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: "*Pseudomonas*: 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

1

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

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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

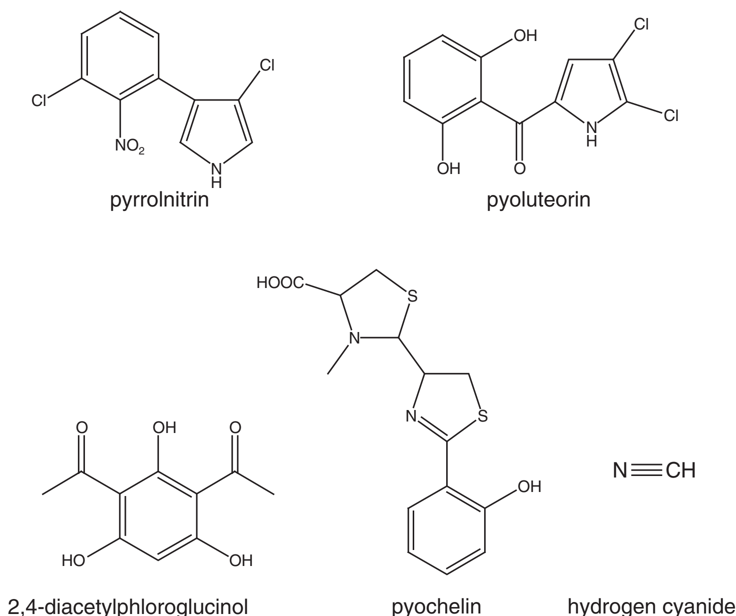

Figure 1. Secondary metabolites produced by *P. fluorescens* Pf-5.

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.

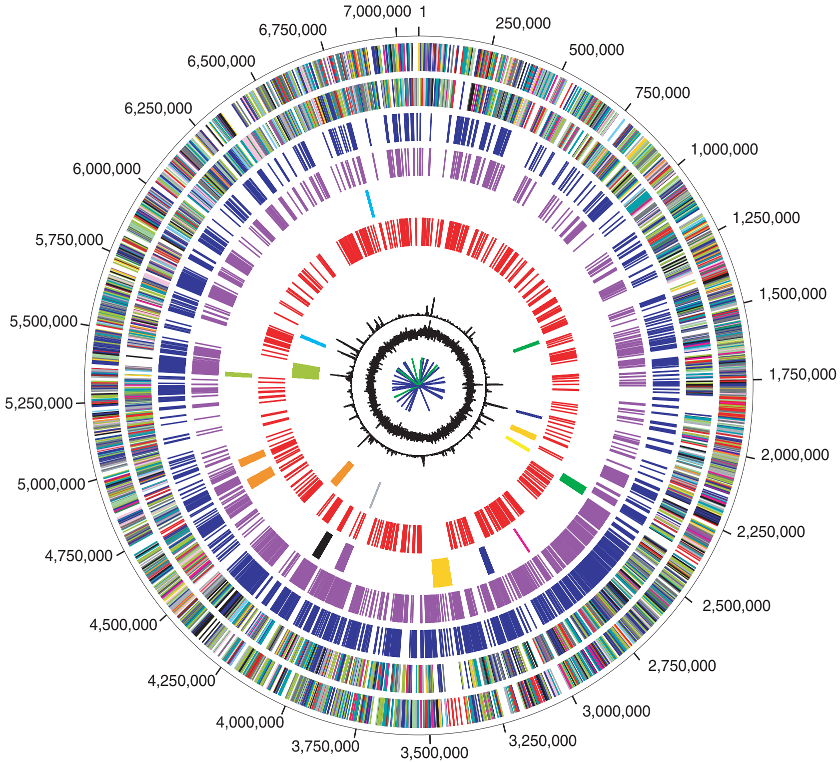

Figure 2. Circular representation of the genome of *P. fluorescens* Pf-5. The outer scale designates coordinates in base pairs (bps), with the origin of replication at 1 bp. The first circle (outermost circle) shows predicted coding regions on the plus strand color-coded by role categories: violet, amino 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*:

