLR5 binding,[37,138] while isolated pili or flagella have been shown to activate NFκB-mediated inflammatory responses.[2] TLR2 signaling is amplified through its association with asialoganglioside gangliotetrasylceramide (aGM1), a putative receptor for pili and flagella.[123]

3.2. Other Potential Adhesins

The multidrug efflux operon MexAB-OprM has been shown to increase *P. aeruginosa* internalization and to enhance virulence in a

leukopenic mouse model. This study linked the ability of *P. aeruginosa* to invade epithelial cells with transmigration though the epithelial monolyaer.[68,88] As discussed in Section 4.2, *P. aeruginosa* lipopolysaccharide (LPS) may function as an adhesin in the setting of binding to the cystic fibrosis transmembrane regulator (CFTR).

4. HOST CELL RECEPTORS FOR BINDING AND INTERNALIZATION

Since the discovery of β1 integrins as the receptor for invasin-mediated entry of *Yersinia* spp. into host cells,[74] it has been appreciated that pathogens subvert diverse receptors and signaling pathways to enter into non-phagocytic cells. Other receptors that have been identified include E-cadherin for internalin-A-mediated entry of *Listeria monocytogenes*,[17] the C-met receptor for internalin-B-mediated entry of *L. monocytogenes*,[17] and Ku-70 for *Rickettsia conorii* entry.[99] In the case of enteropathogenic and enterohemorrhagic *Escherichia coli*, the bacterium translocates its own receptor into the host cell plasma membrane.[85]

4.1. aGM1 May Function as a Receptor for Pili-Mediated Binding

A large number of studies have implicated aGM1 in pilin-mediated binding and invasion, although the magnitude of its contribution remains controversial. *P. aeruginosa* pili bind to glycolipids contained within epithelial cell membranes.[7,92] They show a specificity toward those with the Galβ1-4GlcNAc disaccharide available, aGM1 and asialoganglioside GM2 (aGM2).[56,110,121] This disaccharide moiety is specifically recognized by the C-terminal domain of the PilA subunit,[73,94] the amino acid sequence of which can differ among *P. aeruginosa* strains.[78]

A direct correlation between the presence of aGM1 on host cells and *P. aeruginosa* adherence has been noted, consistent with the role of this glycosphingolipid as a bacterial receptor. Increased bacterial binding to scarified corneal epithelium was shown to be coincident with the presence of greater quantities of aGM1 on these cells, and this adherence was attenuated by addition of an anti-aGM1 monoclonal antibody.[64] Increased expression or exposure of aGM1 on the surface of injured or regenerating epithelium may in part explain the predeliction of this pathogen for damaged epithelium. Pili-dependent *P. aeruginosa* association with immortalized nasal polyp epithelial monolayers was specifically competed with aGM1. Addition of exogenous aGM1 to the apical surface of polarized MDCK cells increased binding, invasion, and type III secretion mediated cytotoxicity of various derivatives of PA103 in a pilin-dependent manner.

The aGM1-enhanced interactions could be blocked by antibodies to aGM1; the antibodies were without effect in the absence of added aGM1.[15] While these studies suggest that aGM1 is capable of functioning as a receptor, the finding that antibodies to aGM1 do not inhibit PA103 binding to MDCK cells in the absence of added aGM1 suggests that receptors other than aGM1 normally mediate adhesion to this cell type. These may include other glycolipids or glycoproteins since mutant MDCK cell monolayers that had altered apical surface glycosylation (concavalin A-resistant or ricin-resistant cells) were previously shown to be less susceptible to PA103-induced cell damage than wild-type MDCK monolayers.[6] The role of aGM1-enhanced binding may be strain specific, as it was not observed to enhance binding of eight clinical strains to MDCK cells.[120]

Interestingly, aGM1 is more prevalent on the surface of primary CF cells and a CF bronchial cell line than on wild-type airway cells, suggesting at least one mechanism by which the lungs of CF patients are more susceptible to *P. aeruginosa* infections.[71,115] *P. aeruginosa* binding to regenerating respiratory epithelial cells isolated from either CF or non-CF patients was inhibited by treatment with anti-aGM1 antisera.[21–23]

4.2. CFTR May Function as a Receptor for Lipopolysaccharide (LPS)-Mediated Adhesion

Several studies implicate a role for the CFTR as a receptor for invasion,[103–106,135,136] although it may be cell type-specific. CFTR is normally found on the apical surface of ciliated cells. Transformed human airway epithelial cells homozygous for the ΔF508 allele of CFTR were defective for the uptake of *P. aeruginosa* compared with the same cell line complemented with the wild-type CFTR gene. LPS mutants or competition by exogenous LPS suggested that LPS-core oligosaccharide functioned as the bacteria ligand for CFTR-mediated epithelial cell invasion. In experimentally infected mice, inhibiting CFTR-mediated endocytosis of *P. aeruginosa* by inclusion in the bacterial inoculum of either free bacterial LPS or CFTR peptide 108–117 resulted in increased bacterial burdens in the lungs. CFTR is found to localize at the site of bacterial binding to the apical surface of polarized respiratory cells, possibly at specialized lipid domains.

Other studies do not support a role for CFTR as a receptor for internalization. *P. aeruginosa* enters cells that express no detectable CFTR, such as A549 and MDCK cells.[109] Binding and internalization of *P. aeruginosa* by two different ΔF508 cell lines was higher than binding and internalization by cells expressing wild-type CFTR.[87] A limitation of these studies is that the mutant cells were not complemented with the wild-type CFTR, leaving open the possibility that the mutant cell lines expressed additional adhesins.

In more recent studies, no difference was observed in the binding of PA01 when added to CFBE41o- (ΔF508) cells stably transfected with control vector or wild-type CFTR, but the complemented cells internalized the bacteria ~10-fold less efficiently, independent of the level of CFTR expression.[20] Experiments designed to test whether expression of CFTR was sufficient to increase entry in MDCK cells led to inconclusive results.[51] CFTR peptides block *P. aeruginosa* uptake in primary human and rabbit corneal epithelial cultures in the presence of serum but failed to do so when added under serum-free conditions in immortalized human corneal epithelial cells.[134]

Very recently it was reported that CFTR is necessary for the clearance of phagocyted *P. aeruginosa* by macrophages.[25] Although macrophages are professional phagocytes, this new function of CFTR might explain persistence of *P. aeruginosa* in CF patients. Alternatively, or in addition, the loss of CFTR may contribute to persistence by virtue of the decrease in internalization into lung epithelial cells, which would normally then be shed into the airways.

4.3. Integrins

Fibronectin and α5β1 have been shown be involved in adherence of *P. aeruginosa* to dedifferentiated respiratory cells in an ex-vivo model of injured airway epithelium. The bacteria colocalized with β1 and α5 integrins.[113] In A549 (human respiratory) and SKOV-3 (human ovarian) cell lines, blocking antibodies to αvβ5 integrins but not to α5β1 diminished PAK adherence to A549 cells and blocking antibodies to vitronectin or to αvβ5 integrins inhibited PAK internalization into A549 or SKOV-3 cells; addition of an excess of vitronectin restored invasion in the presence of vitronectin antibodies. Finally, in the presence of bacteria, the vitronectin fibrillar network disappeared and αvβ5 staining colocalized with adherent bacteria.[96] Using primary monocytes and neutrophils derived from a CR3-deficient individual afflicted with leukocyte adhesion deficiency (loss of CD18 integrin), 5 of 10 tested strains were internalized less efficiently compared to wild-type monocytes and neutrophils.[65]

4.4. Cholesterol

In Chinese Hamster Ovary (CHO) cells and mucin-producing lung epithelial cells lines, adherence did not depend on glycoproteins, glycolipids, or proteoglycans. An ethanol extractable compound, identified as cholesterol and cholesterol esters, was shown to be required for binding. Consistent with this notion, bacterial adherence was reduced in CHO cells

treated with lovastatin or in cholesterol-requiring insect cells grown in cholesterol-deficient medium.[114] These findings indicate that the integrity of the lipid bilayer and its fluidity are essential and may reflect entry through lipid rafts, as described in Section 6.2.

5. INVASION OCCURS PREFERENTIALLY IN INJURED OR INCOMPLETELY POLARIZED CELLS

A key aspect in the pathogenesis of acute *P. aeruginosa* infections is the requirement for preexisting epithelial cell injury.[28] This observation could reflect increased access to the basolateral surface (for example, exposing a receptor or allowing engagement of a basolateral endocytic pathway) or an alteration in the apical surface of injured cells. This phenomenon can be recapitulated in cell culture models of infection.[83,84] Several lines of evidence indicate that *P. aeruginosa* preferentially adheres to and invades the basolateral surface of epithelial cells. For example (i) plating cells at low densities, (ii) disrupting the intercellular junctions of confluent monolayers of primary or immortalized cells by growth in low calcium media or exposure to EGTA, (iii) intentionally damaging epithelial cell monolayers, or (iv) exposure to growth factors such as hepatocyte growth factor enhanced epithelial cell susceptibility to *P. aeruginosa* invasion.[40,102] Likewise, when primary or cultured human bronchial epithelial cells were grown as highly polarized layers by culture on thick collagen gels, internalization was inefficient. In contrast, in cells cultured on thin films, conditions under which the monolayers are poorly polarized, bacterial internalization was greater. Further disruption of the monolayer by addition of a calcium chelator additionally enhanced bacterial internalization. Interestingly, membrane localization of CFTR inversely correlated with invasion efficiency, suggesting that in this model, CFTR is not acting as the primary receptor.[109]

To determine whether enhanced basolateral access explained the increased susceptibility of injured epithelium to *P. aeruginosa* invasion, invasion efficiency was quantified in confluent monolayers of MDCK cells grown on transwell supports for 1 or 3 days. Under both conditions, the monolayers formed polarized monolayers, as evidenced by impermeability to small diffusible molecules and relatively high transepithelial resistance. Interestingly, invasion from either the apical or the basolateral surface was less efficient in day 3 compared to day 1 cells. These findings suggest that internalization is downregulated at both surfaces (apical and basolateral) as cells become more highly polarized.[84] They further support the idea that epithelial cells alter their responses to *P. aeruginosa* as a

function of polarization and suggest a novel way in which epithelial cell responses to pathogens may be altered by epithelial tissue injury.

6. SIGNAL TRANSDUCTION PATHWAYS NECESSARY FOR *P. AERUGINOSA* INVASION

For most if not all bacterial pathogens, entry into non-phagocytic cells involves usurping host receptors, entry pathways, and signal transduction pathways. Indeed, the study of bacterial entry has yielded fundamental insights into host cell biology. Many techniques and approaches have been used for these studies, including pharmacologic inhibitors, overexpression of constitutively active or dominant negative alleles, RNAi-mediated depletion, biochemical assays, and colocalization of relevant host molecules with adherent or internalized bacteria. As reviewed later, application of these strategies has begun to yield insight into the signal transduction pathways usurped by *P. aeruginosa* internalization into mammalian cells.

6.1. Lipid Rafts May Serve as Portals of Entry

Lipid rafts are specialized dynamic regions of the plasma membrane enriched in cholesterol, glycosphingolipids, glycosylphosphatidylinositol-anchored proteins, and some membrane proteins.[11,22] They are thicker and less fluid than the rest of the glycophospholipids in the membrane and can be isolated as a detergent-resistant (Triton-X 100) fraction.[11] Caveolin is associated with a subset of lipid rafts. Rafts are thought to play a role in a diverse array of cellular processes with a common theme of providing sites of local enrichment of molecules that need to interact with each other or to be transported to the same place in a cell.[122] Recently, a number of pathogens have been suggested to enter cells through lipid raft-mediated pathways including echovirus, uropathogenic *E. coli, Chlamydia* spp., *Mycobacteria*, Group A *Streptococcus, Shigella*, and *Campylobacter jejeuni*.[1]

Several studies suggest that lipid rafts may play a role in the internalization of some strains of *P. aeruginosa*.[52] For example, *P. aeruginosa* infection (PA01) triggered the activation of the acid sphingomyelinase and the release of ceramide in sphingolipid-rich rafts. Ceramide reorganized these rafts into larger signaling platforms that were required to internalize *P. aeruginosa*, induce apoptosis, and regulate the cytokine response in infected cells. Failure to generate ceramide-enriched membrane platforms in infected cells results in an unabated inflammatory response, massive release of interleukin (IL)-1 and septic death of mice. Together, these

findings suggest that ceramide-enriched membrane platforms are central to the host defense against this potentially lethal pathogen.

A role for lipid rafts in entry of *P. aeruginosa* into rat primary type I-like pneumocytes as well as a murine lung epithelial cell line (MLE-12) supported by the finding that entry was inhibited by drugs that remove membrane cholesterol and disrupt lipid rafts. Confocal microscopy revealed that intracellular bacteria colocalized with caveolin 1 and 2, lipid raft components. Depletion of caveolin by RNAi impaired *P. aeruginosa* invasion into MLE 12 cells. Inhibition of caveolin tyrosine phosphorylation correlated with a decrease in *P. aeruginosa* entry.

Lipid rafts have also been reported to be important for *P. aeruginosa* internalization by corneal epithelial cells in vivo, in vitro, and after contact lens wear.[133] Contact lens-induced hyptoxia may stimulate the formation of lipid rafts in vivo. Subsequent exposure to *P. aeruginosa* resulted in preferential binding of the bacteria to lipid raft-forming cells. *P. aeruginosa* binding to these corneal surface cells triggered a dynamic process of lipid raft aggregation, leading to bacterial clustering and internalization. In contrast, no bacterial binding was observed in intact rabbit conjunctival epithelial cells, despite the presence of detectable lipid rafts. These findings suggest that the presence of membrane lipid rafts alone is not sufficient to trigger *P. aeruginosa* binding and internalization into corneal epithelial cells.[132] Rather, injury or stress may be a necessary prerequisite.

It is interesting that CFTR and TLR2, a pattern recognization receptor for bacterial surface products, are associated with lipid rafts.[89–123] Using MDCK cells transfected with GFP-CFTR, it was demonstrated that GFP-CFTR and aGM1 colocalized at the site of *P. aeruginosa* adhesion. GFP-CFTR localized to low density Triton-X 100-insoluble fractions in MDCK cells, and this localization was enhanced upon *P. aeruginosa* infection. Extraction of cell surface cholesterol via cyclodextrin treatment of the cells inhibited CFTR entry into rafts, *P. aeruginosa entry*, NF-κB nuclear translocation, and apoptosis. These results suggest that lipid raft localization of CFTR may contribute to host cell signaling in response to *P. aeruginosa* infection.[89]

TLR2 has also been found to be enriched in caveolin-1-associated lipid raft microdomains on the apical surface of airway epithelial cells after infection with *P. aeruginosa*.[123] Other key signaling molecules, including myeloid differentiation protein (MyD88), interleukin-1 receptor-activated kinase-1, and TNF receptor-associated factor 6 were also found associated with the bacterial-induced rafts. The signaling capabilities of TLR2 were enhanced upon association with aGM1, a putative receptor for pili and flagella. Ligation of either TLR2 or aGM1 by ligands with specificity for either receptor by *P. aeruginosa* stimulated IL-8 production through TLR2- and MyD88-mediated activation of NF-κ B.[123]

Nod-1 has recently been shown be required for early cytokine production and to limit bacterial internalization early times postinfection.[127] Together, these studies underscore the potential link between bacterial entry and activation of host defenses.

6.2. Role of the Actin Cytoskeleton

Several early studies demonstrated that invasion of strains (6294, PAK, PA01, PA2, PA3, J1385, or a type III secretion mutant of PA103) is inhibited by cytochalasin D or latrunculin, inhibitors of actin polymerization.[20,31] Rho family GTPases are known to regulate actin polymerization and are activated upon internalization of several bacterial pathogens studied thus far. Toxin B, an inhibitor of Rho, Rac, and Cdc42, inhibited invasion of PAK, PA01, and a type III secretion mutant of PA103.[20,84] There may be some strain dependence, as invasion of strain PA4 in CFBE41o- (ΔF508 CFTR) cells was not inhibited by cytochalasin D or toxin B, whereas invasion of PA01, PA2, PA3, and J1385 entry was inhibited.[20] These results suggest that different strains of *P. aeruginosa* may utilize different pathways for entry. Of note, PA4 was noted able to transmigrate in the presence of gentamycin. While the authors proposed that PA4 transmigration involved internalization, it seems possible that it may reach the basolateral media by passing through paracellular spaces, which could be protected from aminoglycoside antibiotics.

In the case of a type III secretion mutant of PA103, invasion into day 1 MDCK cells was accompanied by activation of Rho, but not Rac or Cdc42. Invasion into day 3 MDCK cells was less efficient, and this correlated with activation of Cdc42 in place of Rho.[84] In other studies utilizing PAK (or PAKΔSΔT), RNAi-depletion studies demonstrate a role for Abl kinase, Crk, Rac and Cdc42 (Pielage, J., Kalman, D., and Engel, J, unpublished results).

6.3. Host Tyrosine Kinases are Activated Upon *P. aeruginosa* Entry

Activation of host protein kinases, including tyrosine and serine/threonine kinases, has been found to accompany the internalization of many microbial pathogens.[116] Consistent with this, *P. aeruginosa* uptake into non-phagocytic cells is accompanied by changes in host protein tyrosine phosphorylation.[29,31] Genistein or Herbimycin, inhibitors of protein tyrosine kinases, block invasion of *P. aeruginosa* strain 6294 into cultured corneal epithelial cells. Some inhibition was seen by Tyrphostin A47 (an EGF receptor inhibitor) and U73122 (a phospholipase C inhibitor).[31]

Bapta-AM and W-7 blocked invasion, suggesting the calcium and calmodulin plays a role. Evidence for a role for Src, a cytoplasmic tyrosine kinase, comes from the finding that (i) invasion was increased in cells lacking Csk, a negative regulator of Src kinase,[32] (ii) Src and Fyn kinase were activated upon invasion of strains 696 or ATCC27853 into Chang or WI-38 cells and (iii) PP1, a specific inhibitor of Src kinase, diminished invasion of these strains.[29] Interestingly, a peptide competitor of the fourth extracellular domain of CFTR prevented Src and Fyn tyrosine phosphorylation, suggesting that entry through a CFTR-associated pathway is linked to activation of these tyrosine kinases.[29]

Using genome-wide RNAi-mediated inactivation, Abl tyrosine kinase, a Src target, has also been shown to mediate entry of type III secretion mutants of PAK or PA103 into HeLa cells (Pielage, J., Kalman, D., and Engel, J, unpublished results). Internalization of PAK (or PAKΔSΔT) was reduced (i) when cells were pretreated with Gleevac, an inhibitor of Abl kinase, (ii) upon depletion of Abl kinase by RNAi, or (iii) in infection of Abl/Arg -/- cells compared to the parental cell line. Gleevac also reduced entry of a type III secretion mutant of PA103. Finally, PAK entry was diminished upon depletion of Crk, Rac, Cdc42, and P21-activated kinase, all molecules known to be downstream of Abl kinase. Direct epistatis studies suggest that Abl kinase initiates this signaling pathway upon PAK internalization.

6.4. Role of Phosphoinositides

Phosphoinositide 3-kinases (PI3Ks) are a highly conserved subfamily of lipid kinases that catalyze the addition of a phosphate molecule specifically to the 3-position of the inositol ring of phosphoinositides to generate PtdIns3P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3).[128] These short-lived phospholipids modulate the actin cytoskeleton and function as scaffolds to which specific effectors that regulate membranes are recruited. Modification of phosphoinositides by kinases and phosphatases permits their precise temporal and spatial control, allowing them to tightly regulate local and transient cellular processes. An emerging theme in bacterial entry by non-phagocytic cells is the modulation of phosphoinositides.[107] For example, *L. monocytogenes* activates PI3K, catalyzing the synthesis of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3; *Shigella* encodes an effector that dephosphorylates PdtIns(4,5)P_2 into PtdIns(5)P; *Yersinia* induces activation of PtdIns(5)P to promote PtdIns(4,5)P_2 formation, and *Mycobacterium tuberculosis* inhibits PI3K.[107]

PI3K has been shown to be necessary for the invasion of epithelial cells by several bacteria, including *Listeria monocytogenes*,[72] *Helicobacter*

pylori,[93] *Escherichia coli* K1 (111) and *Chlamydia* spp.[16] Inhibiting PI3K by chemical means (wortmannin or LY29002) decreased the entry of PAK or a type III secretion mutant of PA103 into MDCK cells without affecting binding. To show sufficiency, exogenous PtdIns(3,4,5)P_3 complexed to a carrier protein was added to the apical membrane. PAK invasion was increased without affecting adhesion.[86]

One of the known targets activated by PI3K is Akt, a serine–threonine kinase. The PI3K/Akt signaling pathway is involved in diverse processes such as vesicular trafficking, mitogenesis, and cell survival.[14] Interestingly, Akt is activated during the entry of several bacterial pathogens.[16,72,98,124] PAK entry correlated with an increase in phosphorylation of threonine 473 on Akt. Inhibition of Akt activity, using a chemical inhibitor or RNAi-mediated depletion, decreased PAK entry without affecting adhesion. Finally, infection of cells transfected with a constitutively active allele of Akt enhanced PAK entry if PI3K was inhibited. Together, these results suggest that the activation of PI3K and subsequent activation of Akt are necessary for PAK entry.

Using a phosphoinositide-binding domain fused to GFP (PH-Akt-GFP) as a spatial probe,[95] large membrane protrusions enriched for PtdIns(3,4,5)P_3 and actin accumulated at the apical surface at the site of PAK binding to day 1 MDCK cells. Commonly, this was observed at the site of cell–cell junctions. Unexpectedly, these protrusions lack apical membrane markers and instead are comprised of basolateral membranes constituents, which are trafficked there from the basolateral surface by transcytosis[86a] (Kierbel *et al.*). The end result is that this bacterium transforms apical membrane into basolateral membrane, creating a local microenvironment that facilitates its colonization and entry into the mucosal barrier. While appearing to benefit the bacteria, the relocalization of some basolateral constituents, such as toll-like receptors, may trigger the innate immune response. The relationship of the PI3K/Akt pathway to other signaling pathways activated coincident with *P. aeruginosa* internalization remains to be explored.

6.5. Host Cell Apoptosis May Influence the Physiologic Consequences of Bacterial Internalization

Modulation of host cell viability is another emerging theme in host pathogen interactions.[38] Some pathogens kill host cells quickly by necrosis. Others induce a more immunologically silent cell death via apoptotic pathways. Finally, some pathogens, particularly obligate intracellular microbes, inhibit apoptosis, allowing for intracellular replication.

P. aeruginosa induces cell death by multiple pathways: a rapid, necrotic cell death through the phospholipase A2 activity of the type III secreted effector ExoU,[62,118] a delayed apoptotic like cell death through the type III secreted effector ExoT (Shafikhani and Engel, unpublished results), a caspase-dependent cell death through ExoS,[3,75,77,80] and a type III secretion but effector-independent apoptotic-like cell death.[60] For at least some strains of *P. aeruginosa*, including PA01 and 762, activation of CD95 (Fas receptor) by CD95 ligand on cultured cell lines or lung epithelium has been shown to induce apoptosis in a type III secretion-dependent manner and to protect animals from infection. In CD95 null (*lpr*) or CD95 ligand null (*gld*) mice, intranasal infection by *P. aeruginosa* resulted in abrogation of bacterial-induced apoptosis but increased sepsis and mortality. Bone marrow reconstitution experiments revealed that the high susceptibility of CD95 or CD95 ligand null mice to *P. aeruginosa* infections was associated with failure of lung epithelial cells to be activated by CD95 and to undergo apoptosis upon infection. It has been proposed that internalization of *P. aeruginosa* without apoptosis of the host cell might permit the bacterium to block maturation of the phagosome, promote intracellular survival and growth of the bacterium, and protect bacteria from the host immune system, leading to higher mortality.[54,76]

In contrast, infection of primary and immortalized corneal epithelial cells results in inhibition of apoptosis by activation of the serine/threonine kinases Akt and ERK1/2 and pro-survival pathways, possibly by enhancing release of the epidermal growth factor receptor ectodomain and activation of the epidermal growth factor receptor.[137] It is possible that inhibition of apoptosis early in infection is beneficial for the intracellular survival of PA, as has been demonstrated for other intracellular pathogens.[55]

7. FATE OF INTRACELLULAR BACTERIA

While much work has focused on identifying the bacterial ligands, putative host receptors, and signal transduction events utilized for *P. aeruginosa* entry, much less is known about the host response and the fate of internalized bacteria. Some human pathogens, including *L. monocytogenesis* and *Shigella flexneri*, survive intracellularly by escaping from a membrane-bound compartment into the cytosol.[129] Others inhibit phagolysosomal fusion, survive, and replicate in unique membrane bound compartments, including *Chlamydia, Salmonella, Yersinia, Mycobacteria*, and *Legionella*.[58] In studies of PA01 entry into cultured respiratory epithelium (CFBE41o- cells), bacteria were observed to enter by a zipper-like mechanism and to at least initially be enclosed in a membrane-bound

vacuole. Some early replication was observed, but bacterial viability at later time points was strain dependent.[20] Others have noted that some strains of *P. aeruginosa* may escape into the cytoplasm and that the LPS structure may modulate intracellular survival.[30] Jin and coworkers demonstrated that internalized PAK survives or replicates more efficiently than internalized PA103UΔT, suggesting that intracellular survival may be strain dependent. The intracellular location of these strains was not reported.[57] PAK also appears to initially survive better in Nod-1-deficient cells, cells defective in sensing intracellular peptidoglycan.[127] In the future, it will be interesting to determine more precisely the sequence of events after internalization – into what intracellular compartment are the bacteria internalized, do all strains escape into the cytoplasm, for how long do the bacteria survive intracellularly, and how does the host cell limit bacterial intracellular survival.

More recently it has become evident that some pathogens may persist intracellularly in a biofilm-like state, serving as an antibiotic-resistant reservoir of bacteria. For example, uropathogenic *E. coli* has been found in "pod"-like structures in the superficial bladder epithelium.[5,79] *Haemophilus influenzae* has been found intracellularly in biopsy samples of airway epithelial cells from individuals with associated with chronic obstructive lung disease.[8] Using electron and confocal microscopy to analyze primary cultures of airway epithelial cells infected with PA01, PAK, or a mucoid clinical isolate, pod-like clusters of intracellular bacteria were observed that persisted in the presence of gentamycin for at least 2 days.[48] Bacterial replication occurred for at least up to 10 h; thereafter, loss of epithelial cells precluded accurate measurement.[48] The intracellular bacteria exhibited heterogeneity in gene expression and relative resistance to host cell permeable antibiotics, including ceftazidime and ciprofloxacin.[48] Interestingly, the intracellular bacteria observed after uptake into polarized MDCK cells through membrane protrusions[86] may have some pod-like and/or biofilm like characteristics (Kierbel and Engel, unpublished results). Together, these findings suggest that *P. aeruginosa* may use epithelial cells as a reservoir for persistence where they may exist in an antibiotic-resistant state that is also protected from host defenses.

8. REGULATION OF INTERNALIZATION

While most strains of *P. aeruginosa* examined thus far are capable of entry into non-phagocytic cells, it is clear that this is a highly regulated process. Initial studies demonstrated that *P. aeruginosa* strains exhibited two distinct invasion phenotypes that correlated with expression of distinct subsets of type III secreted effectors.[42]

8.1. T3SS Effectors can Modulate Invasion

The T3SS is a contact-activated secretion system in which bacterially synthesized effector proteins are directly translocated into eukaryotic target cells.[70] While the secretion and translocation apparatus is highly conserved, the secreted effector proteins differ between species, and are even different among different strains or isolates. The T3SS is found in both clinical and environmental isolates, suggesting that the T3SS is essential to *P. aeruginosa* survival and growth.[36] Four TTS effector proteins have been identified in *P. aeruginosa*, though few if any strains encode or produce all four effectors.[59] ExoU is a potent cytotoxin with phospholipase activity[119] that results in host cell death through necrosis[61]; it is present in about 25% of clinical isolates.[35] ExoY is an adenylate cyclase that requires a host cofactor for activity; its role in virulence remains unknown.[131] One report suggests that expression of either the catalytically active or inactive mutant of ExoY in PA103ΔUΔT was associated with disruption of the actin cytoskeleton in immortalized rabbit corneal epithelial cells. At 2 h but not 4 h postinfection, invasion was decreased 76 and 36%, respectively, for the wild-type and catalytically inactive ExoY.[19]

ExoT and ExoS are highly homologous bifunctional proteins with an N-terminal domain with homology to GTPase activating proteins (GAPs) and a C-terminal domain with ADP ribosyl transferase (ADPRT) activity. Almost all strains examined thus far encode ExoT, while ExoS is usually found in strains that do not contain the ExoU gene.[36] The GAP domains of ExoT and ExoS specifically target Rho family GTPases,[81,90,91] which include Rho, Rac, and Cdc42, modulators of the actin cytoskeleton. The targets of the ADPRT domain of ExoS and ExoT are distinct and nonoverlapping. ExoT has been shown to ADP ribosylate Crk, an SH2-, and SH3- containing adaptor protein that is linked to signaling through Rac.[24,125] ExoS has been shown to ADP ribosylate a number of proteins that are involved in regulating the actin cytoskeleton, including Ras, Rac, Rabs, Ral, and ERM family proteins.[9]

Some strains, exemplified by PA103, encode and produce ExoU and ExoT. These strains are highly cytotoxic, precluding accurate measurement of internalization.[61] Experiments utilizing isogenic mutants that are defective in ExoU and/or ExoT or that are defective in the type III secretion apparatus have revealed that mutants defective in ExoU and ExoT (PA103ΔUΔT or a type III secretion mutant) are approximately 3–10 fold more invasive into cultured epithelial cells or macrophages than an isogenic strain producing ExoT.[18,49,61] Expression of ExoT from PAK or PA103 in PA103ΔUΔT inhibits internalization.[57] These results indicate that ExoT can function as an anti-internalization factor. Together, these

findings suggest that strains that produce ExoU and ExoT appear noninvasive for two reasons. First, and probably of greater biological significance, production of ExoU leads to rapid host cell cytotoxicity. In addition, ExoT inhibits bacterial internalization; however, this effect is only measurable in the absence of ExoU.

A second common genotype is represented by PAK, which produces ExoS and ExoT. It is more invasive than PA103*pscJ* (defective in T3SS), despite producing two potential anti-internalization factors, ExoT and ExoS. The explanation for this apparently paradoxical phenotype may be multifactorial. (i) PAK, more adhesive that PA103, probably because it produces both pili and flagella (PA103 does not produce functional flagella). (ii) There is a delay in the translocation of ExoS and ExoT. Thus, at early times postinfection, PAK and PAKΔSΔT are internalized into HeLa cells with the same efficiency, but internalization of the ExoS- and ExoT-producing strain then plateaus (Pielage, J, Kalman, D., and Engel, J., unpublished results). (iii) PAK appears to survive intracellularly better than PA103. (iv) As discussed in detail in the next paragraph, in some cell lines or perhaps strains, the ability of ExoS to ADP ribosylate and activate Rac may counterbalance and even predominate over the inhibitory effect of its GAP activity.[112] Interestingly, PAK internalization is growth phase dependent, with the stationary-phase cells internalized about 100-fold more efficiently than the exponential-phase cells. This may be explained by the negative regulation of TTS by quorum sensing.[10] Indeed, this growth phase-dependent internalization was not observed in PA103,[57] a LasR-defective strain.[47]

Recent studies suggest that each domain of ExoS and ExoT modulate bacterial internalization by distinct mechanisms. Inactivation of either the GAP or the ADPRT domain of ExoT in PA103ΔU partially reduces its anti-internalization activity. Expression in PA103ΔUT of the closely related T3S toxin, ExoS, diminishes invasion of PA103ΔUΔT into HeLa cells or corneal epithelial cells.[18,49,57] Contradictory effects of ExoS and ExoT on the invasion of PAK have been reported, consistent with reports that ExoS has cell-line dependent effects on cell function.[112] When ExoS harboring a mutation in the ADPRT domain is expressed in PA103ΔUΔT or PAKΔSΔT, there is no decrease in its anti-internalization activity[57] (unpublished results). However, ExoS lacking a catalytically active GAP domain appears to lose anti-internalization activity. This correlates with the ability of its ADPRT domain to activate Rac in at least some cell types[112] (Pielage and Engel, unpublished results). Thus, ExoS may have opposing effects on bacterial internalization. Its GAP domain inhibits Rac-, Rho-, and Cdc42-dependent internalization. However, ADP ribosylation of Rac results in Rac activation, which interferes with the ability of the GAP domain to interact with Rac and

interfering with GAP-mediated Rac inactivation, resulting in increased levels of active Rac1.

8.2. Internalization May Be Modulated by Quorum Sensing

Production of many virulence factors of *P. aeruginosa* is subject to quorum sensing, a density- and growth-phase dependent regulatory system. Invasion has been reported to be regulated by the Las and Rhl quorum sensing systems.[88] QS has also been reported to negatively regulate T3SS.[10,69] Whether the observed effects of QS are a consequence of inhibiting T3SS or whether they represent control at a different level remains to be determined.

9. CONCLUSIONS

The study of *P. aeruginosa* internalization into non-phagocytic host cells is complex and incompletely understood. The fact that this process is highly conserved amongst diverse clinical and environmental isolates together with the ability of the organism to carefully regulate this process suggests that internalization is key to its ability to survive and disseminate in diverse habitats. Strains associated with human infections are capable of being internalized into mammalian cells in vitro. This may allow them access to an immune-privileged site, facilitate transcytosis across the epithelial barrier, and permit the host to diminish bacterial load by desquamation of infected cells. In other settings, for example upon contact with macrophages, *P. aeruginosa* may try to inhibit phagocytic uptake. The role of invasion in the aquatic and plant settings are incompletely explored. Future studies will hopefully further elucidate in further molecular detail the bacterial adhesins, the host cell receptors, the host cell signaling pathways, and the ultimate consequences of entering into non-phagocytic cells. These studies may yield new therapeutic and preventative strategies for controlling these infections as well as enhancing our understanding of the basic biology host-pathogen interactions.

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CATABOLISM AND BIOTRANSFORMATIONS

13

HISTIDINE CATABOLISM AND CATABOLITE REGULATION

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1. INTRODUCTION

Nutritionally versatile Pseudomonads effectively utilize various nitrogenated compounds, including amino acids (such as glutamate, proline, arginine and histidine) as sources of carbon, nitrogen and energy.[19,29,39,63,64,81,86,93,101,102,104,124] The histidine catabolic pathway of *Pseudomonas* was first established in *Pseudomonas fluorescens* followed by *Pseudomonas putida* and *Pseudomonas aeruginosa*.[49,109,110] The genes for the pathway enzymes were then identified and characterized in *P. putida*.[1,15,23,33,38,48] Expression of the *P. aeruginosa* and *P. putida* histidine catabolic enzymes absolutely requires the presence of histidine (or its degradation intermediate urocanate) and is subject to regulation by both carbon and nitrogen,[1,38,49,81] as in *Klebsiella aerogenes* (formerly *Aeromonas aerogenes*) and *Bacillus subtilis* (for reviews, see refs [9,24,57,85]).

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Glucose is the most effective carbon source for *K. aerogenes* and *B. subtilis* and it prevents expression of the histidine utilization (*hut*) operon through cAMP-CAP (catabolite gene activator protein) and CcpA (catabolite control protein A), respectively.[18,62,66] In contrast, succinate and other TCA-cycle carboxylates are preferable to glucose as carbon sources for *Pseudomonas* spp. and they profoundly repress the synthesis of histidine catabolic enzymes and even that of the glucose uptake and metabolic systems.[5,14,49] *Pseudomonas* spp. possess a homologue of CAP, which is called Vfr. The Vfr protein in *P. aeruginosa* regulates the quorum-sensing system as well as the formation of type IV pili and the type III secretion system, without being involved in catabolite control.[19,20,115] *Pseudomonas* spp. do not have CcpA.[106] This group of bacteria therefore must have a novel mechanism of catabolite regulation.

As described in more detail in another chapter,[89] Crc was first identified in *Pseudomonas* as a *c*atabolite-*r*epression-*c*ontrol protein. This protein modulates carbon-responsive expression of the *bkd* operon that encodes branched chain keto-acid dehydrogenase, the *alk* operon consisting of the alkane degradation enzyme genes, and of several other genes involved in the utilization of sugars, hydrocarbons and amino acids, but not that of the histidine catabolic operon.[14,34,35,91] Crc appears to control gene expression post-transcriptionally and in a carbon-source-dependent manner. However, the precise gene control mechanism of Crc remains unknown.[35,89,91] Other factors that can affect the expression of catabolic genes include cytochrome *o* ubiquinol oxidase, IHF (integration host factor), FtsH and (p)ppGpp. These factors are thought to be part of the "physiological control" that modulates the expression of various catabolic pathways to maintain cell physiology under changing nutrient conditions.[89] The only currently known example of a *Pseudomonas* catabolic system that is negatively controlled by a specific carbon source is the *clc* operon of the *P. putida* plasmid pAC27 that encodes the 3-chlorocatechol assimilation enzymes. The binding of fumarate, a TCA-cycle intermediate, to ClcR protein, a positive regulator of the operon, diminishes the transcription activator property of the regulator and hence abolishes transcription of the operon.[54]

This chapter summarizes our current understanding of the histidine catabolic system in *Pseudomonas*, compares it with that of other microorganisms, and highlights the carbon and nitrogen regulation of this system that appears to represent a novel mechanism of catabolite control. Two pairs of two-component regulatory proteins, CbrA–CbrB and NtrB–NtrC, essentially activate expression of the *hut* operon, depending on whether histidine is the source of carbon or of nitrogen. The CbrA–CbrB pair, which is indispensable only when histidine is the carbon source, switches the *hut* operon on or off in the absence or presence of an effective carbon source

(i.e. a catabolite repressor), respectively, thus mediating catabolite control. On the other hand, the NtrB–NtrC regulator pair crucially activates transcription of the *hut* operon in the presence of a catabolite repressor when histidine is the nitrogen source. Since the CbrA–CbrB pair is required for the utilization of a broad range of compounds, including amino acids, sugar alcohols, organic acids and polyamines,[39,52,70] this two-component regulatory mechanism probably participates in the carbon-responsive regulation of many catabolic pathways.

2. HISTIDINE CATABOLIC ENZYMES AND GENES

2.1. Histidine Catabolic Pathways

Histidine catabolism proceeds in four or five enzymatic reactions that overlap in the first three steps (Figure 1). Histidase (the product of *hutH*)[33,121] initially deaminates the α-amino group of histidine to yield urocanate, which is then converted to 4-imidazolone-5-propionate by urocanase (*hutU*).[43,53] Imidazolone propionase (*hutI*)[58] opens the imidazolone ring of

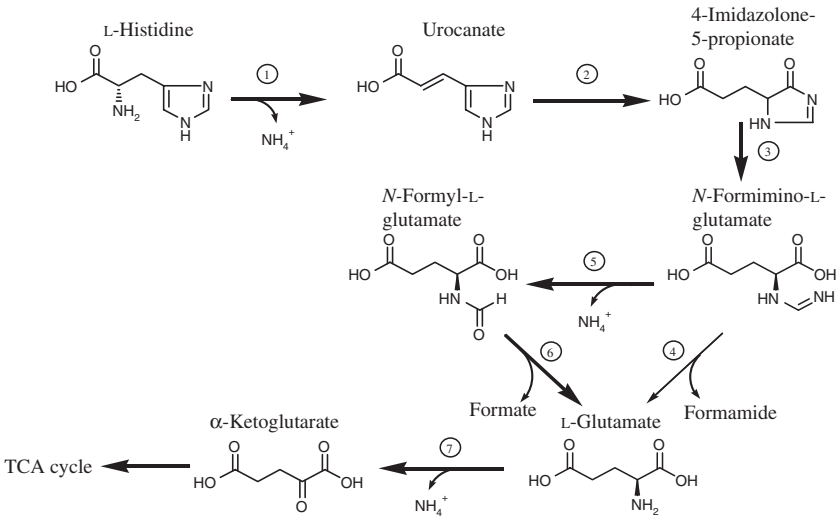

Figure 1. Histidine catabolic pathways. Enzymes catalyzing each reaction are: (1) histidase (histidine ammonia-lyase; EC 4.3.1.3; product of *hutH*), (2) urocanase (urocanate hydratase; EC: 4.2.1.49; *hutU*); (3) imidazolone propionase (EC 3.5.2.7; *hutI*); (4) formimidoylglutamase (EC 3. 5. 1. 49; *hutG*); (5) *N*-formiminoglutamate deiminase (EC 3. 5. 1. 13; *hutF*); (6) *N*-formylglutamate deformylase (*N*-formyl-L-glutamate amidohydrolase; EC 3. 5. 1. 68; *hutG*); and (7) NAD^+-glutamate dehydrogenase (EC 1.4.1.3; *dhB*).

4-imidazolone-5-propionate at the carboxylimide bond, to generate *N*-formimino-L-glutamate in the third reaction. This degradation intermediate at a diverging point can lead to L-glutamate via single- or two-step reactions. Formimidoylglutamase (*hutG*)[43,116] directly converts the intermediate to L-glutamate and formamide. Both the formimidoylglutamase and *N*-formylglutamate deformylase (see below) genes are called *hutG*. These enzymes act on the same scissile formimide linkage at the α-amino group of glutamate, but they have no sequence similarity: formimidoylglutamase is a member of the arginase family (pfam00491),[61] whereas *N*-formylglutamate deformylase (pfam05013) comprises its own small group. We underline the *hutG* of formimidoyl glutamase here to discriminate it from the *hutG* of *N*-formylglutamate deformylase. Some bacteria can use formamide as a nitrogen source through the ancillary functions of urea carboxylase and allophanate hydrolase, which are primarily involved in urea assimilation.[65] Microorganisms that degrade histidine via HutG appear not to possess this enzyme pair and secrete formamide into the medium.[65]

In the two-reaction route, formimidoyl glutamate deiminase (*hutF*)[37] first degrades the diverging intermediate into ammonia and *N*-formyl-L-glutamate, which is successively hydrolyzed by *N*-formylglutamate deformylase (*N*-formyl-L-glutamate amidohydrolase; *hutG*)[16] to L-glutamate and to formate (Figure 1). The HutFG route allows cells to utilize one more ammonia molecules of histidine and it seems to have emerged after the HutG route (see below). Some methylotrophic bacteria and archaea use formate as a carbon source.[55,117] Proteobactria, including *Pseudomonas*, cannot utilize formate[4,27,28,31,114]: some of them may convert it to CO_2 and H_2O via NAD^+-dependent formate dehydrogenase (EC 1. 2. 1. 2)[44,79] and others may accumulate it in the medium as a by-product.[22] L-Glutamate, the terminal product of both pathways, can be used as a precursor in amino acid (alanine, aspartate and glutamine) and protein synthesis, or it can be hydrolyzed via NAD^+-dependent glutamate dehydrogenase[51] to yield ammonia and α-ketoglutarate, which is further metabolized through the TCA cycle as a source of both carbon and energy (Figure 1).

2.2. Distribution of the *Hut* Genes

Genome sequencing projects (http://www.ncbi.nlm.nih.gov;http://mbgd.genome.ad.jp) have discovered that clusters of *hutHUIG* generally occur in both bacteria and archaea such as, β-proteobacteria (*Chromobacterium*, *Ralstonia*, *Bordetella* and *Azoarcus*), γ-proteobacteria (*Hahnella*, *Klebsiella*, *Legionella*, *Salmonella*, *Photobacterium*, *Photorhabdu*s and *Vibrio*), δ-proteobacteria (*Bdellovibrio*), Actinobacteria (*Nocardia* and *Corynebacterium*), Firmicutes (*Bacillus*, *Staphylococcus* and *Streptococcus*),

Fusobacteria (*Fusobacterium*) and Euarchaea (*Haloarcula*, *Halobacterium* and *Picrophilus*). On the other hand, the *hutHUIFG* cluster is restricted to proteobacteria (http://www.ncbi.nlm.nih.gov; http://mbgd.genome.ad.jp) with the exception of *Streptomyces* (Actinobacteria).[45,121] It frequently occurs in the α-division of proteobacteria (*Agrobacterium*, *Bradyrhizobium*, *Brucella*, *Caulobacter*, *Mesorhizobium*, *Rhizobium*, *Rhodobacter*, *Rhodospirillum*, *Silicibacter* and *Sinorhizobium*), where a *hutHUIG* cluster has not been found, and it is less frequent in the β-division (*Burkholderia*, *Ralstonia* and *Rhodoferax*) and γ-division (*Colwellia*, *Pseudomonas*, *Yersinia* and *Xanthomonas*).

The distribution of the *hut* genes implies that the *hutHUIG* set developed before bacteria and archaea diverged and that *hutFG* appeared in an ancestor of the proteobacteria. Each of *P. aeruginosa*, *P. fluorescens*, *Ralstonia eutropha* and *Ralstonia solanacearum* have a *hutG* orthologue in addition to a *hutFG* pair. The *PA3175* gene, a *hutG* orthologue in *P. aeruginosa* PAO1,[106] has formimidoylglutamase activity[61] and a *hutF* knockout mutant of strain PAO1 can still utilize histidine as the sole source of carbon and nitrogen (Itoh *et al*., unpublished results). The *hutG* orthologue therefore probably participates in histidine catabolism. The *hutG* orthologues in bacteria having a *hutHUIFG* cluster might be remnants of the evolution of histidine catabolism, as follows. A *hutFG* pair appeared in an ancient bacterium having the *hutHUIG* system as a by-pass enabling more efficient utilization of ammonia molecules of histidine[38] and ancestral proteobacteria possessed the two routes downstream of *N*-formimino-L-glutamate.

3. ORGANIZATION AND HISTIDINE INDUCTION OF THE *HUT* GENES

The *hut* genes appear to form operons in most microorganisms, except for *Streptomyces coelicolor*, where these genes are scattered over the chromosome.[6,45] Histidine (or its metabolite, urocanate) can induce both the *hutHUIG* and *hutHUIFG* systems in proteobacteria and in *Bacillus*. Little is understood about whether and how other bacteria (*Streptomyces*, *Staphylococcus*, *Streptococcus* and *Fusobacterium*) and archaea (*Halobacterium*, *Haloarcula* and *Picrophilus*) express the *hut* genes in a histidine-dependent manner.

3.1. The *HutHUIG* System

The *Bacillus hutHUIG* genes form an operon together with the upstream *hutP* regulator and downstream *hutM*, a putative histidine transporter gene[60,122] (Figure 2). The *hutIGCUH* genes (in this order) of enteric

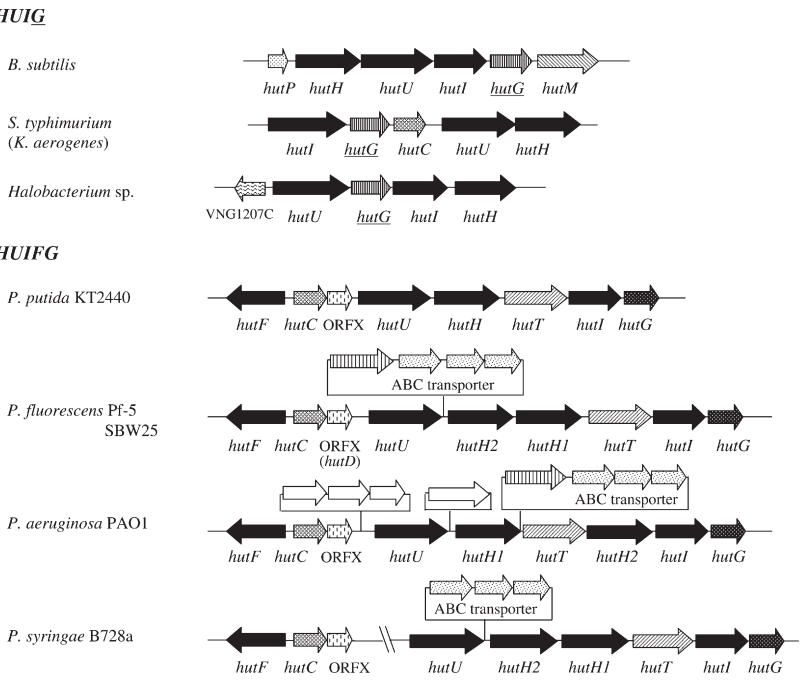

Figure 2. Organization of the *hut* genes. The *hut* genes of *B. subtilis,*[47] *S. enterica* serovar. Typhimurium (*S. typhimurium*)[56] and *Halobacterium* sp.[71] represent the *hutHUIG* system. The nucleotide sequences of the *K. aerogenes hut* genes are not known, but genetic studies support the same gene organization as that in *S. typhimurium*.[10,30,74] The *Pseudomonas hut* genes are shown as representatives of the *hutHUIFG* system. *Pseudomonas* species have the same arrangement of the *hut* genes, except for *P. syringae* in which *hutCF* are located separately (ca. 400 kb). *Pseudomonas* species other than *P. putida* have additional genes of unknown or putative functions at one or three sites (see text). Sequence data are taken from Feil *et al*., (2005),[21] *P. syringae* pv. *syringae* B728a; Nelson *et al*. (2002),[67] *P. putida*; Paulsen *et al*., (2005)[77]; *P. fluorescens* Pf-5; Zhang *et al*., (2006),[124] *P. fluorescens* SBS25; Stover *et al*., (2000),[106] *P. aeruginosa* PAO1.

bacteria (*K. aerogenes* and *Salmonella* spp.) are transcribed in four units[30,56,75] (Figure 2): the *hutUH* genes are co-transcribed from the *hutUp* promoter preceding *hutU*, and *hutIGC* appear to be individually transcribed from their own promoters.[98] Enteric bacteria express the *hut* genes in response to histidine and modulate expression levels depending on the availability of other carbon and nitrogen sources (see below). The *hutUGI* genes of the archaeon, *Halobacterium* sp. lie contiguously without an intergenic space and *hutH* follows after 200 bp.[71] The upstream divergent *VNG1207C* gene encodes a possible transcription regulator of 22 kDa having a helix-turn-helix DNA-binding motif at the C-terminal[71] (Figure 2). However, transcription units and regulation of the archaeon *hut* genes have not been studied.

3.2. The *HutHUIFG* System

Among *Pseudomonas* species, *P. putida* appears to have the simplest organization of the *hut* genes (Figure 2). This organism has another two genes, *hutT* and ORFX, which are also conserved in the *hut* clusters of other *Pseudomonas* species, in addition to the histidine catabolic (*hutHUIFG*) and regulatory (*hutC*) genes. The *hutT* gene encodes a hypothetical 50-kDa histidine transporter, which is indispensable for histidine utilization.[124] The ORFX downstream of *hutC* has been named *hutD,*[124] but the 21-kDa-protein product of this potential gene has no sequence feature suggestive of its function. The gene arrangement of *hutFCUHTIG* is highly conserved among *Pseudomonas* species, but apparently complex genetic events have occurred in some *Pseudomonas* species that include insertions at one or three sites, duplication and subsequent rearrangement of *hutH* (duplicated *hutH* genes are tentatively called here *hutH1* and *hutH2*), or separation of a pair of divergent *hutFC* at a distant (ca. 400 kb) locus (only in *Pseudomonas syringae*) (Figure 2). The sequences inserted between *hutU* and *hutH2* of *P. fluorescens* and *P. syringae* and between the *hutH1* and *hutT* of *P. aeruginosa* carry three or four possible transport genes. The first transport genes of *P. fluorescens* and *P. aeruginosa* specify a 52-kDa-membrane protein of the cytosine/purine/uracil/thiamine/allantoin permease family. The second through fourth of these genes encode an ABC transporter system consisting of a periplasmic amino-acid-binding protein, a membrane permease and an ATP-binding protein (in that order). They also appear between the *hutU* and *hutH2* of *P. syringae* (Figure 2), but the functions of these potential histidine transporter genes remain unknown.

Four extra genes are located in the *hut* region of *P. aeruginosa*. A putative gene between *hutU* and *hutH1* encodes a cytosine/purine/uracil/thiamine/allantoin permease family protein, and three possible genes in the 5′ upstream region of *hutU* specify an ABC-transporter periplasmic amino-acid-binding protein, a homologue of fatty acid desaturase and an ABC-transporter phosphate/ phosphonate periplasmic-binding protein (from upstream to downstream). Their suggested functions and the absence of orthologues in other *Pseudomonas* species favour the notion that these extra genes are irrelevant to histidine utilization.

The HutH1 proteins are similar (>80% amino-acid sequence identity) to each other and to *P. putida* HutH. The HutH2 proteins have homologous sequences (76–82% identity), which differ considerably from the HutH1 paralogues and from the HutH proteins of *P. putida* and other bacteria (≤50% sequence identity). The origin of *hutH2,* as well as the question of whether the HutH2 proteins have histidase activity and participate in histidine degradation, remains unknown.

3.3. Histidine Induction

Two distinct transcription regulators, HutP and HutC, are currently known to mediate histidine-inducible expression of the *hut* genes. HutP is a positive regulator that specifically controls the *Bacillus hutHUIG* systems. In contrast, HutC negatively controls both the *hutHUIG* and *hutHUIFG* systems of proteobacteria.

3.3.1. HutP

The *B. subtilis hutPHUIGM* operon is transcribed from a promoter preceding *hutP*. In the absence of histidine, this transcription is terminated after *hutP*, due to a palindromic sequence between *hutP* and *hutH*, thus preventing futile transcription of the downstream histidine catabolism and transport genes.[24,120] Binding of histidine to HutP renders this antiterminator capable of binding to *hut* transcripts at a nucleotide region overlapping the palindrome, abolishing the termination of transcription and allowing expression of the downstream genes.[46,72] Mutations in the palindromic sequence result in constitutive expression of the *hut* genes.[120] This transcription termination/antitermination mechanism appears to regulate histidine-responsive *hut* gene expression in other *Bacillus* species (*Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus halodurans*)[40,82,111] and the closely related *Geobacillus kaustophilus,*[112] but not in other Gram-positive bacteria, suggesting that HutP is specific to the *Bacillaceae*.

3.3.2. HutC

HutC is a transcriptional repressor protein of the GltR protein family[1,88,96] and in the absence of histidine, it represses the expression of both *hutHUIG* and *hutHUIFG* systems in proteobacteria. Proteins of this family have an N-terminal helix-turn-helix DNA binding (D-b) domain and a C-terminal domain for binding effectors and oligomerization (E-b/O). Urocanate is the physiological effector for the HutC repressors of enteric bacteria (*K. aerogenes* and *Salmonella enterica* serovar *Typhimurium*, *Salmonella typhimurium*) and *Pseudomonas* spp.[36,59,81] and hence mediates histidine induction of the *hut* operons. Imidazole propionate, a non-metabolizable analogue of urocanate, can also serve as an inducer.[95]

The *hut* genes of *K. aerogenes* (and probably of *S. typhimurium*) are transcribed in four units, *hutC*, *hutIG*, *hutG* and *hutUH*.[10,98,100] These transcriptional units, except for *hutC* itself, are under the control of HutC and urocanate.[97] The HutC-operator site (*hutUo*), a sequence having dyad symmetry with one mismatch (5′-ATGCTTGTACAAGTAT-3′), is located between the −35 and −10 sequences of the *hutUp* promoter of *hutU*[73] (Figure 3). HutC-binding to this operator site therefore must hamper

σ^{70}-RNA polymerase holoenzyme (RNP) access to the promoter. Urocanate abolishes the interaction of HutC with *hutUo* through binding to the E-b domain, which is thought to induce a conformational change in the D-b domain.[36,88,98]

The *hut* genes of *P. putida* are also expressed in the transcriptional units, *hutC*, *hutF*, *hutG* and *hutUHTIG*. As in the enteric bacteria, all transcriptional units other than *hutC* are inducible by urocanate.[36,38] However, the expression of *hutG*, but not that of *hutUHTIG* and *hutF*, is also activated by *N*-formyl-glutamate, the substrate of the HutG enzyme.[36] As *N*-formyl-glutamate prevents HutC binding to the *hutG* promoter region *in vitro*, it appears that *N*-formyl-glutamate also serves as an effector of HutC and interacts with the repressor at a site different from that of urocanate.[36,37] A HutC-binding region of about 40 bp determined by DNaseI footprinting contains a core dyad symmetrical 5′-ATGCTTGTATG TACAAGTA-3′ motif[36] that is very similar to a HutC-binding sequence (HutC-BS) in *K. aerogenes hutUp*[73] (Figure 3). The *HutUp* of *P. putida* is a σ^{70}-dependent promoter, like that of *K. aerogenes*, and *P. putida* HutC-BS covers the −35 sequence of the promoter. Notably, the *hutUp*

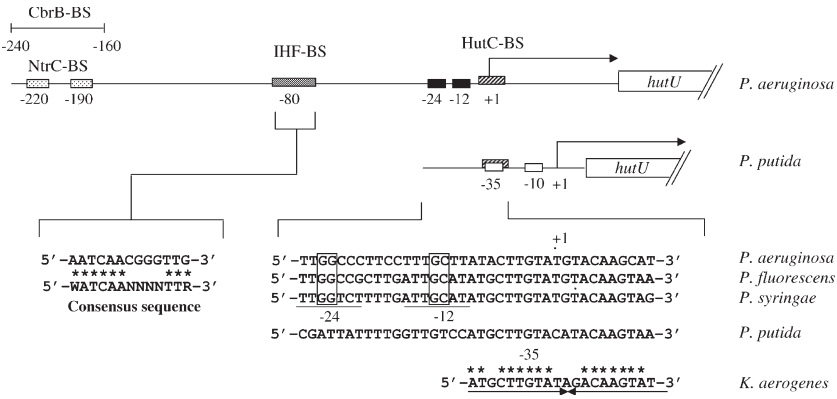

Figure 3. *Cis*-acting regulatory elements in the *hutU* promoter regions of *P. aeruginosa* and *P. putida* and HutC-binding sequences among *Pseudomonas* species. HutC-BS, IHF-BS, NtrC-BS and CbrB-BS denote the binding sequences of HutC, integration host factor (IHF), NtrC and CbrB, respectively. Deletion analysis showed that the DHA region between −240 and −160 is important for CbrB-dependent expression of a *hutU-lacZ* fusion (Itoh *et al*., unpublished results), but CbrB-BS has not been identified. Numbers below binding sequences indicate nucleotide positions relative to transcription initiation start sites (+1) of *hutU* in *P. putida* PRS1[36] and in *P. aeruginosa* PAO1 (Itoh *et al*., unpublished data): the σ^{54}-dependent *hutU* of *P. syringae* Lz4W is transcribed 2-bp downstream from the +1 site in *P. aeruginosa* PAO1.[41] The consensus binding sequences of *K. aerogenes* HutC and *P. aeruginosa* IHF are taken from Osuna *et al*. (1994)[73] and from Wozniak (1994),[118] respectively, and the nucleotide sequences are from the GenBank (http://www.ncbi.nlm.nih.gov).

promoters of other *Pseudomonas* strains have –24 and –12 sequences (5′-YTGG-[N_8]-TTGCW-3′) that are typical of a σ^{54}-dependent promoters[12,41,69] (Figure 3). HutC-BSs of *P. aeruginosa*, *P. fluorescens* and *P. syringae* overlap the transcription start sites (Figure 3). Thus, *Pseudomonas* HutC proteins inhibit transcription from *HutUp* at the point where the σ^{70}-RNP holoenzyme has access to the promoter (in *P. putida*) or where transcription is initiated by the σ^{54}-RNP holoenzyme (in *P. aeruginosa*, *P. fluorescens* and *P. syringae*).

The Antarctic psychrotrophic bacterium *P. syringae* Lz4W has two σ^{70}-dependent promoters around 90- and 140-bp upstream of the σ^{54}-dependent promoter.[41] Transcription from the upstream and downstream promoters is activated at low temperature (4°C) and at both low and high (4°C and 22°C) temperatures, respectively. These promoters have a unique sequence of 5′-CAAAA-3′ at the –10 regions, which appears important for the initiation of transcription at low and high temperatures. The upstream promoter has an extra 5′-CAAAA-3′ sequence at the –15 region (a half turn away from the –10 region) that might facilitate increased expression of the gene at low temperature.[41] The upstream cold-response promoter with the extra CAAAA sequence, but not the downstream promoter, is perfectly conserved in *P. syringae* B728a,[21] although the physiological significance of cold-responsive expression of the *hut* operon remains elusive.

4. HISTIDINE CATABOLIC PATHWAY: CARBON AND NITROGEN REGULATION

The carbon- and nitrogen-responsive regulation of *hut* operon expression is part of the global regulatory mechanisms that control diverse metabolic and physiological functions according to nutritional status, growth rate and phase. Cellular concentrations of metabolites and effector nucleotides (ATP, cAMP, GTP, etc.) that determine the amounts or properties of the global regulatory proteins should vary depending on carbon and nitrogen sources. We briefly examine the carbon and nitrogen regulatory systems of the *K. aerogenes* and *B. subtilis hut* genes and compare them with that of *P. aeruginosa*, which likely includes a unique mechanism of carbon catabolite repression and a close link between carbon and nitrogen regulation.

4.1. Carbon Control

A CAP–cAMP complex and CcpA mediate the repression of *hut* operon expression by glucose in *K. aerogenes* and *B. subtilis*, respectively. Glucose favours dephosphorylation of the PTS enzyme II^{Glc} (EII^{Glc}) in *K. aerogenes*. The non-phosphorylated form of EII^{Glc} inhibits glucose

uptake (surface exclusion) and deactivates adenylate cyclase that catalyses cAMP synthesis. In the absence of glucose, EII^{Glc} is phosphorylated and thereby adenylate cyclase is activated, leading to an increase in cellular concentrations of cAMP (for a review, see ref 92). The CAP–cAMP complex enhances transcription from the *hutUp* promoter through binding to –82-bp upstream of the transcription start point.[73]

In *B. subtilis*, CcpA negatively controls multiple catabolic pathways including histidine degradation, in the presence of a rapidly metabolizable carbon source (such as glucose, fructose or mannose). Fructose-1, 6-bisphosphate (FBP), a glycolytic intermediate of hexoses, stimulates the ATP-dependent phosphorylation of the HPr protein of the PTS system. Phosphorylated HPr (HPr-P) becomes capable of binding to CcpA, an interaction stimulated by FBP.[18] The HPr-P/CcpA complex binds to a *cre* (*c*atabolite *r*epression *e*lement) site located in *hutP* between +203- to +216-bp and thus hampers transcription of the downstream *hut* catabolic genes.[24,120] A *glnA* (glutamine synthetase) mutation partially relieves CcpA-mediated catabolite repression and inactivation of *tnrA* (a global nitrogen regulatory gene) suppresses such relief.[123] The activities of nitrogen-related enzymes would indirectly affect carbon catabolite repression by altering cellular levels of carbon metabolites.[123] The regulatory protein AbrB, which regulates a wide variety of genes encoding alternative pathways of metabolism and energy production in response to nutrient depletion during transition to the stationary phase,[26,107] also partially relieves CcpA-dependent catabolite repression.[25]

In *P. aeruginosa*, the CbrA–CbrB two-component system critically activates expression of the *hut* operons in the absence of succinate (or other carbon catabolite repressors). Two-component systems in general, monitor changes in environmental nutrient availability, osmotic pressure, oxygen tension or cell population density, and regulate metabolic pathways as well as the cellular functions necessary for environmental adaptation.[76,105] The activities of the CbrA and CbrB proteins appear to be regulated by a catabolite repressor, although whether this occurs directly or indirectly remains unknown. As a consequence, this protein pair likely participates in carbon catabolite control of the *P. aeruginosa hut* operon.

4.2. The CbrA–Cbrb Two-Component Regulatory System

The *cbrAB* genes were originally identified in *P. aeruginosa* PAO1 as a two-component regulatory system that is essential for the utilization of amino acids (histidine, arginine, proline and alanine), polyamines (putrescine, spermidine and spermine) and sugar alcohol (mannitol), as carbon sources.[52,70] This gene pair also appears to regulate the metabolism

of some sugars (glucose and fructose), organic acids (citrate and isocitrate) and the synthesis of lipase A.[17,70,90] Furthermore, this gene pair is involved in the metabolic regulation of the type-III secretion system and its effectors, exoenzymes S and T.[87]

CbrA has the N-terminal membrane domain of a putative sensor and the C-terminal cytoplasmic domain of protein histidine kinase, which is homologous to the NtrB family of proteins. CbrB, the cognate response regulator, belongs to the NtrC family of proteins. Transcriptional regulators of the NtrC-family bind to DNA regions far upstream from their promoters to activate transcription.[80] By analogy with other bacterial two-component regulatory proteins,[76,105] a signal input into the sensor domain is thought to activate the kinase activity of the C-terminal domain that undergoes autophosphorylation at a histidine residue (His-766 in CbrA). Subsequent phosphotransfer to CbrB at an aspartate residue (Asp-52 in CbrB) would render this transcriptional regulator active. The phosphorylated (active) response regulator binds to a target sequence (see below) via the C-terminal helix-turn-helix (HTH) DNA-binding domain.[108]

When histidine is the sole source of carbon or of carbon and nitrogen, a *cbrB* mutation (as well as a *cbrA* mutation) completely abolishes transcription of the σ^{54}-dependent *hutUp* promoter, and thereby the utilization of this amino acid as the carbon source.[70] Succinate, a catabolite repressor, diminishes CbrB-dependent transcription from *hutUp*. A deletion between nucleotides –240 and –160, relative to the transcription initiation site, abolished CbrB-dependent transcription (Itoh *et al.*, unpublished results). This DNA region harbors an NtrC-binding site (Figure 3; see Sections 5–4). Although the precise CbrB-binding sequence remains to be determined, CbrB likely directly activates transcription from the *hutUp* promoter.

Both *P. fluorescens* and *P. syringae* also appear to have the σ^{54}-dependent *hutUp* promoter (Figure 3). By contrast, *P. putida* has the σ^{70}-dependent *hutUp* promoter[36] (Figure 3). In *P. putida*, the CbrA–CbrB and NtrB–NtrC (see Section 5–4) two-component regulatory systems may not play any role in histidine catabolism by *P. putida*. Alternatively, this species might have intermediate regulatory factors, such as the Nac protein of enteric bacteria (see Section 5–4), which are under control of the two-component systems and activate transcription from relevant σ^{70}-dependent *hutUp* promoters. The mechanism regulating the *P. putida hutUp* seems to differ from those of its closely related species.

4.3. Nitrogen Control

Different nitrogen sources affect expression of the *hut* operon depending on the bacterium. Amino acids prevent expression of the

B. subtilis hut operon and ammonia prevents that of the counterparts of *K. aerogenes* and *P. aeruginosa*.

Amino acids repress synthesis of the Hut enzymes and histidine transport proteins of *B. subtilis* by inhibiting transcription from the *hutP* promoter of the *hut* operon, *hutPp*, through the CodY protein,[3,25,120] which is a global regulatory protein in low G + C Gram-positive bacteria.[103] This regulatory protein is highly active as a transcriptional repressor in cells rapidly growing in rich medium and becomes inactive in nutrient-depleted medium or during the transition from the exponential to the stationary phase, thus being involved in the regulation of diverse stationary-phase specific phenotypes.[103] Two types of effector molecules, branched-chain amino acids (isoleucine and valine) and GTP, modulate CodY activity.[50,83] Binding of the effectors to CodY enhances the affinity of the C-terminal HTH DNA-binding domain to target DNA regions.[42,50,99,103] Amino-acid depletion activates the (p)ppGpp synthetase activity of the ribosome-bound RelA protein, which catalyzes the transfer of the pyrophosphate group from ATP to the 3′-hydroxyl group of GTP,[13] resulting in a reduction of the cellular GTP pool. Genes under CodY control are therefore repressed when intracellular pools of GTP and isoleucine-valine are high (nutrient rich) and derepressed when the cellular pools of the effectors are depleted by amino acid limitation. The operator sites of CodY have been determined in the *dpp* (dipeptide permease) and *fla* (flagella) promoters, between the transcription initiation start site and the –10 region.[7,94]

4.4. The NtrB–NtrC Two-Component Regulatory System

The NtrB–NtrC (also called NR_{II}–NR_{I}) two-component regulatory system (for reviews, see refs [57, 69]) regulates a set of nitrogen assimilation genes, including *hut* and *glnA* (glutamine synthetase), and it appears ubiquitous in proteobacteria (http://mbgd.genome.ad.jp). NtrB (synonym GlnL) has both histidine kinase and phosphatase activities. This transmitter autophosphorylates and donates the acquired phosphate group to the response regulator NtrC (synonym GlnG). The phosphorylated (active) from of NtrC (NtrC-P) can be the substrate of the NtrB phosphatase, thus primarily determining the activity of the response regulator.

PII (encoded by *glnB* and *glnK* in enteric bacteria and by *glnK* in *Pseudomonas* spp.) and uridyltransferase/uridylyl-removing enzyme (UTase/UR; product of *glnD*) function as the sensors of carbon and nitrogen status in the cells, which reflect carbon and nitrogen supplies in the medium and modulate the phosphatase activity of NtrB. The following model was developed based on studies using *K. aerogenes* and *E. coli*. It is probably also valid in *Pseudomonas* spp., although little mechanistic detail in these organisms is understood.[113]

Both α-ketoglutarate (α-KG) and ATP promote the uridylylation of PII by UTase, whereas Gln enhances the deuridylylation of PII-UMP by UR and inhibits UTase activity (Figure 4). Cellular concentrations of α-KG and ATP are high and those of Gln are low under nitrogen-limited (or carbon excess) conditions, whereas during nitrogen excess (carbon limitation) the situation is reversed. Therefore, PII-UMP dominates over non-modified PII under nitrogen limitation. In this way PII-dependent activation of the phosphatase of NtrB is prevented, increasing the amount of NtrC-P. Under an excess of nitrogen, the level of non-modified PII increases and autophosphorylation of NtrC is inhibited, reducing NtrC-P levels. Among a variety of nitrogenated compounds that can be utilized by *P. aeruginosa*, ammonia is the most efficient nitrogen source and the strongest effector of the NtrB–NtrC system of this bacterium, as in *Enterobacteriaceae*.[69]

Escherichia coli B/r cells growing aerobically in minimal salt medium with glucose and ammonia as carbon and nitrogen sources, respectively, use about half of the glucose for energy production and the other half for cell component synthesis.[68] *E. coli* cells contain about 1 and 5 mmoles of nitrogen (as ammonia) and carbon (as glucose)/g of dry weight, respectively.[84] Histidine catabolism through the HutHUIG and HutHUIFG pathways yields two and three ammonia molecules, respectively, and 1 mole of α-ketoglutarate (Figure 1). Thus, histidine is a nitrogen-rich substrate.

In fact, ammonia alone has essentially no effect on the expression of the *K. aerogenes* and *P. aeruginosa hut* operons[59,81] and a defect of *ntrC* (or *ntrB*) has no effect on growth when histidine is the sole source of carbon and nitrogen. However, when an efficient carbon source (catabolite repressor) is present together with histidine, ammonia exerts a powerful repressive effect on the *hut* operons, and the *ntrB* and *ntrC* genes become essential. In *P. aeruginosa* succinate represses transcription from the *hutUp* by 50–70%.[49] The remaining transcription is not due to "partial" catabolite repression. Mutations of *ntrB* or *ntrC* or high concentrations (10 mM) of ammonia almost completely diminish the residual transcription. The catabolite repressor succinate would increase the cellular pools of α-KG and ATP, that is, cellular signals of carbon excess that activate the NtrB–NtrC system (Figure 4).

NtrC-P activates σ^{54}-dependent promoters through interacting with operator sites at about 100-bp upstream of the promoters.[57] DNaseI-footprints have determined *P. aeruginosa* NtrC-binding sites at nt –220 (5′-CCCG-CAATTGCGC TGAA-3′) and –190 (5′-GGCCCCAAACCGGTGC G-3′) (Itoh *et al*., unpublished results) relative to the transcription start point (Figure 3). These binding sites have similarity (underlined) to the consensus NtrC-binding site of *E. coli* (a 6-bp palindrome with a 5-bp space; 5′-TGCACCAAAATGGTGCA-3′). The NtrC-type transcription activators requires the IHF that binds between the promoter and NtrC-

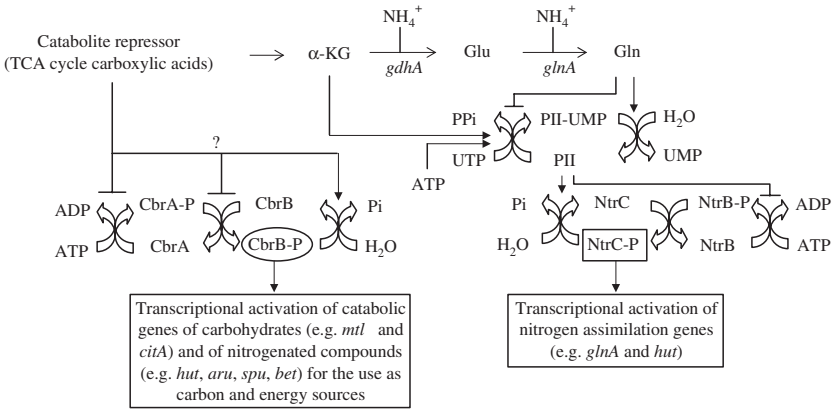

Figure 4. Regulatory circuits of the CbrA–CbrB and NtrB–NtrC two-component systems. CbrA autophosphorylates with ATP (CbrA-P) and transfers the phosphate moiety to CbrB to form CbrB-P, an active form of the transcriptional regulator. A catabolite repressor (TCA-cycle intermediates) might inhibit any step(s) of this phosphorelay or promote CbrB-P dephosphorylation, thus controlling CbrB-P levels. Similarly, phosphotransfer from NtrB-P (autophosphorylated NtrB) to NtrC results in the formation of transcriptionally active NtrC-P. According to the *Klebsiella* model of nitrogen regulation,[57,69] PII protein (the *glnK* product in *Pseudomonas*) regulates NtrC-P levels by modulating the kinase and phosphatase activities of NtrB. The uridylylation of PII though the uridylyltransferase activity of GlnD protein is stimulated by α-ketoglutarate (α-KG) and ATP (intracellular signals of carbon-rich status). Uridylated PII (PII-UMP) cannot regulate the PII phosphatase activity. On the contrary, glutamine (Gln) is a signal of nitrogen excess that enhances the removal of uridylyl by GlnD. Nitrogen-limited (or carbon excess) conditions elevate NtrC-P levels by promoting the uridylation and hampering the deuridylation of PII. Under carbon-limitation (absence of a catabolite repressor), CbrB-P activates the expression of diverse catabolic genes to supply carbon sources. Under nitrogen-limitation (absence of ammonia), NtrC-P directly promotes the expression of *glnA*, which encodes glutamine synthetase of the low-ammonia pathway, and indirectly suppresses the expression of *gdhA,* which encodes anabolic $NADP^+$-glutamate dehydrogenase of the high-ammonia pathway.[8,11,57] In enteric bacteria, Nac mediates NtrC-P dependent expression of the *hut* operons and other nitrogen assimilation genes.

The functions of the other genes are as follows: *aru*, arginine and ornithine utilization via the succinyltransferase pathway; *bet*, betain utilization; *citA*, citrate transport; *hut*, histidine utilization; *glnA*, glutamine synthetase; *mtl*, mannitol utilization; *spu*, polyamine utilization.

binding sites to bend the DNA, so that the transcription activator interacts with σ^{54}-RNP holoenzyme at the promoter.[57,80] A DNA sequence (5′-AATCAACGGGTTG-3′) located at nt −80 resembles the consensus IHF-binding site (5′-WATCAANNNNTTR-3′) in the upstream regions of the σ^{54}-dependent *algD* and *algT* genes involved in the regulation of alginate biosynthesis of *P. aeruginosa*.[118,119]

NtrC-dependent transcription of the *P. aeruginosa hut* operon starts at the same site as that of CbrB-dependent transcription (Itoh *et al.*,

unpublished results). The common σ^{54}-dependent promoter is thus used for carbon- and nitrogen-responsive transcription of the *hut* operon (Figure 3). By contrast, the *hut* operon of *K. aerogenes* is indirectly controlled by the NtrB–NtrC system via the Nac (*n*itrogen *a*ssimilation *c*ontrol) protein. This protein belongs to the LysR family of proteins that activates σ^{70}-dependent promoters.[8,69,97]

4.5. The *Ntrc251* Mutation and Catabolite Regulation

In a *cbrB* mutant of *P. aeruginosa* PAO1, the ability to utilize histidine (a Hut$^+$ phenotype) is spontaneously recovered at a high frequency (ca. 10^{-5}). These Hut$^+$ (pseudo)-revertants can utilize other amino acids (arginine, proline and alanine), but not sugars (such as glucose), sugar alcohols (mannitol) and organic acids (citrate), and require glutamate for optimal growth on succinate and other carbon sources. Like enteric bacteria,[57,85] *P. aeruginosa* PAO1 synthesizes high levels of anabolic NADP$^+$-glutamate dehydrogenase (the *gdhA* product) and low-levels of glutamine synthetase (the *glnA* product) under nitrogen-rich conditions (20 mM ammonia).[113] Under nitrogen limitation (1 mM ammonia or 20 mM nitrate), expression profiles of these enzymes are reversed through the functions of the NtrB–NtrC system; GdhA synthesis is repressed 25-fold and GlnA synthesis is enhanced 10-fold in nitrate medium. A *cbrB* mutation has no effect on the nitrogen-regulation of these enzymes. However, regulation of the nitrogen assimilation genes (*gdhA*, *glnA* and *hut*) in Hut$^+$ revertants is altered. They produce GdhA at repressed levels and GlnA at induced levels, independently of the nitrogen status in the medium. Due to low GdhA expression levels, this revertant cannot produce sufficient glutamate, which supplies about 85% of the cellular nitrogen,[57] and hence requires exogenous glutamate for optimal growth.

One Hut$^+$ revertant analysed in detail had a C to T transition at nt 251 of *ntrC* causing an amino-acid change of Ala-84 to Val (Itoh *et al*., unpublished results). The N-terminal receiver (recognition) domain of NtrC contains five β-sheets (β1 to β5) flanked by five α-helices (α1 to α5).[27] Ala-84 is distal to the β4 sheet (Pro-75 to Ile-80 corresponding to Pro-74 to Ile79 of *E. coli* NtrC) and faces the conserved phosphorylation site, Asp-55 (Asp-54 in *E. coli* NtrC), which is distal to the parallel β2 sheet (Gln-48 to Ile-52). Some mutations in the receiver domain, for example, a deletion of Glu-10 or a substitution of Lys-104 to Gln, confer resistance upon the response regulator to dephosphorylation via the NtrB-PII complex.[78] Furthermore, the His-84 residue (His-85 of *P. aeruginosa* NtrC adjacent to the mutation residue) is thought to determine the hydrolysis rate of NtrC-P by the NtrB–PII complex.[32] The Ala-84-to-Val mutant NtrC

might have a conformation that mimics the phosphorylated (active) form or confers resistance to dephosphorylation by the NtrB–PII complex. In this way, NtrC251 might replace CbrB as an activator of the *hut* operon.

Succinate represses HutH synthesis by *P. aeruginosa* PAO1 to around 25%. This remaining expression absolutely depends on the functions of NtrB and NtrC and is almost completely (below 1%) repressed by ammonia. HutH protein synthesis and a *hutU-lacZ* translational fusion in the *cbrB ntrC251* mutant are no longer subject to catabolite repression and to ammonia regulation. The altered regulation of the nitrogen assimilation genes in the *cbrB ntrC251* mutant is in agreement with the notion that NtrC251 has an active structure similar to that of NtrC-P or is constantly phosphorylated.

5. CONCLUSIONS

In *P. aeruginosa*, the CbrA–CbrB or NtrB–NtrC two-component regulatory proteins activate transcription of the histidine utilization (*hut*) operon when histidine is the source of either carbon or nitrogen, respectively (Figure 4). The response regulators CbrB and NtrC of the NtrC family proteins[12,57,108] bind to around –200 relative to the transcription start site of the *hutU* gene (Figure 2) and activate transcription from the same σ^{54}-dependent promoter of *hutU* (Figure 3). The HutC repressor blocks the transcription initiation site and prevents transcription of the *hut* operon when histidine (or urocanate) is absent.

Like other two-component response regulators,[76,105] CbrB must be phosphorylated to exert its transcriptional activator function. Because the function of CbrB is required only when an effective carbon source (catabolite repressor such as succinate, other TCA-cycle intermediates or glucose) is absent, it can be postulated that the response regulator should become phosphorylated in the absence of a catabolite repressor. The catabolite repressor might therefore inhibit either CbrA autophosphorylation or subsequent phosphorelay from CbrA-P to CbrB (Figure 4). Alternatively, it might stimulate dephosphorylation of CbrB-P. Since the N-terminal membrane domain of CbrA has homology to PutP Na^+/proline transporters,[70] this domain might have a dual function as transporter and as a sensor. If CbrA indeed has a transport function, then the question arises of how this would relate to carbon catabolite repression. Clearly, the catabolite-control mechanism of the *hut* operon in *P. aeruginosa* is distinct from that in *K. aerogenes,* which involves the cAMP and CAP system,[92] and from that in *B. subtilis*, where CcpA negatively controls the *hut* operon in response to a catabolite repressor.[24] The indispensability of the CbrA–CbrB system for the utilization of other amino acids

(arginine/ornithine and alanine), polyamines (putrescine, spermidine and spermine), mannitol, citrate and a phenolic compound as well for the synthesis of lipase A,[2,52,70,90] suggests that this system also participates in carbon-responsive control of the catabolic enzymes of these compounds and the degradative enzyme lipase; the LipQ and LipR two-component regulatory proteins controlling the lipase A gene are the counterparts of CbrA and CbrB, respectively.[90] The mechanism linking catabolite repression to the CbrA–CbrB system and the machinery that adjusts the phosphorylation levels of CbrB appear pivotal to this novel catabolite-control system, but in fact remain to be elucidated.

Ammonia-responsive regulation of the *P. aeruginosa hut* operon is apparently mediated via the NtrB–NitrC two-component regulatory system much like that in *K. aerogenes*.[57] PII protein probably senses intracellular concentrations of α-ketoglutarate, ATP and glutamine, and then communicates information about the cellular status of carbon and nitrogen to NtrB, which controls the amounts of the phosphorylated (active) form of NtrC, a transcriptional activator (Figure 4). The *hut* operons of *P. aeruginosa*, *P. fluorescens* and *P. syringae* have a σ^{54}-dependent *hutUp* promoter, which is directly activated by NtrC-P, whereas *P. putida* has a σ^{70}-dependent *hutUp* (Figure 3) like *K. aerogenes*.[57,69] In enteric bacteria (including *K. aerogenes*) NtrC-P activates transcription of the σ^{70}-dependent *hutUp* via Nac protein.[8,57,97] Because *P. putida*, as well as other *Pseudomonas* spp., does not have a *nac* homologue, this bacterium might have an intermediate regulator that executes a function similar to that of Nac.

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14

SYNTHESIS AND DEGRADATION OF POLYHYDROXYALKANOATES

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Abbreviations: PHA: Polyhydroxyalkanoate, GAPs: Granule associated proteins, scl-PHAs: Short-chain-length PHAs, mcl-PHAs: Medium-chain-length PHAs, PHB: Polyhydroxybutyrate, CoA: Coenzyme A, ACP: Acyl carrier protein, GFP: Green fluorescent protein, P(HB-*co*-HA): Poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoates), PHO: Poly-3-hydroxyoctanoate, PHV: Poly-3-hydroxyvalerate, SCP: single-cell-protein.

1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are optically active biopolyoxoesters composed of (*R*)-3-hydroxy fatty acids which represent a complex class of storage polyesters. They are synthesized by some Archaea and by a wide range of Gram-positive and Gram-negative bacteria in aerobic and anaerobic environ-

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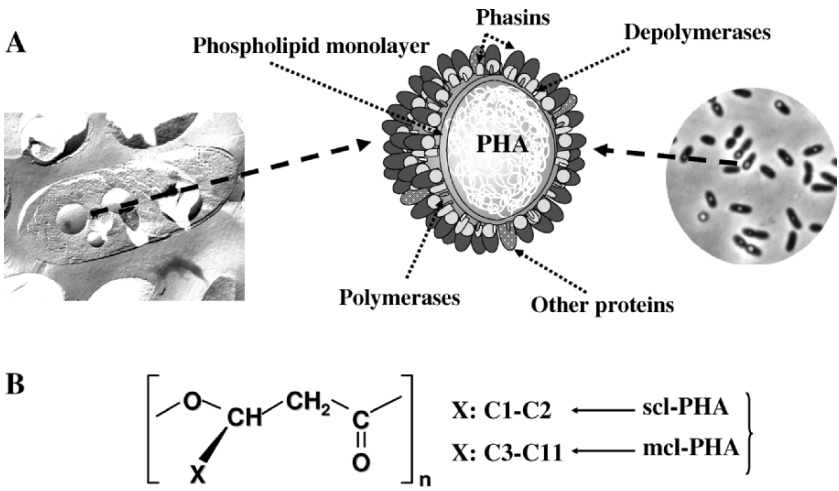

Figure 1. Polyhydroxyalkanoates (PHAs) structure and composition. (a) Freeze-fracture electron (*left*) and phase-contrast micrographs (*right*) of *P. putida* KT2440 mcl-PHA-producing cells. A model of the PHA granule structure is shown (*center*). PHA granules are composed by PHA coated by a monolayer of phospholipids and granule-associated proteins (GAPs). The most abundant proteins in the granules are phasins, which may play a similar role to oleosins (avoiding granule collapse in plant seeds), some of them acting also as transcriptional regulators. Some phasins show modular composition indicated as dark (bullet) and light (circle) gray domains. Polymerases (or synthases) and depolymerases are also associated to the granule. (b) Chemical structure of PHAs. PHAs are generally composed of (*R*)-β-hydroxy fatty acids, where the pendant group (X) varies from methyl (C1) to undecyl (C11). The best-known PHAs are PHB (X = methyl), P(HB-*co*-HV) (X = methyl or ethyl), and P(HO-*co*-HH) (X = pentyl or propyl).

ments.[5,54,55,123] PHAs are accumulated as inclusions in the bacterial cytoplasm in response to inorganic nutrient limitations when the microbes are cultured in the presence of an excess carbon source[55,100] (Figure 1).

At present, PHAs are classified into two major classes (Figure 1): short chain length PHAs (scl-PHAs) with C4–C5 monomers and medium chain length PHAs (mcl-PHAs) with C6–C14 monomers.[55] They were first described by Lemoigne, who observed that *Bacillus megaterium* produced an intracellular polymer that contained hydroxybutyrate monomers, later called polyhydroxybutyrate (PHB),[50] which is the most widely produced scl-PHA by bacteria.[100,103]

Several decades later, other related biopolyesters with longer side chains (mcl-PHAs) began to be seen.[20,31] Their production in the laboratory was first reported for *Pseudomonas putida* GPo1 (formerly known as *Pseudomonas oleovorans* GPo1).[15] Depending on the organism, PHA production can reach levels as high as 90% of the cell dry weight.[54] When the environment becomes more hospitable, the PHAs are biodegraded to the corresponding monomers, which are used as carbon and energy sources.[38]

PHA granules can be observed intracellularly as light-refracting granules by phase-contrast microscopy and as electron-clear deposits by transmission electronic microscopy of thin sections (Figure 1). Freeze-fracture electron microscopy has added another dimension to the structural analysis of PHA granules. No fixation is needed, and granules can be visualized nicely within the cell cytoplasm, showing details of granule interactions with the cellular cytoplasm and cytoplasmic membranes.[15,18,73]

First investigations of purified scl-PHA granules from *Bacillus*[25] and mcl-PHA granules from *P. putida* GPo1[15] demonstrated that the granules contained protein and lipid materials besides PHA. It was clear that PHAs, being highly hydrophobic polymers, must be separated from the cell cytoplasm, suggesting that the lipids found associated with the PHA probably derived from membrane structures that surrounded the PHA.[15,18] The nature of the PHA–lipid membrane interaction and the structure of this membrane have been the subject of considerable debate in the PHA field. Freeze-fracture electron micrographs of slowly growing *P. putida* GPo1 have shown that the boundary (lipid) layer around the large PHA granules formed in such cells is too thin to be a bilayer membrane, leading to the conclusion that PHA granules must be surrounded by a lipid monolayer.[73] When such cells are fractured, fracture faces arise due to the separation of this phospholipid monolayer from the hydrophobic polyester granule. Similar observations have been reported for freeze-fractured PHA granules of *Bacillus cereus*.[18]

Subsequent structural analyses revealed that the phospholipid monolayer is interspersed with a set of granule-associated proteins (GAPs)[101] so that complete granules consist of a mass of PHA, enveloped by a lipoprotein monolayer. GAPs include (i) PHA synthases, involved in the polymerization of PHAs; (ii) intracellular PHA depolymerases, responsible for polymer degradation; (iii) phasins, the main GAP components, which play structural roles; and (iv) other proteins including transcriptional regulators (Figure 1).[61,74,101]

The number and size of the granules, their monomer composition, and macromolecular structure vary depending on the producer organism.[5,54,55,123] The many different PHAs that have been identified to date are primarily linear, head-to-tail polyesters composed of chiral 3-hydroxy fatty acid monomers, the hydroxyl-substituted C3 thus far having been found to be exclusively in the *R* configuration[55,75] (Figure 1). The alkyl substitution group at this position ranges from methyl to undecyl depending on the producer strain, and can be saturated, aromatic, unsaturated, halogenated, epoxidized, or branched.[54,102] The mechanical and physicochemical properties such as stiffness, brittleness, melting point, glass transition temperature, and resistance to organic solvents depend on monomer composition.[55,118]

In this chapter, we provide an updated overview of the metabolism of PHAs in pseudomonads and their biotechnological applications.

2. METABOLIC PATHWAYS INVOLVED IN PHA SYNTHESIS

In most bacteria, such as the paradigmatic *Ralstonia eutropha* H16 strain, PHB is synthesized in a three-step reaction starting with acetyl-CoA (Figure 2). In the first step, two acetyl-CoA molecules are condensed in a reaction catalyzed by a 3-ketothiolase. Then, the acetoacetyl-CoA generated is stereoselectively reduced to (*R*)-3-hydroxybutyryl-CoA by a NADPH-dependent acetoacetyl-CoA reductase. Finally, the (*R*)-3-hydroxybutyryl-CoA monomers are polymerized by a PHB synthase, releasing PHB and free CoA as end products (Figure 2).[99]

Most of the *Pseudomonas* species rely on the β-oxidation pathway, a route first suggested in the early 1970s for organisms present in activated sludge,[113] and fatty acid *de novo* synthesis to convert fatty acid or carbohydrate intermediates, respectively, into different (*R*)-3-hydroxyacyl-CoAs.[34,47] These metabolites are used as substrates by the PHA synthases,

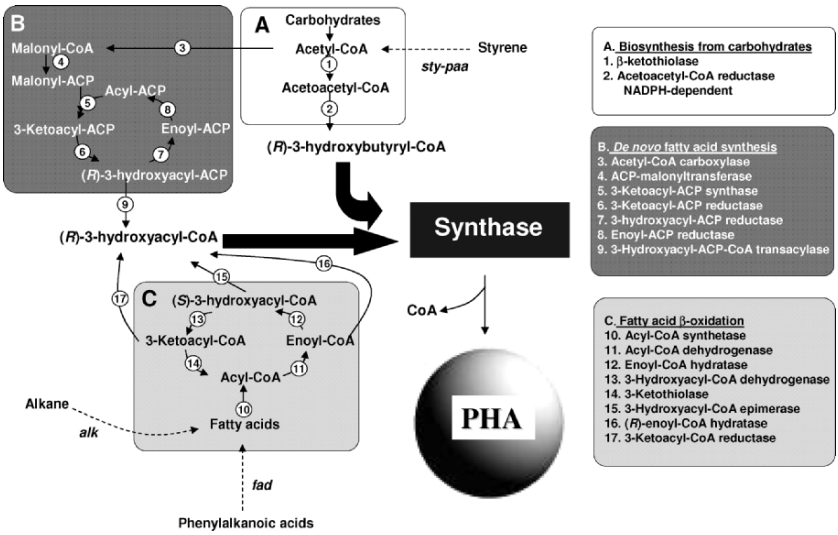

Figure 2. Metabolic pathways involved in PHA biosynthesis of pseudomonads. (a), (b), and (c) are central pathways that converge in the central intermediate (*R*)-3-hydroxyacyl-CoA which is recruited by the PHA synthase to be incorporated into the polyoxoester by the concomitant release of CoA. Pathway (a) is rarely found in some *Pseudomonas* species like *Pseudomonas* sp. 61-3. Discontinuous arrows indicate the peripheral routes that, in particular strains, connect to central pathways to generate the (*R*)-3-hydroxyacyl-CoA intermediates: *alk* genes encoding alkane oxidation pathway from *P. putida* GPo1; *sty–paa* linked pathways for styrene and phenylacetic acid degradation from *P. putida* CA-3; *fad* genes encoding phenylalkanoic acids β-oxidation pathway in *P. putida* U.

which catalyze the committed step of mcl-PHA biosynthesis and finally end up in PHA polymer (Figure 2).[81] *In vitro* studies showed unequivocally that the ultimate substrate for the PHA synthase are the (*R*)-3-hydroxyacyl-CoA metabolites.[13,46] The β-oxidation intermediates that are converted into (*R*)-3-hydroxyacyl-CoA from fatty acids have not yet been established in detail.[83] To produce mcl-PHAs in recombinant *Escherichia coli* cells expressing a PHA synthase–coding gene, ongoing β-oxidation has to be slowed down either by adding acrylic acid (a β-oxidation inhibitor) or by using specific mutants deficient in certain steps of the β-oxidation.[75,76,77,84] In the same vein, PHA production was greatly increased in *P. putida* U β-oxidation mutants where more substrate was apparently available for PHA synthesis (see later).[64,65] These observations suggest that the PHA polymerase competes with the enzymes of the β-oxidation complex for their substrates.[84] PHA formation from glucose is linked to fatty acid biosynthesis (Figure 2). Glucose is oxidized to acetyl-CoA without involvement of the fatty acid β-oxidation pathway. Instead, the (*R*)-3-hydroxyacyl-acyl carrier protein ((*R*)-3-hydroxyacyl-ACP) intermediates of the fatty acid biosynthesis route are directed towards PHA biosynthesis by the transacylase reaction catalyzed by PhaG.[82] This specific transacylase catalyzes the transfer of the (*R*)-3-hydroxyacyl moiety of the respective ACP thioester to CoA (Figure 2). It is worth noting that, despite the fact that the gene encoding this metabolic pathway–linking enzyme is not located in the *pha* cluster, it is coregulated with the latter.[32,82]

Although most mcl-PHA intermediates are obtained through fatty-acid β-oxidation by the different species of the genus *Pseudomonas*,[54] mcl-PHAs synthesized from different carbon sources or even containing different monomers can also be obtained through other pathways (Figure 2).[15,23,108] The choice of a particular route seems to be a strain-specific trait. As examples, *P. putida* GPo1 is able to generate mcl-PHA from *n*-alkanes and 1-alkenes by the alkane oxidation pathway which is encoded on the OCT plasmid,[15,111] whereas *P. putida* CA-3 accumulates aliphatic PHA from styrene and phenylacetic acid, two compounds which are degraded to succinyl-CoA and acetyl-CoA through two linked pathways, *sty* and *paa*.[63,66,108,114]Additionally, accumulation of unusual polymers (aromatic mcl-PHA) has been reported in different *Pseudomonas* species.[2,11,19,22,23,53,54,64,65] In contrast to other species of PHA-producing bacteria, *P. putida* U was also able to synthesize aromatic or mixtures of aliphatic and aromatic PHAs when it was cultured in a chemically defined medium containing *n*-phenylalkanoic acids or *n*-phenylalkanoic plus *n*-alkanoic acids as the carbon sources.[23,64,65] Furthermore, most of these polyesters (poly(3-OH-*n*-phenylalkanoates)), which contain monomers with carbon chain lengths that range from 5 to 10 carbon atoms, have different physicochemical properties and characteristics, opening up a

plethora of potential biotechnological applications.[2,54] The synthesis of these monomers requires the participation of a β-oxidation pathway which constitutes one of the upper routes of the phenylacetyl-CoA catabolon (see chapter 6).[53,66] This β-oxidation pathway involves the participation of a transport system coupled to an acyl-CoA synthetase (FadD) which catalyzes the activation of *n*-phenylalkanoic and *n*-alkanoic acid ($n > 4$) to their acyl-CoA derivatives. Later, an acyl-CoA dehydrogenase (FadF), which requires the participation of an electron-transferring flavoprotein (FadE), catalyzes the introduction of a double bond in the β-position. Finally, a protein complex (FadBA) with five enzymatic activities (enoyl-CoA-hydratase, 3-OH-acyl-CoA dehydrogenase, *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase, 3-OH-acyl-CoA epimerase, and 3-ketoacyl-CoA thiolase) catalyzes the removal of two carbon units of the acyl-chain being processed (Figure 2).[64] Genetic studies revealed that disruption or deletion of some of the β-oxidation genes handicaps the growth of *P. putida* U in chemically defined media containing either *n*-alkanoic or *n*-phenylalkanoic acids with an acyl moiety longer than C4. However, all these mutants regained their ability for degrading *n*-alkanoic acid as a result of the induction of a second β-oxidation pathway (βII) which cannot be used for the complete catabolism of *n*-phenylalkanoates. The accumulation of metabolic intermediates from *n*-phenylalkanoates drove these mutants to become bioplastic overproducers.[54,64,65] Thus, the mutation or the deletion of either *fadA*, *fadB*, or both genes in *P. putida* U has allowed the synthesis of a huge number of biodegradable polymers, other than those accumulated in the wild type, eliciting a strong intracellular accumulation of unusual copolymers.[2,54,64,65]

Pseudomonas sp. 61-3 is an exception among the pseudomonads in that it is able to accumulate PHB through a pathway similar to that of *R. eutropha*, as well as a heteropolymer containing a mixture of monomers (see later).[58]

3. GENETIC ORGANIZATION OF THE *PHA* GENES

The organization of the mcl-PHA biosynthetic genes (*pha*) was reported in 1991 in the strain *P. putida* GPo1 (Figure 3) and was further completed in 1999 with the description of the phasin-coding genes *phaI* and *phaF*.[36,74] The *pha* cluster is well conserved among the mcl-PHA producer strains (Figure 3). It is composed of (i) two synthase coding genes (*phaC1* and *phaC2*) involved in PHA synthesis; (ii), a depolymerase encoding gene (*phaZ*) responsible for PHA mobilization (see later) and the *phaD* gene encoding a transcriptional regulator.[43,79] The *phaF* and *phaI* genes are transcribed divergently to the other *pha* genes, and encode the phasins that play both regulatory and functional roles (see later).[74]

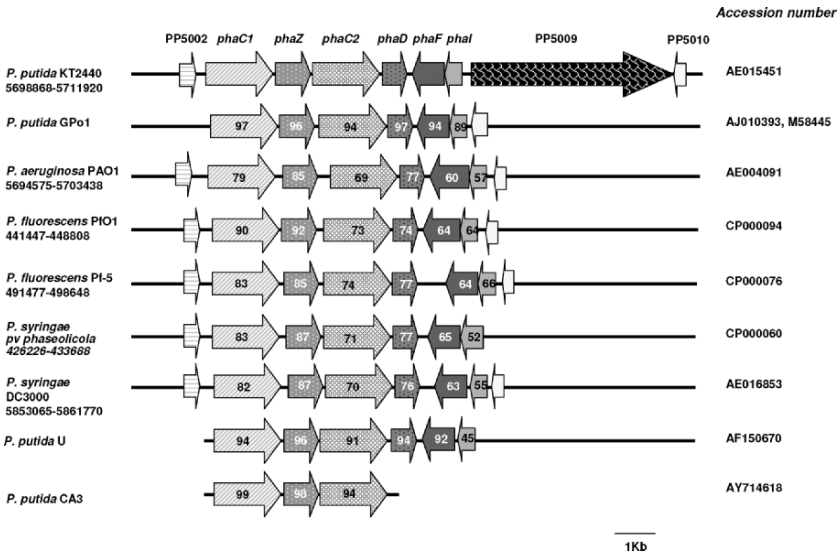

Figure 3. Comparative representation of the *pha* gene clusters and flanking regions from *P. putida* KT2440, *P. putida* GPo1, *P. aeruginosa* PAO1, *P. fluorescens* PfO1, *P. fluorescens* Pf-5, *P. syringae* pv *phaseolicola*, *P. syringae* DC3000, *P. putida* U and *P. putida* CA3. Names of the genes and of the function-unknown open reading frames (ORFs) are indicated at the top of the figure. PP5002, PP5009, and PP5010 are ORFs not directly related to PHA biosynthesis. PP5009 is only present in *P. putida* KT2440 genome. Arrows indicate the different genes, their relative sizes, and the transcriptional direction. Numbers inside the genes represent the percentage of protein identity of their respective products with respect to that of *P. putida* KT2440 strain. When complete genomes are provided, coordinates are shown under the name of the strain. GenBank accession numbers are indicated on the right of the figure. The *phaC1* and *phaC2* genes encode two synthases and are separated by the *phaZ* gene that encodes an intracellular depolymerase. *phaD* gene encodes a transcriptional regulator. *phaF* and *phaI* genes code for phasins.

Some particular strains of *Pseudomonas* are able to synthesize PHB and a heteropolymer containing a mixture of monomers.[55] A first example of such a double synthesis process was provided by introducing the PHB synthesis genes into *P. putida* GPo1, and establishing conditions which allowed the simultaneous production of both scl-PHA and mcl-PHA.[72] A striking result of these experiments was that although blends appeared to be formed, there was no PHA blending within individual granules. Two distinct granules coexisted in single cells: scl-PHA granules that contained more than 98% hydroxybutyrate monomers and mcl-PHA granules that contained 12% hydroxyhexanoate and 88% hydroxyoctanoate monomers, with less than 1% hydroxybutyrate monomers. Thus, although individual cells produced two distinct PHA synthesis machines capable of producing

scl-PHA and mcl-PHA, the two polymers essentially never formed a single granule. Clearly, the two polymers do not mix easily, not only *in vitro*, but also *in vivo*.[72]

Another interesting example, involves a native strain, *Pseudomonas* sp. 61-3, which is able to simultaneously produce PHB and poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoates) (P(HB-*co*-HA)). In this strain also, two different synthesis machines encoded by the *phb* and *pha* genes, respectively are involved in PHB and P(HB-*co*-HA) biosynthesis.[58] The genes encoding the enzymes β-ketothiolase ($PhbA_{Ps}$), acetoacetyl-CoA reductase ($PhbB_{Ps}$), PHB synthase ($PhbC_{Ps}$), and the putative transcriptional activator ($PhbR_{Ps}$) are located in the *phb* cluster. The two PHA synthases ($PhaC1_{Ps}$ and $PhaC2_{Ps}$) and the intracellular PHA depolymerase ($PhaZ_{Ps}$) are similar to those reported in other pseudomonads.[58] Here also, freeze-fracture electron microscopy studies revealed that the two types of PHAs are stored in separate inclusions in the same cell.[56]

4. SYNTHASES

4.1. The Key Enzymes for Polyester Biosynthesis

PHA synthases, also referred to as PHA polymerases, are the key enzymes for the biosynthesis of these polyesters that transform the soluble monomeric substrates (*R*)-hydroxyacyl-CoA thioesters into an insoluble polymer with the concomitant release of CoA.[81,103] These soluble enzymes turn into amphipathic enzymes upon covalent catalysis of polyester-chain formation.[103] More than 60 PHA synthase genes have been cloned from 45 species of bacteria and broadly categorized into four different classes based on their *in vivo* substrate specificities, primary amino acid (aa) sequences, and subunit composition.[81] As a general characteristic, class I (e.g., *R. eutropha*), class III (e.g., *Chromatium vinosum*), and class IV (e.g., *B. megaterium*) PHA synthases are preferentially active towards (*R*)-3-hydroxyacyl-CoAs containing three to five carbon atom acyl chains. Class II PHA synthases (PhaC1 and PhaC2) are mainly found in pseudomonads and preferentially use medium chain length 3-hydroxyalkanoates containing 6 to 14 carbons as substrates.[23,35,36,106,107] However, there are some exceptions. Thus, *Pseudomonas* sp. 61-3 contains PhaC1 and PhaC2 variants that are very similar to the class II PHA synthases but that exhibit a broader substrate range, being able to polymerize both medium and short chain length monomers.[58] Moreover, this strain also contains a class I PHA synthase ($PhbC_{Ps}$) that polymerizes short chain length 3-hydroxyalkanoates.[58]

Although the crystal structure of a PHA synthase has not been reported, a threading model of the *P. aeruginosa* mcl-PHA synthase

PhaC1 was developed based on mutational analysis and on the homology to some members of the α/β-hydrolase superfamily. The serine that is known to be involved in covalent catalysis and that acts as a catalytic nucleophile in lipases is replaced by a cysteine in synthases. In this threading model, residues Cys-296, Asp-452, and His-453 appear to form part of the catalytic center of class II PHA synthases.[3,81]

Class I and class II PHA synthases share less than 40% amino acid sequence identity and the residues involved in substrate specificity could not be identified by sequence comparison analysis. As an alternative, a rational protein evolution process, based on known three-dimensional models, has been followed to suggest changes that might alter the enzyme specificity.[4] The substrate specificity of the *P. putida* GPo1 PhaC1 was successfully altered by semirandom mutagenesis in conserved regions. Four PhaC1 synthases were created which exhibited a drastic increase in their ability to incorporate short chain length monomers into PHA.[97] Mutations based on an *in vitro* evolutionary engineering approach that enhanced the activity and altered the substrate specificity of the *Pseudomonas* sp. 61-3 synthase have also been reported.[57]

Although PHB synthases have been studied and characterized in detail *in vitro*,[24,103] much remains to be learned about mcl-PHA synthases. Thus, the relationship between polymerase levels, polymerase activity, and PHA accumulation has not yet been established. Recently, Ren *et al*. provided some data for synthase levels and activities in *P. putida* GPo1 and *E. coli* and concluded that optimal activity of the PHA synthase requires still undefined factors which are available in *P. putida* but not in *E. coli*.[85]

A coupled enzymatic system involving the participation of a commercially available acyl-CoA synthetase and PHA synthase was used for the *in vitro* synthesis of mcl-PHA.[78] These experiments showed that the synthesis of PHA granules require neither a previously synthesized PHA polymer nor phasins (see later).[78]

4.2. Granule Formation

PHA synthases are not only involved in the biosynthesis of PHA but they also play an important role in granule formation.[80]

Two models of PHA granule formation have been described in bacteria: (i) the micelle model and (ii) the budding model (Figure 4). In the micelle model, a self-assembly process is initiated resulting in the formation of insoluble cytoplasmic inclusions with a phospholipid monolayer which contains covalently attached polyester synthases at the surface.[80] The micelles are expanded and appear as intracellular granular structures that can be easily observed in phase-contrast microscope preparations. This model is supported by the fact that PHA granules can be formed

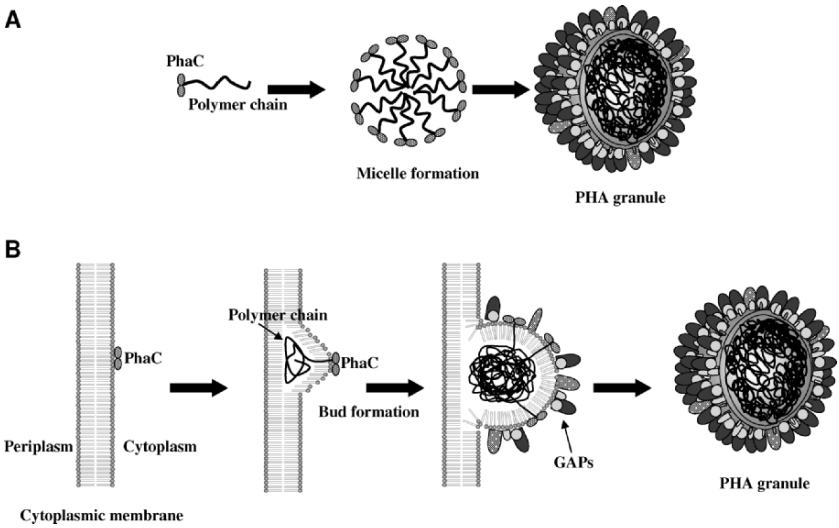

Figure 4. Models for granule formation. Two models of PHA granule formation have been proposed: (a) the micelle model and (b) the budding model. (a) In the micelle model, soluble enzyme converts monomer-CoA to oligomers, which remain enzyme-bound. At a critical oligomer length and enzyme–oligomer concentration, the enzyme–oligomer complexes form micelles with the enzyme located at the interface, separating the PHA from the cytoplasm. The hydrophobic polymer can then be extruded into a hydrophobic environment. (b) In the budding model, the PHA polymerase is attached to the cytoplasmic membrane. The oligomers produced remain also enzyme-bound, but the hydrophobic environment in which the polymer grows is the space between the phospholipid monolayers of the cytoplasmic membrane.

in vitro in the absence of membranes.[24,55,78] However, electron microscopy studies have shown the existence of membrane-like material surrounding PHA granules,[105] providing experimental support for the budding model. Recent fluorescence microscopy studies employing green fluorescent protein (GFP)-labeled polyester synthase enabled *in vivo* monitoring of PHA granule formation, as well as establishing its subcellular localization.[67] In these studies, GFP was fused to the N terminus of class I and class II PHA synthases, respectively, without affecting PHA particle formation. Early stage granules were found to be localized at the cell poles, suggesting that granule formation starts at this position according to the budding model. In cell division impaired *E. coli* strain, localization of granule formation was found to be dependent on nucleoid structure suggesting that nucleoid occlusion occurred.[67] In summary, the *in vivo* studies using GFP-labeled PHA synthase from *P. aeruginosa* PAO1 supported the budding model by localizing granule formation close to the cytoplasmic membrane at the cell poles.[67] These results are in agreement with those obtained for *R. eutropha*.[37]

5. DEPOLYMERIZATION

When bacteria are cultured under carbon limitation, the accumulated PHA can be biodegraded to the monomers which are reutilized by the cells as carbon and energy sources.[54,55] PHA degradation is performed in bacteria by at least two different pathways (Figure 5). One involves an intracellular degradation process, whereas the other is carried out extracellularly.[38] When intracellular degradation occurs, the accumulated PHA is hydrolyzed when the producer microorganism requires a carbon source. In this case, the breakdown of the polymer is carried out by intracellular depolymerases which seem to be anchored to the PHA granule.[21,38]

In extracellular degradation, exogenous PHA is utilized as a carbon and energy source. The source of this extracellular polymer is the PHA released by PHA-accumulating microorganisms that cease dividing and undergo lysis. These PHA granules are spread into the environment and hydrolyzed by secreted enzymes into water-soluble oligomers and monomers. The ability to degrade extracellular scl-PHA is widespread among bacteria. Thus, many extracellular scl-PHA depolymerases have been characterized in depth over the last decade and more than 20 genes have been identified.[38]

Extracellular mcl-PHA degrader microorganisms are less frequently found in the environment. The enzyme of *Pseudomonas fluorescens* GK13 is the prototypical extracellular mcl-PHA depolymerase (Figure 5).[92,93,94] This protein, from here on referred to as poly-3-hydroxyoctanoate (PHO) depolymerase, was shown to hydrolyze PHO and poly(3-hydroxydecanoate-*co*-3-hydroxyoctanoate) whereas it was unable to degrade PHB or poly-3-hydroxyvalerate (PHV). Furthermore, this enzyme hydrolyzes PHO even if the polymer contains unsaturated monomers or when it is cross-linked by physical modification.[12] This depolymerase seems to be mcl-PHA-specific although it also degrades soluble esters (such as *p*-nitrophenylacylesters) with six or more carbon atoms in the acyl moiety.[94] The dimeric ester of 3-hydroxyoctanoic acid was identified as the main product of enzymatic hydrolysis of PHO. The gene encoding this depolymerase ($phaZ_{Pfi}$), comprises 837 bp and is transcribed as a monocistronic mRNA of about 950 bp from a putative sigma 70-like promoter 32 bp upstream of the ATG start codon. The deduced protein (278 amino acids) reveals the existence of a leader peptide at its N terminus. When expressed in *E. coli*, the mature depolymerase starts with Ala-23, whereas the mature enzyme purified from *P. fluorescens* GK13 starts with either Leu-34 or Arg-35 resulting in proteins of 26,687 or 26,573 Da, respectively. The primary structure revealed it to be a strongly hydrophobic protein, not similar to scl-PHA extracellular depolymerases except for a small region in the neighborhood of a lipase-box at positions 170–174 (GISSG), and the presence of two

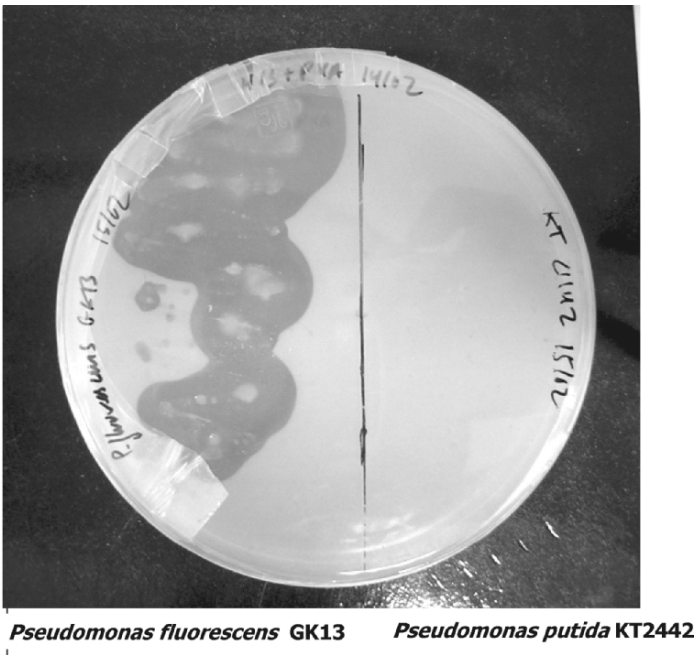

Figure 5. Comparative growth of *P. putida* KT2442 and *P. fluorescens* GK13 on mcl-PHA as the sole source of carbon. *P. fluorescens* GK13 (*left*) is able to use external mcl-PHA as the sole source of carbon when the polymer is included in solid agar media due to an extracellular depolymerase activity. In contrast, *P. putida* KT2442 (*right*), cannot grow because it produces an intracellular PHA depolymerase. The appearance of transparent-clearing zones around the growing bacteria is a typical characteristic of the strains that secrete extracellular depolymerases that hydrolyze PHA to water-soluble products.

well-conserved residues (Asp-228 and His-260). Site-directed mutagenesis confirmed these amino acids to be essential for activity,[38] probably constituting a catalytic triad typical of the α / β-hydrolase superfamily.[6] The three catalytic triad amino acids (Ser-172, Asp-228, His-260) are located in the C-terminal region of the protein, different from all known scl-PHA depolymerases. It has been suggested that the N terminus of the protein constitutes a polymer-binding site.[92,93] This idea is supported by the fact that PCR-induced mutagenesis of the PHO depolymerase gene led to a collection of mutants in the N-terminal region that are unable to depolymerize PHA but are still able to hydrolyze esters of *p*-nitrophenol.[38]

In contrast to extracellular PHA depolymerization, intracellular degradation of previously accumulated PHA is poorly understood. However, during the past 5 years, analysis of the genomes of *R. eutropha* (strain H16) and other PHB producer strains as well as the cloning and

characterization of several PHB depolymerases and oligomer hydrolases, revealed that PHB hydrolysis is a very complex process.[1,26,44,45,70,88,122]

Regarding intracellular mcl-PHA degradation, the only example described so far is that of the PHA mobilization in the genus *Pseudomonas*. A first mcl-PHA depolymerase was found to be encoded by a gene (*phaZ*), located between the two mcl-PHA synthases (*phaC1* and *phaC2*) of *P. putida* GPo1. *phaZ* was found to show little or no homology to known sequences, except for a decapeptide that contained the typical lipase fingerprint Gx_1Sx_2G (VNV**IGVSWG**G) consistent with the notion that the protein is in fact a lipase.[36] At the time there were no examples of lipases with V and W in the variable x_1 and x_2 positions, but this has meanwhile changed with the emergence of Family V bacterial lipolytic enzymes, characterized by a (GVSWG) lipase box, clearly placing the mcl-PHA depolymerase in this group.[6] It has also been suggested that the active center of the enzyme might be related to that of serine esterases, based on the fact that self-hydrolysis of mcl-PHA granules in a strain of *P. oleovorans* was blocked by serine esterase inhibitors.[21]

The likely role of the mcl-PHA depolymerase in intracellular PHA degradation was initially suggested by the isolation of a *P. putida* GPo1 mutant (GPp500) which accumulated mcl-PHA, but was unable to degrade the polymer in the stationary phase, while such degradation was always seen in the parent strain. Evidently the mutant carried a defective depolymerase.[36] Similar conclusions were drawn for related strains such as *P. putida* U by mutagenesis and complementation techniques,[23,89] with the general conclusion that mutants in *phaZ* were defective in mcl-PHA degradation and, therefore, in mcl-PHA mobilization. Very recently, PhaZ has been purified and biochemically characterized as a true intracellular mcl-PHA depolymerase ascribing, definitely, this activity to the *phaZ* product (de Eugenio et al. doi:10.1074/jbc.M608119200).

6. THE PHOSPHOLIPID–PROTEIN ENVELOPE AND THE PHASINS

There has been much discussion on possible functions of the phospholipid–protein envelope around the presumably naked PHA mass. The simplest interpretation is that it is difficult to imagine a naked and highly hydrophobic granule existing in the bacterial cytoplasm without attracting hydrophobic lipids, proteins, and possibly other molecules with apolar surfaces or residues. The binding of amphipathic compounds would increase the apparent solubility of the PHA granule in the cytoplasm, so that it is not surprising that such granules are proposed to be enveloped by a lipid monolayer,[73] with hydrophobic fatty acid tails bound to the PHA surface and

hydrophilic lipid head groups facing into the cytoplasm. A lipid monolayer interspersed with protein around the PHA granules would reduce direct contact of PHAs with water, preventing the transition of the polyester from the amorphous liquid state to a more stable crystalline form.[38,73] Moreover, it has been suggested that the phospholipid coat acts as a protective barrier preventing cellular damage that would be caused by the interaction of PHAs with internal structures or with cytosolic proteins.[101,104]

Phasins have been identified in several microorganisms and are thought to fulfill a similar function to that of oleosins in pollen and seed plants.[52,59,69,74,91,117] These proteins generate an interphase between the cytoplasm and the hydrophobic core of PHA granules.[101] They are widespread among bacteria, sharing similar functions but differing in their primary structures. It has been proposed that phasins consist of a hydrophobic domain which associates with the surface of the PHA granules and a hydrophilic domain which is exposed to the cytoplasm.[69] The amphiphilic layer stabilizes the PHA granules and prevents them from coalescing.[70,71] Other functions proposed for phasins are: to control the number and surface of granules, to protect the host cell by contributing to coverage of the hydrophobic surface of the polymer, to prevent protein misfolding on the hydrophobic granule,[70,71] or to serve as a storage source of nitrogen.[59]

P. putida strains contain two phasins of 15 kDa (PhaI) and 26 kDa (PhaF) that are conserved in all mcl-PHA producers[74] (Figure 6). PhaF is a modular bifunctional protein which behaves as a structural protein and also as a transcriptional regulator[74] (Figure 6). The N-terminal region (142 aa) presents a 58.6% similarity with the complete PhaI phasin (139 aa) and it works as a functional domain (BioF domain, see later) able to bind PHA granules[61] and acting in harmony with PhaI as a major structural protein (Figure 1). The transcriptional regulatory function has been ascribed to its C-terminal domain (see Section 7). The hydropathy plot of BioF shows a clear hydrophobic region of nine residues (IWLAGLGIY) located in the first third of the BioF domain at amino acid positions 25 to 33. Remarkably, this region is conserved in all PhaF-like proteins (Figure 6), suggesting that the motif plays a fundamental role in the interaction with the PHA granule. However, the detailed molecular mechanism that controls the interaction of phasins with the phospholipid envelope is still unknown.[61]

Mutant strains lacking phasins usually produce less amount of PHA than their parental strains (leaky phenotype) showing in addition that PHA granules altered in their number and size.[71,117] It has been reported that the absence of PhaF affected neither the PHA content nor the granule formation of *P. putida* GPo1 grown in nitrogen-limited batch cultures. However, in nitrogen-limited continuous cultures, the PHA content of these mutants was reduced threefold when compared to that of the wild-type strain. In contrast to the later stages of PHA formation in batch

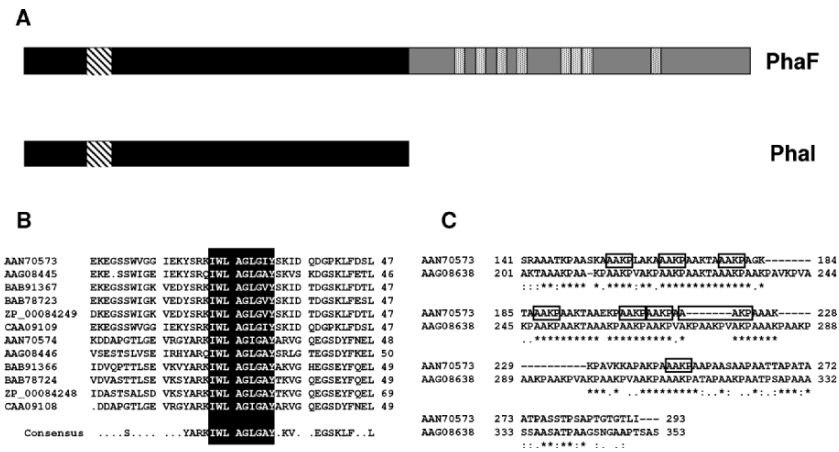

Figure 6. Comparative representation of phasins. (a) Domain architecture of the phasins PhaF and PhaI. The N-terminal domain of PhaF shows a significant similarity to the PhaI protein. Hatched (in N-terminal domain) and dotted boxes (in C-terminal domain) indicate the regions highlighted in sequences of panels (b) (black shading) and (c) (white boxes), respectively. (b) Multiple sequence alignment of N-terminal regions of PhaF and PhaI proteins in *Pseudomonas* strains. The numbers at the right indicate the position of the residues in the complete amino acid sequence of the protein. A consensus sequence was deduced for positions at which the residues were identical in at least 7 of the 12 compared sequences. Black shading indicates the hydrophobic region that is conserved in all PhaF-like proteins (PhaF and PhaI). The accession numbers correspond to PhaF-like proteins from the following microorganisms: AAN70573, *P. putida* KT2440; AAG08445, *P. aeruginosa* PA01; BAB91367, *Pseudomonas* sp. strain 61-3; BAB78723, *P. chlororaphis*; ZP_00084249, *P. fluorescens*; CAA09109, *P. putida* GPo1; AAN70574, *P. putida* KT2440; AAG08446, *P. aeruginosa* PA01; BAB91366, *Pseudomonas* sp. strain 61-3; BAB78724, *P. chlororaphis*; ZP_00084248, *P. fluorescens*; CAA09108, *P. putida* GPo1. (c) Multiple sequence alignment of the C-terminal regions of PhaF (AAN70573) protein from *P. putida* KT2440 and AlgP (AAG08638) protein from *P. aeruginosa* PAO1. The numbers at the right indicate the position of the residues in the complete amino acid sequence of the protein. White boxes indicate the AAKP repetitions in the PhaF protein.

fermentation, in continuous cultures cells are capable of dividing while PHA is also formed. How granules are generated in newly formed cells under these conditions is still an open question.[74]

Very recently, Luengo and coworkers showed that the deletion of the *phaF* gene in *P. putida* U has no affect on the synthesis of aliphatic PHAs but, surprisingly, prevents the formation of aromatic polyesters.[89]

P. putida BMO1 double mutants in the genes encoding PhaF (GA2) and PhaI (GA1) or a mutant in PhaI alone exhibited a PHA leaky phenotype and the composition of the accumulated PHA was shifted towards hydroxyacids of 12 carbon atoms.[110] The double or single mutants did not exhibit any significant differences in their phenotypes, which may indicate

that the PHA leaky phenotype is due to the lack of PhaI or due to a disturbance in the arrangement of the PHA inclusion boundary. The changes in polymer composition could represent changes in metabolite pools. In contrast to the phasin mutant of *R. eutropha* (*phaP*$^{-}$),[117] no alterations of the PHA granule size was found in *P. putida* mutant strains. However, both *phaP*$^{-}$ *R. eutropha* as well as *phaI P. putida* mutants accumulated less PHA than did the wild-type strains.[110,117]

In the case of *Pseudomonas* sp. 61-3, which produces two types of polyester inclusions, PHB and (P(HB-*co*-HA), PhaF and PhaI were present only in P(HB-*co*-HA) inclusions but not in PHB granules. Separation of proteins associated with the PHB granules showed the presence of two major bands corresponding to proteins of 24 kDa (GA24) and 48 kDa. GA24, which was also found in P(HB-*co*-HA) inclusions, is not similar to other sequenced proteins. The 48 kDa protein is highly identical to the porin D precursor of *P. aeruginosa*. Whether the presence of this porin in the PHB granule is a consequence of contamination of the PHB inclusion fraction by cellular outer membrane material is still an open question.[56]

7. REGULATION OF THE PHA PRODUCTION

Regulation of PHA metabolism is very complex, since it is exerted (i) at the enzymatic level, by cofactor inhibition and availability of the metabolites, and (ii) at the transcriptional level, by specific and global transcriptional regulatory factors.[39,55,80] The regulation of PHA biosynthesis in pseudomonads has only been studied to a very limited extent. In particular, very little is known about the regulation of mcl-PHA production at the enzymatic level.[39,55] In PHB-producing bacteria such as *R. eutropha*, the intracellular concentrations of acetyl-CoA and free CoA play a central role in the regulation of polymer synthesis.[30,48,96] Furthermore, PHB synthesis is stimulated by both high intracellular concentrations of NAD(P)H and high ratios of NAD(P)H/NAD(P)[30,39] via inhibition of citrate synthase activity, facilitating the metabolic flux of acetyl-CoA to the PHB synthetic pathway. It has been shown that *P. putida* strains rely on the β-oxidation and fatty acid *de novo* biosynthetic pathways to provide the substrates for mcl-PHA synthesis by the PHA synthases (see Section 2). Therefore, it can be reasonably assumed that regulatory factors that affect the activity of the enzymes of these metabolic pathways will also be involved in the regulation of PHA biosynthesis. In fact, it is thought that the PHA synthases compete with the β-oxidation complex for substrates.[39] Similarly, when an alginate-overproducing mutant of *P. aeruginosa* that also produces PHA was cultured in gluconate, the PHA accumulation by the alginate overproducer decreased in comparison

to that of the wild type, despite the fact that there was no difference in *pha*C1 transcription in these strains, indicating that alginate biosynthesis competes with PHA biosynthesis for a common precursor of both biopolymers, most likely acetyl-CoA.[68]

Luengo and coworkers have recently examined the effects of the overexpression of different GAPs on polymer synthesis. They observed that the overproduction of PhaC1 synthase in a mutant strain of *P. putida* U, in which the whole *pha* locus had been deleted, affects both the number and size of the granules (granule numbers increased while granule size decreased) indicating that the quantity of polymerase in this bacterium could affect the number of polymerization nuclei. Alternatively, the lack of phasins led to altered granule sizes and numbers. Moreover, they also showed that the wild-type phenotype was restored by the overexpression of both PhaC1 and PhaF.[89]

The transcriptional regulatory systems of *pha* genes of *Pseudomonas* species have been scarcely studied and mainly incomplete and spread information is available for some of the best-known PHA producer strains. In this section, a detailed draw of the accumulated knowledge about this issue is presented.

As a general concept, two different levels of transcriptional regulation can be distinguished in bacterial metabolic pathways.[17] First, there are specific regulatory mechanisms that drive the expression of relevant pathway genes when a related inducer, i.e., a pathway substrate, an intermediate metabolite or a pathway product, is present. Second, there are global or superimposed regulatory systems that adjust the expression of a pathway gene cluster to the general physiological status of the cell. The latter is driven by global regulators such as alternative RNA polymerase sigma subunits.[17]

A specific transcriptional activator that belongs to the AraC/XylS family, called $PhbR_{Ps}$, was found in *Pseudomonas* sp. 61-3,[58] a strain that is capable of producing a PHB and (P(HB-*co*-HA) as discussed earlier. $PhbR_P$ is specific for the regulation of the transcription of the genes required for synthesis of the PHB homopolymer and not for the P(HB-*co*-HA) polyesters.

Although the *pha* gene organization and products are highly conserved in the mcl-PHA producer strains (Figure 3), the DNA sequences of the *pha* cluster intergenic regions differ considerably in such species.[39] Consequently, it is not surprising that transcriptional factors might trigger different effects. The gene *phaD* of the *pha* cluster (Figure 3) belongs to the TetR family of transcriptional regulators.[79] It was shown in *P. putida* GPo1 that the *phaD* product plays an important role in mcl-PHA biosynthesis in this organism.[43] A mutation of this gene affected polymer accumulation in several ways. First, mcl-PHA production was reduced to less

than 20% of that in the wild type. Second, the number of PHA granules increased. Since PhaD is not associated with the PHA granules, this effect has been ascribed to an altered expression of phasins.[43] The protein pattern of the PHA granules changed and PhaI protein was not detectable as a GAP. Very recently, it has been demonstrated that PhaD is a transcriptional regulator that specifically drives the expression of the PhaI and PhaF proteins in response to the carbon source for growth. The *phaD*-dependent expression of the phasins, PhaI and PhaF, could explain the phenotype of the *phaD* mutant.[98] In *P. putida* U, the deletion of *phaD* reduces the synthesis of PHA as well. This effect was not seen when the *phaD* mutant was transformed with a plasmid-overexpressing *phaF*, suggesting that, in this bacterium, PhaD could be involved in the regulation of *phaF* expression as a transcriptional activator.[89]

In *P. putida* GPo1, two promoters have been reported upstream of the *phaC1* gene (Pc_1 region),[36,112] with transcriptional start sites 198 and 112 bp upstream of the *phaC1* ribosomal-binding site.[112] Data from mRNA analysis showed no transcripts larger than 3 kb, suggesting that either *phaC1*, *phaZ*, *phaC2*, and *phaD* do not form part of the same transcription unit, or that an mRNA processing event is involved. Probing for *phaC1* and *phaZ* gene transcripts revealed a hybridization band corresponding to a *phaC1* transcript and an additional band corresponding to a *phaC1Z* transcription unit.[74] Consequently, *phaC1* and *phaZ* might be transcribed in the same unit, as also reported for the homologous system of *P. aeruginosa*.[106] Thus, part of the transcript stops at the end of the *phaC1* gene and part continues to the end of *phaZ*. This is in agreement with the presence of a putative transcription terminator found downstream of the *phaZ* gene.[36]

The environmental conditions that influence the expression driven by the Pc_1 promoter region in *P. putida* GPo1 include, besides nitrogen limitation, the nature of the carbon source present in the medium. This strain is an exception among the pseudomonads in that it is able to accumulate PHA from alkanes and alkanoates but unable to do so from precursors such as glucose or other carbohydrates.[55] When citric acid or glucose are used as the carbon source, Pc_1 is less active than in the presence of octanoic acid. This response could have evolved as a defense mechanism against the wasteful production of PHA-associated proteins in the absence of an appropriate substrate.[74]

Expression of *phaF* and *phaIF* transcripts is regulated differently in this strain (see later) which suggests the possible presence of two promoters, one located upstream of the *phaI* gene and the other located upstream of *phaF*. This difference in the expression of *phaF* and *phaIF* transcripts has also been described for *P. putida* KT2440 and *P. aeruginosa*.[32,33,80]

As mentioned in Section 6, PhaF is a modular bifunctional protein. The N-terminal domain behaves as a structural protein or phasin. The C-terminal domain consists of 114 aa with nine copies of the AAKP motif characteristic of histone H1-like proteins found in eukaryotes and prokaryotes[74] (Figure 6) and functions as a transcriptional regulator (see Section 6). The best-studied member of this protein family is AlgP, a protein involved in the regulation of alginate production in *P. aeruginosa*[16,60] (Figure 6). Medvedkin and coworkers demonstrated the DNA-binding ability of artificial peptides containing the AAKP motif. They proposed that this motif consists of a single α-helix turn followed by a bend imposed by the proline residue and stabilized by the high alanine and lysine content.[60] The Lys residues are readily positioned to form hydrogen bonds with DNA phosphates and proteins containing this motif form a right-handed superhelix able to bind to the major groove of B-DNA.

The Medvedkin model applies to PhaF and is compatible with a possible transcriptional regulatory function of this phasin.[74] The involvement of PhaF in the *pha* transcriptional regulatory system was first demonstrated in *P. putida* GPo1[74] (Figure 7). Disruption of the *phaF* gene leads to increased expression of *phaC1*, suggesting that *phaF* acts as a negative regulator of *phaC1* expression in this strain. A model as shown in Figure 7 was proposed for this strain as a result of the *pha* cluster mRNA analysis. The *phaF* gene can be transcribed to generate two different mRNAs, one containing only *phaF* and the other containing both *phaI* and *phaF* genes. The *phaF* transcript can be observed in the wild-type strain even when glucose or citrate, which cannot be used as PHA precursors by this strain, is used as growth substrate. However, expression of the *phaIF* transcript appears to be dependent on the presence of fatty acids such as octanoic acid (PHA precursors) in the culture medium. Under these growth conditions, *phaIF* transcript expression is also stimulated by the PhaD regulator.[98] This regulatory system implies a permanent presence of PhaF in the cells (Figure 7). When either glucose or citrate is supplied as the carbon source, PhaF is not attached to granules simply because they are never generated from such substrates in *P. putida* GPo1. Under those conditions, PhaF could bind to DNA, turning off the expression of *phaC1* (or *phaC1Z*) and *phaIF* (or *phaD*) transcription units (Figure 7). Although the DNA-binding ability of peptides containing AAKP repeats has been sufficiently demonstrated,[60] there is no evidence pointing to direct binding of PhaF to the DNA promoter regions of *phaC1* or *phaIF*. Moreover, an indirect regulatory effect of PhaF on the expression of the *pha* genes by driving the effect of other still indeterminate transcriptional factors cannot be excluded. In the presence of octanoic acid, PhaF is attached to the granule and the transcription rates of *phaC1*, *phaI*, and *phaF* increase significantly leading to the formation of new granules (Figure 7). The question of whether PHA

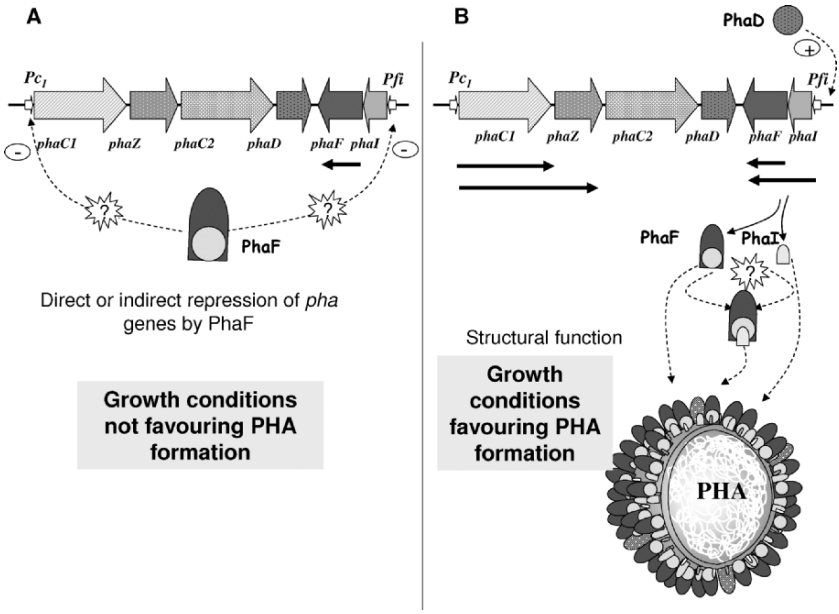

Figure 7. Proposed model for the regulation of the PHA synthesis in *P. putida* GPo1. Filled arrows indicate the direction of gene transcription. The *phaC1*, *phaF*, and *phaIF* transcripts are marked as black arrows. Discontinuous arrows means unknown mechanisms. Modular composition of PhaF is indicated as dark (bullet) and light (circle) gray parts. (a) Repression of the expression of the *phaC1* gene and the *phaIF* operon when *P. putida* GPo1 is cultured in medium containing citrate or glucose as the carbon source. Under these growth conditions, *phaF* is transcribed and it exerts a negative regulatory function in gene transcription. (b) Induction of the expression of *phaC1*, *phaI*, and *phaF* genes in the presence of octanoic acid and PhaF association with the PHA granule. Under PHA-producing conditions, PhaF protein binds to nascent granules (with or without PhaI proteins) and transcription from *Pc1* and *Pfi* promoters occurs. PhaD has been proposed as an activator of the *Pfi* promoter.

granules, octanoic acid, or even PhaI protein play inducer roles and change the conformation of PhaF to a form that binds to the granule awaits further research.[74]

In *P. putida* KT2440, the expression of *phaF* and *phaIF* transcripts are regulated differently as well. Although PHA accumulation was RpoN-independent in this strain, it has been demonstrated that the transcription of the *phaF* gene is driven by this global regulator. RpoN controls the expression of the *phaF* transcript according to the nitrogen availability in the culture medium, while *phaFI* expression is nitrogen-dependent but RpoN-independent. This is in agreement with a putative RpoN-dependent promoter detected upstream of the *phaF* gene. An additional putative

RpoN-dependent promoter was proposed to control the expression of the *phaC1* gene. However, experimental data supporting this assumption has not been published and the *phaC1* gene is transcribed independently of RpoN or nitrogen availability. In this strain, PhaF works as a repressor of *phaC1* and *phaIF* transcripts as has been proposed for *P. putida* GPo1.[32,33,80]

There are at least two described promoters involved in the expression of the *pha* genes in *P. aeruginosa*. Both promoters are upstream of the *phaC1* gene and resemble the consensus sequences for RpoN- and RpoD-dependent promoters. It is not clear whether a third promoter upstream of the *phaC2* gene in *P. aeruginosa* is active.[106]

In *P. aeruginosa* PAO1, PHA accumulation from gluconate requires a functional RpoN sigma factor, whereas PHA accumulation when cells were grown on fatty acids was only reduced in the absence of RpoN.[106] This effect has been explained by the RpoN-dependent expression of the *phaG* gene encoding the transacylase[32,33,80] (Figure 2).

Unlike in other bacteria, the *pha* locus in *P. putida* U seems to be integrated by six different functional units (*phaC1*, *phaZ*, *phaC2*, *phaD*, *phaI,* and *phaF*).[89]

A gene encoding a putative global regulatory protein was identified in *P. putida* KT2442. This putative regulator is similar to the sensor component of the two component regulatory systems GacS/GacA,[39,55] containing a "histidine protein kinase" and "response regulator" domain. In accordance with these data, it has been described that a GacS transmembrane sensor kinase homologue regulates the alginate and PHB polymer production in *Azotobacter vinelandii*.[10] Finally, the involvement of the RpoS alternative sigma factor in the PHA depolymerization regulatory system has been proposed in *P. putida* GPo1, suggesting that a very complex regulatory system affects the PHA cycle and pointing to a possible role for this polymer in global regulatory networks related to stress tolerance.[87]

8. BIOTECHNOLOGICAL APPLICATIONS

Environmental pollution caused by synthetic polymer wastes has been recognized as a large problem because those compounds, derived from fossil fuels, are extremely stable, and do not really enter into the degradation cycle of the biosphere. In contrast, PHAs can be obtained from renewable resources, explaining, at least in part, why these biodegradable and recyclable thermoplastic polymers have been extensively studied for the past three decades.[118] The specific properties needed for applications of bioplastics have been widely discussed and reviewed.[51,54,55,118,123]

8.1. Historical Perspectives of the Industrial Synthesis of PHAs

PHB was first described 80 years ago,[50] but despite several reports on its occurrence, metabolism, and production in various organisms from the 1950s onwards,[31] PHB was essentially forgotten for nearly half a century. Industrial interest in PHB and later in P(HB-*co*-HV) (a copolymer of PHB and PHV) developed in the 1970s, mostly because of newly available industrial fermentation plants for the production of materials.

This development was driven by an enormous interest in single cell protein (SCP). A worldwide food shortage, especially in the poorest countries of the developing world, was believed to be imminent,[9,95] and many industrial fermentation plants were planned and built all over the world for the production of SCP from oil, methane, or various waste products.[40,41]

As it turned out, SCP produced from oil failed to meet the expected quality criteria, the expected food shortage failed to happen, and the notion of SCP as an important protein source faded away. However, experience with SCP encouraged several companies to explore the production of other microbial materials.[42] One of these was ICI (the British Imperial Chemical Industries) which also worked on PHB and P(HB-*co*-HV),[7] and began producing Biopol in the late 1980s in quantities up to 1,000 tons per year at a price around 15 US$/kg.[49,116]

Meanwhile, starting in the 1970s, other PHAs had been seen. Wallen and Rohwedder studied PHB in sludge, and although they found mostly C4 and C5 monomers, they also reported the presence of some C6 and C7 hydroxyalkanoate monomers.[113] A decade later, with improved analytical tools, Findlay and White identified 11 different monomers in marine sediments.[20]

Production in the laboratory of PHA variants that contained mostly medium chain length monomers was first reported for *P. putida* Gpo1 grown on *n*-octane in two-liquid phase media[15] reminiscent, interestingly, of SCP production on *n*-paraffins.[41] It soon became possible to modify the composition of these materials at will,[8,47] which led to a greatly increased number of biopolymer variants and considerable enthusiasm about possible applications.[118]

Despite all of this progress in the laboratory, Biopol was produced on a scale sufficient for the development and testing of applications, but not for bulk-scale products. Even this modest start did not last long. In 1993, ICI and its new Zeneca unit demerged. The fledgling Biopol activities followed Zeneca, but with Zeneca concentrating on pharmaceuticals and shaping itself for a future merger with Astra, the biopolymer activities no longer fit and were sold to Monsanto in 1996.[7] Monsanto, which saw great potential in producing P(HB-*co*-HV) in plants, underestimated the

difficulties of achieving this, and abandoned its PHA activities in 1998, selling what was left of its PHA project to Metabolix.

The industrial birth of PHB was missed, to say the least. However, although the Biopol that was produced by ICI, Zeneca, and Monsanto remained too expensive for large-scale industrial applications, it was a happy resource for academic and industrial polymer scientists who studied and applied this material during the few years of its availability. Gradually, other industries, predominantly located in Brazil[1] and China[2], now entered the field. The material cost has been brought down and is said to hover around 3 US$/kg P(HB-*co*-HV).

Metabolix, the only serious long-term player in the PHA industry for now, owns a considerable portion of the intellectual property related to PHA production and applications. The company has invested in high end medical applications of the material on the one hand, but is also working with Archer Daniels Midland on the production of large amounts of material from corn starch. This places Metabolix squarely at the center of major developments towards the use of biomass for new materials and chemicals.[3]

The present embryonic industrial development of PHAs is based on sporadic scientific findings from the 1920s to the 1980s. None of the research behind these findings was planned. It happened because there were scientists playing around with various microorganisms. Industrial developments were triggered mostly by experience with large-scale SCP plants, and the hopes for plant-based new materials.

8.2. Current Research Perspectives in PHA Metabolism

In recent years, the development of functionalized mcl-PHA has attracted much interest because of the ease of chemical modification of these materials.[27,28,29] Thus, tailored olefinic mcl-PHAs were produced from mixtures of octanoic acid and 10-undecenoic acid in *P. putida* GPo1[29] and different aliphatic and mixtures of aliphatic and aromatic PHAs have been produced in *P. putida* U and derived strains.[2,23,29,54,64,65,66] Substituents in the side chains of PHAs can be modified chemically, for instance by cross-linking of unsaturated bonds.[28] This variation in the length and composition of the side chains and the ability to modify their reactive substituents is the basis for the diversity of the PHA polymer family and their vast array of potential applications.[54]

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[1]See http://www.copersucar.com.br/institucional/ing/empresa/tecnologia_ historico.asp
[2]See http://www.iupac.org/publications/ci/2004/2606/cc2_010604.html
[3]See http://www.bic.searca.org/news/2006/mar/bra/29.html

PHAs are biocompatible materials that could become important in medical applications.[109,123] Currently, these polyesters are being considered as potential biomaterial for medical applications such as sutures, implants, urological stents, neural- and cardiovascular-tissue engineering, fracture fixation, treatment of narcolepsy and alcohol addiction, drug-delivery vehicles, cell microencapsulation, support of hypophyseal cells, or as precursors of molecules with antirheumatic, analgesic, radiopotentiator, chemopreventive, anthelmintic, or antitumoral properties (those containing aromatic monomers or those linked to nucleosides).[123]

Biotechnological applications based not only on the material, but on the PHA synthesis and degradation apparatus have also been described. As an example, there is growing interest in the study of the PHA hydrolysis for the development of sustainable processes for the production of chiral intermediates.[119,120,121] Since the 3-hydroxyalkanoic acids monomeric units of PHA are enantiomerically pure and of *R*-configuration,[47,55,75] they are potentially interesting starting materials for fine chemical synthesis. An *in vivo* method for the production of chiral hydroxyalkanoic acid monomers from PHA by *P. putida* strains has recently been reported.[14,86] Furthermore, overexpression of the gene encoding the depolymerase PhaZ in *P. putida* U prevents the accumulation of these polymers as storage granules.[90] A genetically engineered strain of *P. putida* U (Δ*fadBA–phaZ*) was designed to efficiently bioconvert (more than 80%) different *n*-phenylalkanoic acids into their 3-hydroxyderivatives, which are excreted into the culture broth.[90] Very recently, an *in vitro* hydrolytic method for the production of chiral hydroxyalkanoic acid monomers based on the use of PhaZ from *P. putida* KT2442 has been developed (de Eugenio et al. doi:10.1074/jbc.M608119200).

The use of the PHA-binding domain of PhaF as a polypeptide tag (BioF) capable of binding a BioF-fused protein to bioplastic granules has recently been described.[61] This method can be used to selectively immobilize recombinant proteins on mcl-PHAs simultaneously with their biosynthesis in the bacterial cell. PHA-immobilized enzymes can be isolated by a simple centrifugation step, and a highly purified soluble protein can be released from the support by a mild detergent treatment (Figure 8). The usefulness of these properties has been demonstrated by constructing different chimeric fully functional proteins.[61] Furthermore, this tool has been established as an environmentally friendly way to deliver active proteins to the environment such as a Cry1Ab derivative insecticide.[62]

Conversion of polystyrene to PHA has been reported as an approach for recycling petrochemical products. The procedure involves the pyrolysis of polystyrene to styrene oil, followed by the material conversion of the styrene oil to PHA by *P. putida* CA-3.[115]

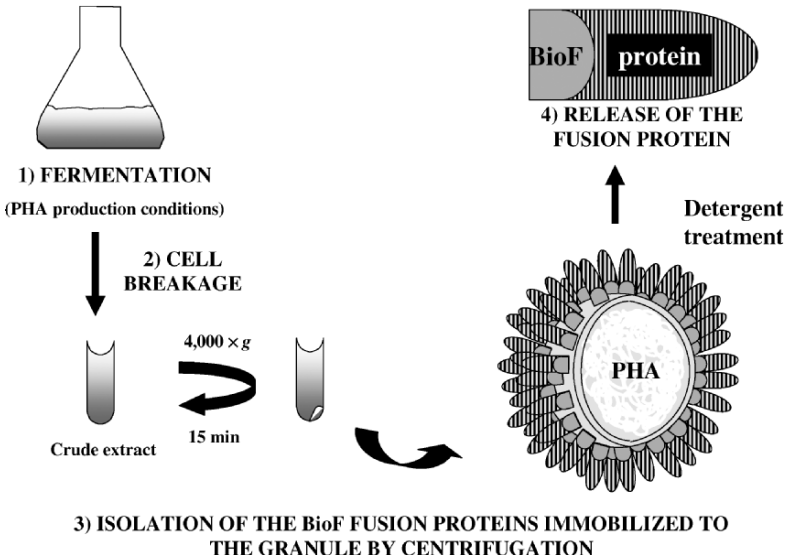

Figure 8. Schematic protocol for the immobilization and purification of proteins fused to PHA. The N-terminal domain of the PhaF phasin from *P. putida* GPo1 was used as a polypeptide tag (BioF) to anchor fusion proteins to PHAs. (1) A *P. putida* recombinant strain producing a BioF fusion protein is fermented under PHA production and protein induction growth conditions. (2) Cells are harvested and broken by a fourfold French Press passage. (3) The soluble fraction is discarded after a brief centrifugation of the crude extract. Precipitated PHA granules contain BioF fusion proteins associated to their surfaces. (4) Fusion proteins can be released from the PHA granules with a mild detergent treatment.

The fact that PHAs nicely fit the environmental ambitions of the 1980s and 1990s did not lead to significant markets for the material. It is only now, after another 20 years of much work on material variations and properties, reduced production costs, and most important, the increasing cost of oil, that a sustainable PHA market might at last develop.

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15

QUORUM-QUENCHING ACYLASES IN *PSEUDOMONAS AERUGINOSA*

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1. INTRODUCTION

The β-lactam acylases, mostly found by screening samples from natural sources, represent a unique family of heterodimeric N-terminal nucleophile hydrolases. Interestingly, most of the strains found producing β-lactam acylases are *Pseudomonas* species. We have shown that these enzymes show high selectivity towards the acid side chain, but are far more promiscuous with regard to the amine moiety of the substrate. It is therefore highly unlikely that the industrially relevant deacylation of β-lactam compounds has evolved specifically in nature. Interestingly, no less than four putative acylases of the N-terminal nucleophile family have been identified in the *Pseudomonas aeruginosa* PAO1 genome. We have investigated these four putative acylases of which one could be expressed in *Escherichia coli*. Interestingly, the enzyme was found to catalyse the hydrolysis of acyl-homoserine-lactones as reported for *Ralstonia* acylase suggesting a role in quorum quenching. In a bioassay, the purified acylase was shown to degrade

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acyl-HSL signal molecules with side chains ranging from 11 to 14 carbons at physiologically low concentrations confirming quorum-quenching activity. The discovery of quorum-quenching acyl-HSL acylases in the genome of *P. aeruginosa* POA1 may explain infection progression and offer new targets for anti-bacterial therapy.

2. NTN-HYDROLASES AS β-LACTAM ACYLASES

In search for an enzyme capable of hydrolyzing Penicillin G, in 1960 an enzyme was described which later on was used extensively for the commercial production of 6-aminopenicillanic acid (6-APA), the most important intermediate for the industrial production of semisynthetic penicillins.[1] This first enzyme was named penicillin acylase (EC 3.5.1.11) and later on numerous bacterial species have been described as penicillin G acylase-producing strains, including *E. Coli*, *Kluyvera citrophila* and *Alcaligenes faecalis*.[2,4] Recombinant DNA methods have been applied not only to increase the yields of commercially used penicillin G acylases,[5] but also to decipher the complex processing of these enzymes.[6] The penicillin G acylase of *E. coli* ATCC11105, a paradigm for this enzyme class, was found to be produced as a large precursor protein, which is transported into the periplasm and further processed to the mature protein constituting a small (α) and a large (β) subunit. Not only this heterodimeric structure, but also the role of the N-terminal serine residue as a nucleophile for the deacylation reaction is evolutionarily preserved resulting in renaming these enzymes to N-terminal nucleophile (NTN) hydrolases[7] comprising the much larger family of β-lactam acylases.

Whereas the conversion of penicillin G requires an enzyme with specificity for the aromatic phenyl acetate side chain, the processing of the second largest β-lactam fermentation product, cephalosporin-C, would require the cleavage of aminoadipyl, an aliphatic side chain, from the β-lactam nucleus. Since no enzyme capable of performing a one-step deacylation was found,[8] a two enzyme-mediated reaction has been introduced to produce 7-aminocephalosporanic acid (ACA). In this process, D-amino acid oxidase converts amino-adipyl into glutaryl and a glutaryl acylase performs the enzymatic deacylation to cephalosporin-C. The glutaryl acylases (EC 3.5.1.-) can be obtained from several *Pseudomonas* species.[8–13]

Interestingly, the specificity of the β-lactam acylases is mainly directed towards the acyl side chain and the enzyme is not selective to the β-lactam moiety at all. Penicillin G acylase can also hydrolyze phenylacetyl-leucine,[4] and cephalosporin acylase can also hydrolyze glutaryl-leucine.[14] This has further raised the question on the natural role of NTN- hydrolases in Gram-negative bacteria as the hydrolysis of β-lactam

can not be considered as an evolutionary advantage. 6-APA en 7-ACA are for most Gram-negative bacteria more bacteriocidal as the non-hydrolyzed precursors. The discovery that one of the members of this NTN-hydrolase family can hydrolyze acyl-homoserine lactone compounds has shed some new light on the possible physiological role of these enzymes.[13,15] The presence of no less than four related genes encoding putative NTN-hydrolases in the genome of *P. aeruginosa*[16] has stimulated us to investigate the function of acylases in quorum sensing.

3. BACTERIAL ACYLASES (AND LACTONASES) AS QUORUM-SENSING INTERFERING ENZYMES

P. aeruginosa and most Gram-negative bacteria use acyl-homoserine lactones (AHLs) as signal molecules. In general, AHL consists of a homoserine lactone moiety and an acyl chain. The length and substitutions of acyl side-chains vary amongst the species. Two signal molecules, *N*-(3-oxododecanoyl)-L-homoserine lactone (3O–C_{12}-HSL) and *N*-butyryl-L-homoserine lactone (C_4-HSL) have been studied in great detail in *P. aeruginosa*. The common acyl groups of AHLs identified so far vary from 4 to 18 carbons in length; they may be saturated or unsaturated, and with or without a C-3 substitution (usually hydroxy- or oxo-).[17]

As quorum sensing has a contribution to the modulation of some target genes, including virulent factors, it is of great interest to study means of interference with quorum-sensing systems. Interruption of quorum sensing is also called quorum quenching. Since AHLs may also act directly as virulence factors in mammalian hosts (review by ref [18,19]) degradation of AHLs may be interesting not only to stop the quorum-sensing system, but also to directly reduce the virulence effects.

Quorum quenching can be accomplished in several ways (i) by blockage of AHLs production, (ii) by degradation of AHL signal molecules, or (iii) by interference with the signal receptor activation. Several enzymes have already been described to be competent to degrade the signal molecules. Lactonases inactivate AHLs by hydrolyzing the ester bond of the lactone ring of the molecules to yield acyl-homoserine. Acylases cleave the amide bond that joins the lactone moiety and acyl side-chain (Figure 1). All of the resulting breakdown products are not active as signal molecules.

Some of the enzymes degrading AHL were shown to act as quorum-quenching enzymes under physiological conditions.[13,20–22] Constitutive expression of the AHL lactonase and acylase in quorum-sensing bacteria was shown to abolish or dramatically reduce AHLs accumulation.[23–25] Additionally, degradation of AHL signal molecules by lactonases or acylases was shown to have impact on expression of quorum-sensing regulated genes.

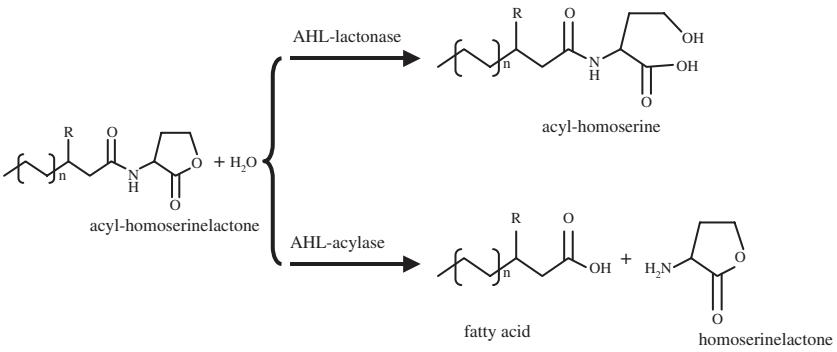


Figure 1. Degradation of homoserine lactone molecules via two alternative enzymatic systems: lactonase (*top*) and acylase (*bottom*).

Several bacteria were found to produce AHLs lactonases. AiiA of *Bacillus* sp. strain 240B1 is the first AHLs lactonase that was found.[23] Other members of genus *Bacillus* were also confirmed to produce AiiA homologues, i.e. *Bacillus cereus*,[26] *Bacillus mycoides* and *Bacillus Thuringiensis*[27] and *B. thuringiensis* subsp. *Kyushuensis*.[28] In addition, several AiiA homologues were produced by other bacteria, AhlD (*a*cyl-*h*omoserine *l*actone degradation) by *Arthrobacter* sp. and AhlK by *Klebsiella pneumoniae* KCTC2241.[29] *Agrobacterium tumefaciens* was found to produce two AiiA homologues, AttM and AiiB.[20,21]

Although lactone opening of AHLs was shown to interfere with quorum sensing, there may be a complication in practice. The breakdown product of AHL lactonase, which is a ring-opened acyl-HSL molecule, spontaneously undergoes ring formation in acidic environment (review by ref 30). Therefore, the deacylation process might be preferred, since fatty acid generated by the process is metabolized and the reaction cannot revert.[31] In addition, the other acylase-cleavage product, homoserine lactone (HSL), is a potential growth inhibitor for several bacteria. It suppresses for instance the growth of *E. coli* and *Arthrobacter* strain VAI-A[32,33] giving an additional effect against bacterial growth.

AHLs cleaved by strains producing acylases give homoserine lactone and fatty acid. The latter one is afterward metabolized. Thus, AHLs can be used as a sole carbon and energy source. *Variovorax paradoxus* has been reported to degrade 3OC6-HSL and C4-, C6-, C8, C10 and C12-HSL through deacylation process and to use these as sources of carbon, energy and nitrogen.[31] An acylase originating from *Ralstonia* strain XJ12B shows homology to a number of other acylases and N-terminal hydrolases of the NTN-hydrolase super family. This acylase, AiiD, inactivates both long- and short-chain AHLs.[25] Other acylases that showed activity towards both

short- and long-chain AHLs were produced by *Rhodococcus erythropolis* W2 and *Streptomyces* sp. strain M664. *R. erythropolis* degrades AHL through both oxidoreductase and amidolytic activities.[34] *Streptomyces* sp. produces AhlM, an AHL acylase, which exhibits broad substrate specificity, including activity on penicillin G.[22] Furthermore, the two acylases from *P. aeruginosa*, PvdQ (PA2385) and QuiP (PA1032), were shown as well to have a potential as quorum-sensing interfering enzymes. These enzymes degrade long acyl-chain-HSLs and not HSLs with short side chains.[13,15,35] PvdQ and QuiP are discussed in more detail later in the chapter.

4. ACYLASES IN THE PSEUDOMONADACEAE FAMILY

So far, five sequences of characterized AHL acylases have been deposited to the National Center for Biotechnology Information (NCBI) database. They are *Ralstonia* sp. strain XJ12B AiiD[25] (accession number AAO41113), *R. erythropolis* W2 QsdA[34] (accession number AAT06802), *P. aeruginosa* PAO1 PvdQ[13,15] (accession number NP_251075), *P. aeruginosa* PAO1 QuiP[35] (accession number NP_249723) and *Streptomyces* sp. strain M664 AhlM[22] (accession number AAT68473). Two of them belong to the Pseudomonadaceae family. To investigate the distribution of genes homologous to AHL acylase sequences amongst Pseudomonadaceae genomes, the *Ralstonia* sp. AiiD amino acid sequence was used as a query against the completed genomes of Pseudomonadaceae members available on the National Center for Biotechnology Information (NCBI) database. The reason for this is that AiiD belongs to NTN cephalosporin acylases and shows highest similarity to PvdQ and QuiP compared to other AHLs (data not reported here). Subsequently, it was used as a blast request sequence using the Blastp program (http://www3.ncbi.nlm.nih.gov/BLAST/). The alignments for all of sequences were created using the ClustalV method in MegAlign 5.07 (DNA Star, USA).

The analysis identifies at least 27 amino acid sequences distributed over Pseudomonadaceae members deduced from open reading frames (ORFs) with significant identity scores to AiiD (July 22, 2006). Many of them are deduced protein sequences with undemonstrated functions yet and classified as members of the penicillin acylase family or proteins related to penicillin acylase. Most of them have been tentatively annotated as amidases or acylases.

The sequences of the three AHL acylases of non-Pseudomonadaceae members included for reference show an identity in range of 11–39% to all of the homologues sequences. Each genome of Pseudomonadaceae members has at least two distinct AiiD homologues genes. One homologue from each

strain exhibits identity with *Ralstonia* AiiD ranging from 20 to 39%. Moreover, the conserved structural elements of these homologues show that they all belong to NTN-hydrolase cephalosporin acylase family (http://www.ncbi.nlm.nih.gov/ Structure/cdd/wrpsb.cgi). In the *P. aeruginosa* PAO1 genome, there are four AiiD homologues, including PvdQ and QuiP. Two other distinct putative AiiD homologues in *P. aeruginosa* PAO1 genome, PA1893 and PA0305, will be discussed in the next part of the chapter.

A phylogenetic analysis of these AiiA homologues shows that they fall into three clusters (Figure 2). The first cluster covers *Ralstonia* AiiD,

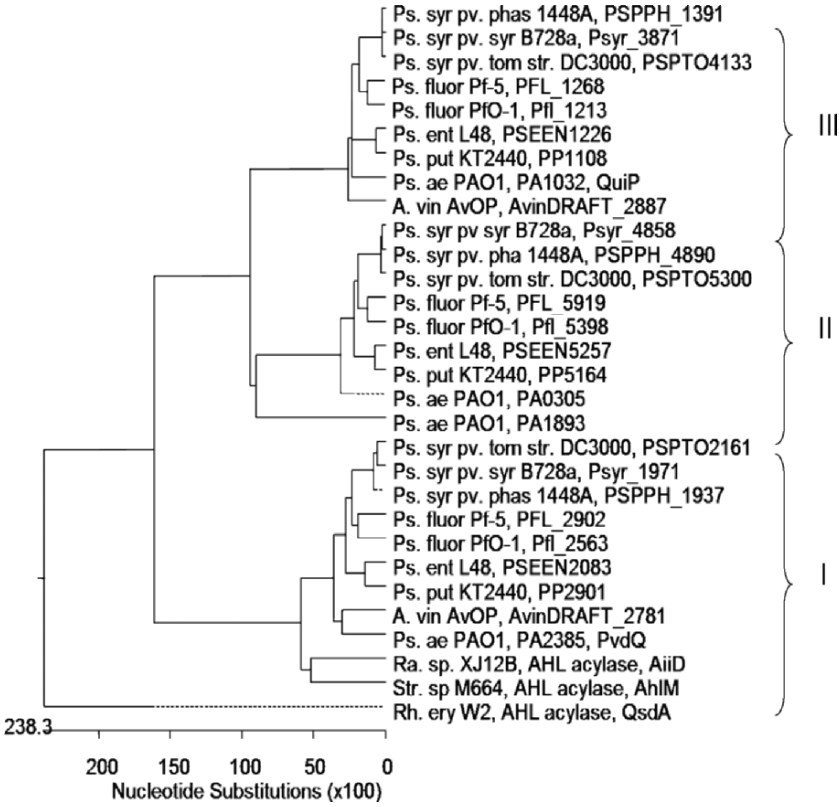


Figure 2. Phylogenetic tree of Pseudomonadaceae AHL acylases and AHL acylases homologues of known function based on the protein alignment of *Ralstonia* strain XJ12B AiiD. The phylogenetic tree was made with the DNASTAR sequence analysis software (DNASTAR Inc.). Distances are shown below the tree. The abbreviations are reported in the phylogenetic tree as follows: Ps for *Pseudomonas*, pv. for *pathovar*, syr for *syringae*, phas for *phaseolitica*, tom for *tomato*, fluor for *fluorescens*, ent for *entomophila*, put for *putida*, ae for *aeruginosa*, A vin for *Azotobacter vinelandii*, Ra sp for *Ralstonia* sp., Str sp for *Streptomyces* sp. and Rh ery for *R. erythropolis*. The numbers after the strain name refer to the locus tag.

Streptomyces AhlM and PvdQ. Moreover, a homologue of each representative strain that shows similarity with one or more of the three AHL acylases emerges in the first cluster. They are envisaged to be prospective AHL acylases.

The second and the third cluster are only distantly related to cluster 1 and they harbour a number of cephalosporin and penicillin acylases. Interestingly, QuiP which resides in cluster 3 also hydrolyzes AHLs. In conclusion, it seems that in the genome of every Pseudomonadaceae member there might be at least one potential AHL acylase.

5. ACYLASES IN *P. AERUGINOSA*

When searching for acylases homologues (NTN hydrolases) within *P. aeruginosa* PAO1 genome, one can find four candidates: *pvdQ* (PA2385), *quiP* (PA1032), PA0305 and PA1893 (see Figure 5). The identification numbers of the genes come from the *Pseudomonas* genome project (www.pseudomonas.com). The products of *pvdQ* and *quiP* genes were shown to encode acyl-homoserine lactone acylases.[13,15,35] PA0305 and PA1893 encode hypothetical proteins of unknown function (www.pseudomonas.com). The PvdQ precursor shares 37% amino acid identity with aculeacin A acylase (AAC) from the high-GC Gram-positive organism, *Actinoplanes utahensis*[36,37] and 39% with acyl-homoserine lactone acylase AiiD from *Ralstonia* XJ12B.[25] PvdQ and PA1893 precursors also share a significant match (25%) at the peptide level with *Pseudomonas* sp. SY-77 glutaryl acylase SY-77,[14] for PA0305 it is 22% and for QuiP it is 20%. Moreover, QuiP and PA0305 share 27–26% amino acid identity with cephalosporin acylase acyII from *Pseudomonas* sp. SE83[9] and 22–21% with aculeacin A acylase from *A. utahensis*.[36,37] Table 1 presents an overview of amino acids identity of *P. aeruginosa* acylases homologues with known acylases.

5.1. PvdQ (PA2385)

PvdQ is the first described N-terminal nucleophile hydrolase, with biochemical properties similar to β-lactam acylases, found in *P. aeruginosa* PAO1. Figure 3 shows the *pvdQ* gene localization on a *P. aeruginosa* chromosome. PvdQ enzyme was found to have acyl-homoserine lactone acylase activity towards long acyl-HSLs.[13,15] The earliest report about microbial acyl-HSL acylase with quorum-quenching potential can be found in a publication of Lin *et al*.[25] An identified enzyme, *aiiD*, found in *Ralstonia*

Table 1. Amino acid sequence identity of *P. aeruginosa* PAO1 acylases homologues with known acylases. Protein sequences were aligned using the SECentral (Clone Manager, version 6.00 and Align Plus 4, version 4.10, Scientific and Educational Software, Cary, NC).

Amino acid identity in %	PvdQ (PA2385)	QuiP (PA1032)	PA0305	PA1893
SY-77 glutaryl acylase *Pseudomonas* sp. SY-77	25	20	22	25
AiiD acyl-homoserine lactone acylase *Ralstonia* XJ12B	39	20	21	23
AAC aculeacin A acylase *A. utahensis*	37	22	21	23
PGA penicillin G acylase *E. coli*	18	22	24	22
Cephalosporin acylase (acyII) *Pseudomonas* sp. SE83	23	27	26	29

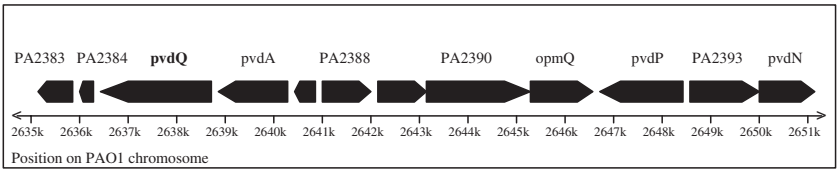


Figure 3. Schematic representation of *pvdQ* gene localization within *P. aeruginosa* chromosome.

strain XJ12B was cloned and expressed in *E. coli* and *P. aeruginosa*. The expression of *aiiD* in *P. aeruginosa* resulted in reduced elastase and pyocyanin production, and decreased ability to swarm suggesting quorum-quenching activity of AiiD.[25] This finding stimulated a search for other acylases with quorum-quenching properties. The second described bacterial acyl-homoserine lactone acylase is *pvdQ* (PA2385), found in *P. aeruginosa*.[13,15,35,38] AiiD and PvdQ share a significant amino acid identity of 39%.

The *pvdQ* gene is translated as a precursor consisting of four parts: a putative signal sequence, the α-subunit, a spacer peptide, and the β-subunit, whereas the active enzyme consists of only the α- and β-subunits of 18 and 60 kDa, respectively. PvdQ is a periplasmic protein. The β-subunit of PvdQ starts with a serine (Ser) residue at position 217(aas), which is characteristic for β-lactam acylases.[13] Figure 4 presents an alignment of PvdQ with other acylases within conserved residues region. It is known that the N-terminal residue (Ser) of β-subunit in NTN-hydrolases plays an important role in enzyme autocatalytic processing as well as in enzymatic activity. Rational randomization at this position resulted in leucine, valine, lysine, arginine or aspartic acid mutant. All the PvdQ mutants could be

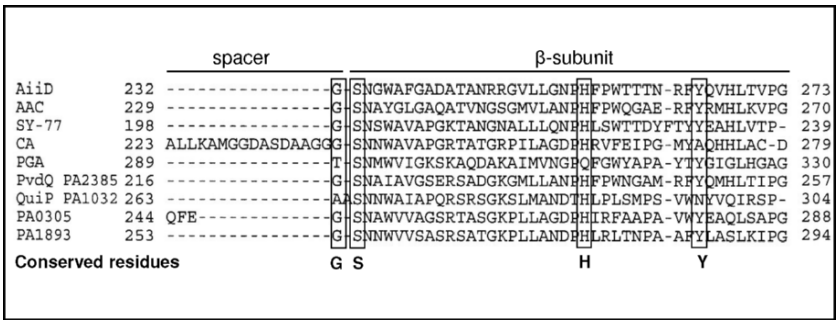


Figure 4. Sequence alignment of the conserved regions of several acylases and their homologues. Acylases homologues used in the alignment include: AiiD acyl-homoserine lactone acylase (794 aas) from *Ralstonia* strain XJ12B[25]; AAC aculeacin A acylase (786 aas) from *Actinoplanes* utahensis[36,37]; SY-77 glutaryl acylase (720 aas) from *Pseudomonas* sp. SY-77[14]; CA cephalosporin acylase (774 aas) from *Pseudomonas* sp. SE-83[9]; PGA penicillin G acylase (846 aas) from *E. coli*[6]; PvdQ(PA2385) (762 aas),[38,39] QuiP(PA1032) (847 aas),[35] PA0305 (795 aas) and PA1893 (809 aas) from *P. aeruginosa* PAO1. The boxed residues represent the important residues for the autocatalytic processing and activity of known acylases.

expressed in *E. coli* from the plasmid, as the wild-type PvdQ. However, for mutants protein processing was impaired (only precursor polypeptide was present). This result confirms the importance of 217Ser residue for PvdQ autocatalytic processing and supports the fact that PvdQ is NTN-hydrolase (Krzeslak *et al*., unpublished data).

The *pvdQ* gene lies in a pyoverdin operon.[38,39] Pyoverdin is one of *P. aeruginosa* siderophores and it is important in iron uptake. For growth, *P. aeruginosa* needs iron as an essential cofactor. The bioavailability of iron is limited by its low solubility in nature.[40,41] *P. aeruginosa* overcomes this problem by synthesizing high-affinity iron-chelating molecules, siderophores that provide the organism with iron under the most nutritionally dilute conditions.[42] *P. aeruginosa* produces two chemically unrelated siderophores, pyoverdins (PVD)[43,44] and to a limited extent pyochelin.[45,46] These siderophores function as powerful iron chelators, solubilizing and transporting iron through the bacterial membranes via specific receptor proteins at the outer membrane level.[47,49] It makes pyoverdin essential for *P. aeruginosa* growth and virulence. In general, pyoverdins consist of three distinct structural parts, a dihydroxyquinoline chromophore responsible for their fluorescence and colour, a peptide chain comprising 7–8 amino acids bound to the carboxyl group of the chromophore, and a small dicarboxylic acid connected via an amide bond to the amino group of the chromophore.[50] Pyoverdins of a single strain have the same peptide but may differ in the nature of the acyl group. The

acyl chain of pyoverdin is made of a dicarboxylic acid residue, which can be either succinate, or its amide form, or α-ketoglutarate or glutamate, depending on the producing strain or growth conditions.[51] The mechanism for formation of the pyoverdin chromophore was illustrated by Dorrestein *et al*.[52] For the biosynthesis of pyoverdin, two regions of the *P. aeruginosa* chromosome are important: *pvd* locus and *pvc* locus. The *pvc* locus (*pvcA, pvcB, pvcC* and *pvcD* genes) is involved in the synthesis of the chromophore part of pyoverdin and the *pvd* locus (*pvdQ, pvdA, fpvI, fpvR, pvdP, pvdO, pvdN, pvdM, pvdF, pvdE, fpvA, pvdIJ, pvdD, pvdH, pvdL, pvdG, pvdS, pvdY* and *pvdX* genes) in the synthesis of the peptide chain. The genes involved in the acyl chain formation are not characterized yet.[53–55] The function of *pvdQ*, *pvdP, pvdY, pvdO* and *pvdX* genes in the pyoverdin biosynthesis pathway still remains unknown. Nevertheless, it was shown that their expression occurs under iron-limiting conditions[56] in concert with the onset of pyoverdin synthesis under these conditions.

The *pvdQ* presence in the *pvd* locus suggests an involvement in the pyoverdin biosynthesis. *Lamont et al*. indicated that *pvdQ* plays a role in pyoverdin synthesis as pyoverdin is not produced in *pvdQ* negative strain.[38] The *pvdQ* gene has no PvdS-dependent promoter but it has an iron starvation box implying that transcription of this gene takes place under iron restriction.[51] Both, *pvdQ* and *pvdA*, were found in *P. aeruginosa* strains producing type I, type II and type III pyoverdin,[38] suggesting that they are required in biosynthesis of all three types of pyoverdin.

Initially, it was speculated that PvdQ could be involved in degradation of AHLs acyl-homoserine lactones as a carbon source[15] as Huang *et al*. indicated that PAO1 can utilize its quorum-sensing molecules (AHLs) as a sole energy source essential for growth. Additional experiments demonstrated that *pvdQ* knockout mutants still were able to grow in a medium supplemented with the long AHL (3-oxy-C12-HSL) as a sole energy source.[15] In addition, the examination of *pvdQ* mRNA expression levels showed that there was no increase in mRNA levels when cultures were utilizing different carbon sources[35] suggesting that acyl-HSL utilization is not solely depending on PvdQ and therefore, it was proposed that another enzyme is involved in AHLs utilization. Extensive investigation of PvdQ that was done by Sio *et al*.,[13] proved that PvdQ degrades efficiently AHLs with side chains ranging from 11 to 14 carbons but not short-chain acyl-HSLs. The substituent at the 3′-position of the side chain did not affect its activity. In addition, Sio *et al*. found that overexpression of PvdQ in *P. aeruginosa* or exogenous addition of PvdQ to *P. aeruginosa*-growing cultures inhibits or delays the accumulation of signal molecules (3-oxy-C12-HSL and 2-heptyl-3-hydroxy-4(1H)-quinolone) and thereby decreases expression of several virulence factors, like elastase and

pyocyanin, important in *P. aeruginosa* pathogenicity. Hence, it can act as quorum-quencher *in vitro*.[13] It was also demonstrated that PvdQ has no activity against β-lactams[13] as other members of the NTN-hydrolase family.

Up to now, our knowledge is still incomplete to define the physiological role of PvdQ. Several experiments showed that *pvd*Q is needed for biosynthesis of pyoverdin and *pvdQ* expression is up-regulated under iron-limiting conditions.[39] There is a possibility that one of the precursors in the pyoverdin biosynthetic pathway may serve as a substrate for PvdQ. However, this has not been shown yet. The difficulty to determine the physiological role of PvdQ also comes from the diversity in *P. aeruginosa* bacterial forms (biofilm, planktonic), the variations in growth conditions, nutrients availability and other environmental factors.

5.2. QuiP (PA1032)

PA1032, recently named as *quiP* (for *q*uorum signal *u*tilization and *i*nactivation *p*rotein), is a second *P. aeruginosa* acylase homologue identified up to now.[35] In Figure 6 the localization of *quiP* on genomic DNA of *P. aeruginosa* is shown. Protein sequence analysis predicts QuiP to have significant similarity to acylases; QuiP shows 20–27% identity on a peptide level with other acylases (Table 1). As characteristic for N-terminal nucleophilic hydrolases, the gene encodes a polypeptide that undergoes post-translational maturation to result in a heterodimeric mature protein.[57] However, the post-translational processing of the QuiP precursor has not been demonstrated yet. An alignment of the conserved region of known acylases with QuiP shows that at position 297 there is asparagine (N), whereas for most acylases it is tyrosine, a strongly conserved residue (Figure 4). The *quiP* gene was cloned into an inducible plasmid and expressed in *E. coli,* and only the unprocessed form of the protein could be detected; the separate α- and β-subunits were not visible. Visible, unprocessed QuiP was a 90-kDa protein. When analysed by LC-MS/MS, the generated peptides matched with *in silico* prediction.[35] Signal peptide prediction indicates that protein will be translocated to the periplasm, as observed for other acylases.

Phenotypic analysis of *quiP* transposon mutant (mutant ID33050; Washington collection www.genome.washington.edu/UWGC/) showed that a strain carrying the transposon insertion was impaired in growth on decanoyl-HSL when compared to the wild type. QuiP complementation studies showed that cells could restore acyl-HSL-degrading potential when QuiP was constitutively expressed from a plasmid. It was also observed that constitutive *quiP* expression in *P. aeruginosa* resulted in a remarkable decrease of 3OC12HSL accumulation. An analogous observation was

made when *quiP* was expressed in *E. coli* from the inducible plasmid; cell extracts were able to degrade acyl-HSLs. In addition, the study of *quiP* mRNA expression levels for cultures grown on C10HSL, decanoate or succinate as a carbon source demonstrated an increase in mRNA level for cultures grown on C10HSL. These findings indicate that QuiP is involved in acyl-HSL utilization.[35] In particular, QuiP displays specificity towards long (C7HSL, C8HSL, C10HSL, 3OC12HSL and C14HSL) but not short acyl-HSLs. Furthermore, *quiP* was not picked up by microarrays as a gene regulated by quorum sensing.[58,59]

Concluding, recent results show that QuiP has acyl-HSL activity and it implies its involvement in signal decay in *P. aeruginosa*. However, it is not yet known under which physiological conditions *quiP* is expressed and what the main function of QuiP is.

5.3. PA1893

PA1893 is the third acylase homologue within the *P. aeruginosa* PAO1 genome. Its localization on the chromosome is shown in Figure 5 and in detail in Figure 6. PA1893 Orf shares 29% amino acid identity with cephalosporin acylase (acyII) from *Pseudomonas* sp. SE83[9,59] and 25% with *Pseudomonas* sp. SY-77 glutaryl acylase.[14,59] PA1893 peptide sequence was partially aligned with other acylases (Figure 4). As can be seen from this alignment, PA1893 has conserved residues known to be important for autocatalytic processing and enzymatic activity of known NTN-hydrolases. According to the *in silico* prediction, PA1893 is a polypeptide of 809 aa (89.7 kDa) that has, at the N-terminal, a hydrophobic stretch of 29 amino acids encoding a signal peptide. The predicted β-subunit of 556 aa (61.8 kDa) starts with a serine (Ser) residue at position 254. With respect to similarity to other acylases, PA1893 is predicted to undergo post-translational modification, thus to consist of α- and β-subunit as a mature protein.[57,60] However, protein maturation and subunit identification have

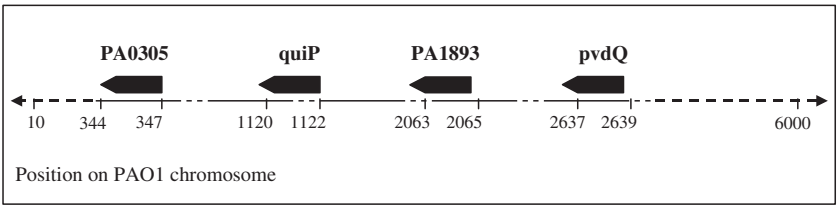
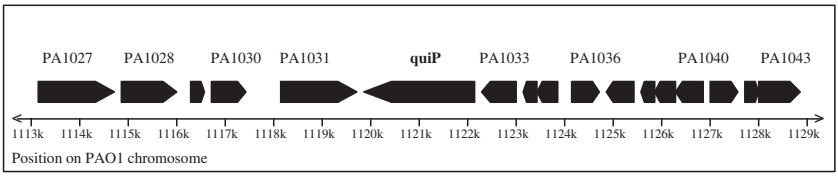
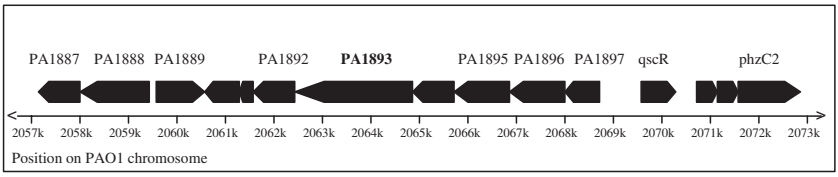


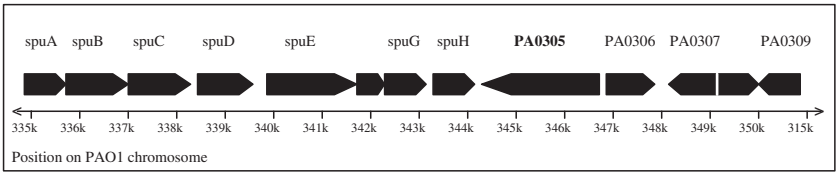
Figure 5. Chromosomal localization of four genes encoding (putative) NTN-hydrolases in the *P. aeruginosa* genome. The labels represent kilobasepairs.[16]



(A)



(B)



(C)

Figure 6. Detailed schematic representation of *quiP* (a), PA1893 (b) and PA0305 (c) gene localization within *P. aeruginosa* chromosome.

not been shown yet, due to problems with protein solubility.[35] Yet, by lowering the growth temperature (17°C) PA1893 was found to be partly soluble and it was found to undergo the autocatalytic processing moderately (precursor and β-subunit were detectable; Krzeslak, unpublished data).

PA1893 transposon mutant (mutant ID7831; Washington collection www.genome. washington.edu/UWGC/) was grown on decanoyl-HSL to study its ability to degrade acyl-HSLs. Results of this experiment showed that there was no significant growth differences in comparison to the wild-type strain.[35] In addition, it was observed that mRNA expression level of PA1893 was up-regulated when *Pseudomonas* cultures were grown in a medium supplemented with C10HSL as a carbon source in comparison to decanoate and succinate.[35] Microarray experiments provide information that PA1893 is a quorum-activated gene.[58,59]

Still, further studies have to be performed to elucidate the biological role of PA1893 in *P. aeruginosa*.

5.4. PA0305

The fourth acylase homologue in *P. aeruginosa* genome is PA0305. Figure 6 presents the PA0305 localization on the genome. Similar to the three other acylase homologues, PA0305 also is predicted to encode a NTN-hydrolase that undergoes post-translational modification.[57,60] PA0305 shows 26% amino acid similarity to cephalosporin acylase (acyII) from *Pseudomonas* sp. SE83[9,59] and 24% to penicillin G acylase from *E. coli*.[6] The degree of PA0305 identity on a peptide level to other known acylases can be found in Table 1. Sequence alignment of β-subunit shows that PA0305 has at the position 248-serine (S), at position 270-histidine (H) and at position 280-tyrosine (Y), residues highly conserved among NTN-hydrolases (Figure 4). *In silico* analysis predicts that the gene is transcribed as a precursor peptide consisting of a signal sequence, an α-subunit, a spacer peptide and a β-subunit, while the active enzyme consists of just the α- and β-subunits. PA0305 is a polypeptide of 795 aas (87.3 kDa) that has a signal peptide of 25 aas at the N-terminus. The predicted β-subunit starts with a serine residue (S) at the position 248 and consists of 548 aas (59.9 kDa).

There is little known about the physiological role or enzymatic activity of this enzyme. So far, the PA0305 Orf was picked up by PCR from PAO1 chromosomal DNA and expressed in *E. coli*.[35] However, only an unprocessed PA0305 polypeptide could be seen on SDS-PAGE gel up to now. To investigate any involvement of PA0305 in acyl-HSLs utilization, PA0305 transposon mutant (mutant ID32876; Washington collection www.genome.washington.edu/UWGC/) was examined for its ability to grow on decanoyl-HSL as a growth substrate. The growth phenotype of PA0305 transposon mutant did not show any significant difference when compared to the wild type strain.[35] Therefore, PA0305 is not implicated in direct acyl-HSLs utilization in *P. aeruginosa*. Moreover, there was no increase in PA0305 mRNA expression level when cultures were grown utilizing C10HSL as a carbon source, relatively to growth on decanoate and succinate.[35]

Similar to PA1893, the physiological role of PA0305 is unidentified. Therefore, additional studies have to be completed to have a better understanding of the function of PA0305 for *P. aeruginosa*.

6. WHAT FOR *PSEUDOMONAS* MAY NEED ACYLASES?

Although many NTN-hydrolases have been identified and characterized in different microorganisms so far, their physiological role most of the time remains unclear. Regarding the abundance of acylases, it is undoubtedly

evident that there is a rationale for bacteria to have these types of enzymes. In general, acylases are responsible for deacylation of antibiotics (penicillins, cephalosporins), AHL compounds or cyclic lipopeptides. As acylases hydrolyze an amide bond that links a ring-like nucleus to a side chain (aromatic or aliphatic), their substrates are molecules with an amide bond. Yet, acylases can be quite specific acting on a very few substrates. Interestingly, it was observed that penicillin G acylase can also hydrolyze cephalosporin-G and even phenylacetic-leucine.[61] Also glutaryl-cephalosporin acylase SY77 was found to be very specific for the acyl side chain, only glutaryl, and very promiscuous for the acid moiety hydrolyzing even glutaryl-serine and glutaryl-leucine.[14] From the 3D structure it becomes obvious that the acyl side chain is fully buried within the active site cleft, whereas the acid moiety has evolved limited interaction with the enzyme[62] (see Figure 7). This raises the possibility that the NTN-hydrolases are broad-spectrum towards the acid moiety and small-spectrum with respect to the acyl side chain. Within β-lactam acylases there are some indications that penicillin acylases could act as scavengers for nutrient compounds,[63] whereas there are no hints on such a role for cephalosporin acylases. QuiP and PvdQ are indicated to be involved in acyl-HSL scavaging and whether the primary function of PvdQ is in pyoverdin biosynthesis is not completely clear yet. Results from studies with a PvdQ-negative *P. aeruginosa* strain that is impaired in pyoverdin production[38] may suggest a possible direct involvement of PvdQ in

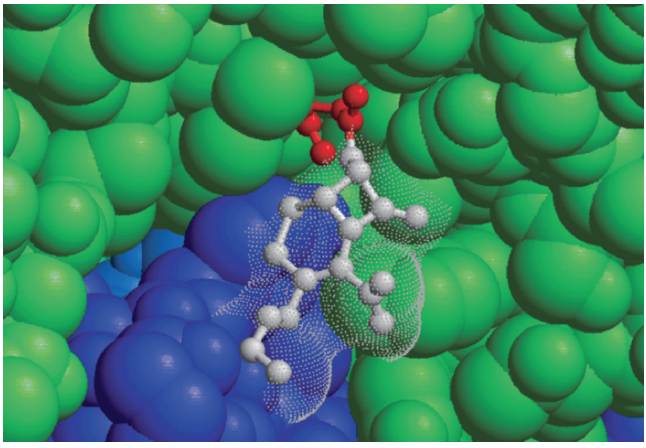


Figure 7. Solvent accessibility of glutaryl cephalosporin in complex with *Pseudomonas* SY-77 Ntn-hydrolase. It can be observed that the acyl side chain (red) is fully covered by the β-chain (green) and α-chain (blue) residues, whereas the cephalosporin moiety (white) is readily accessible to the solvent (small dots).

pyoverdin biosynthesis; however, up to now there are no clear-cut leads for this. The observed pyoverdin-negative phenotype of *ΔpvdQ* PAO1 strain can simply be the indirect effect of *pvdQ* deletion on pyoverdin production. The fundamental question is whether *P.aeruginosa* needs acylases to be able to perform its physiological processes or to regulate its physiological processes. For example, the disruption of *P. aeruginosa* acylases genes, separately in each gene, doesn't have a lethal effect on bacteria. Nevertheless, mutants display some phenotypic changes.[13,35] As mentioned earlier, two *P. aeruginosa* acylases (PvdQ, QuiP) were shown to have acyl-HSL activity, but is their primary biological role in *P. aeruginosa* to degrade 3OC12HSL signal molecule as a sole energy source or to regulate its own quorum-sensing activity? Some bacteria breakdown AHL and metabolize them whereas other strains might just degrade them so that other bacterial partners in a community can utilize these products.[33] Perhaps, these enzymes also control interactions within microbial communities, like between quorum-sensing bacteria and AHL-degrading bacteria. Does *P. aeruginosa* need acylases for bacterial networking, for virulence, to be able to compete with other bacteria for limited resources or for environmental adaptation? What stimulates acylases expression? Is the level of acylases expression different for the planktonic form than for biofilm form? Does *P. aeruginosa* need acylases to degrade its own signal molecules, or signal molecules of other bacteria? PvdQ and QuiP are quite specific towards long-chain acyl-HSLs,[13,35] so would that be the best strategy for bacteria to utilize these enzymes as communication interferers – quorum quenchers – between bacteria? Could acylases play a dual role: termination of other bacteria and utilization of disrupted signal molecules as a carbon source? It is known that quorum-sensing plays an important role in the virulence of many pathogenic bacteria, therefore it serves as a target for anti-pathogenic treatments.[64] In *P. aeruginosa* it was shown that inactivation of quorum-sensing systems dramatically reduces virulence.[65] Thus, while investigating the physiological role of acylases, from *P. aeruginosa* and other species, in their natural environment there is a huge temptation to look at them as enzymes with a potential for antimicrobial therapies. As one of the possibilities to interfere with bacterial quorum sensing is to employ acyl-HSL acylases or lactonases capable to degrade quorum-signal molecules, few simple experiments, with a promising outcome, have been already done to examine the quorum-quenching activity of AHL-inactivating bacteria.[23,24,29,66] At present, the role that acylases play in their natural ecological environment remains still unclear, and the physiological function for PvdQ and QuiP in AHL degradation remains to be proven. Possibly, acylases have assigned more complex tasks than just simple degradation. Therefore, there are many questions to be answered in order to understand what *Pseudomonas*, as

well other species, needs acylases for. This knowledge could give as a deeper insight into the role of acylases and into their potential role in antibiosis.

7. CONCLUSIONS

Naturally occurring acylases within the *P. aeruginosa* genome, as well as within other microbial genomes, are intriguing enzymes with puzzling biological functions, however with a clear biotechnological and pharmaceutical potential. Interfering with quorum sensing opens up to start a new era of antimicrobial therapies as an alternative to traditional antibiotics. In particular, their strength lies in a possibility to design targeted enzymes for degrading specific HSL molecules. Clearly, still more studies need to be performed in order to understand and realize what these enzymes mean for *P. aeruginosa,* and consequently what usefulness they can bring for us. The more we know about these enzymes, the more we understand about physiology of *P. aeruginosa* and the broader range of possible applications it provides. Identification and further characterization of *P. aeruginosa* acylases represent an interesting research topic for the near future and may lead us to novel therapies to fight infections.

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