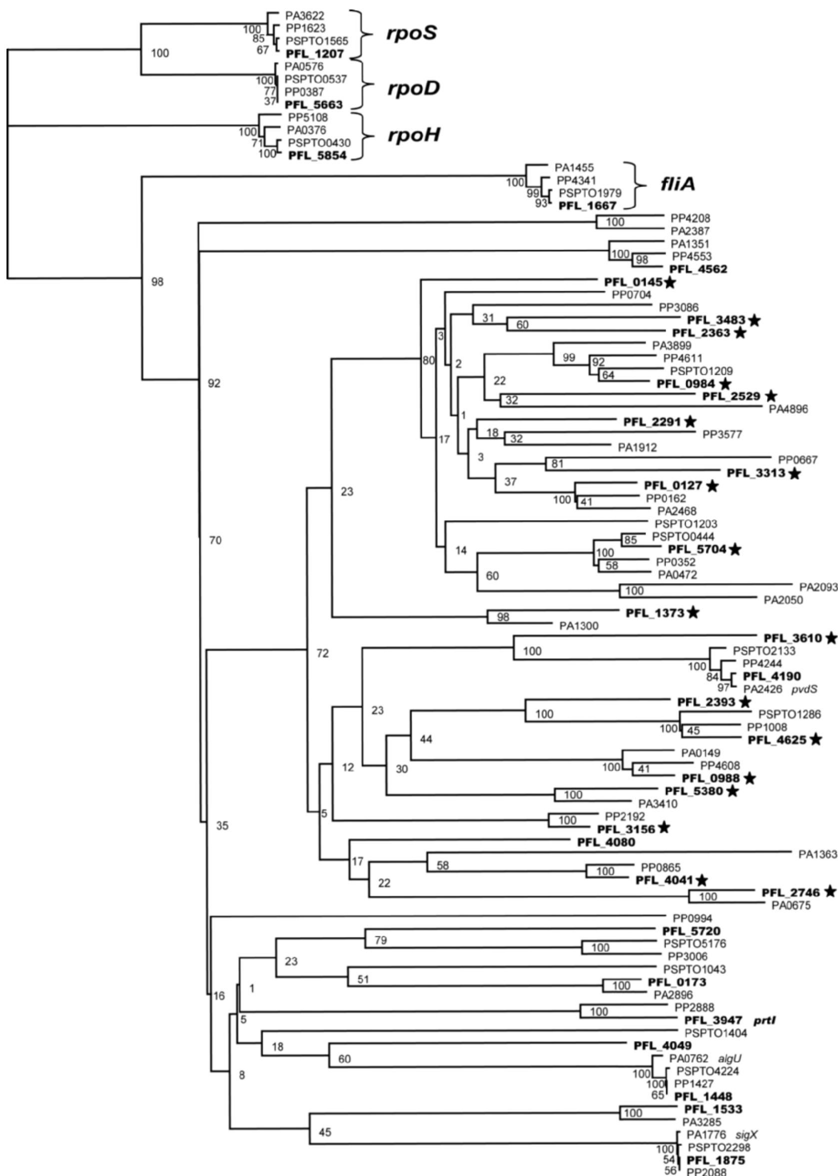

Figure 3. Phylogenetic relationships among the sigma 70 factors in four species of *Pseudomonas*. Predicted peptide sequences of sigma factors in the sigma 70 family were compared among *P. fluorescens* Pf-5 (bold), *P. aeruginosa* PAO1, and *P. putida* KT2440, and *P. syringae* pv. tomato DC3000. Genes 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).

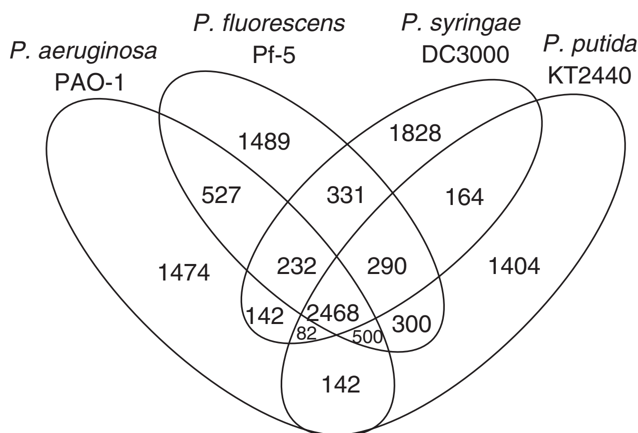

Figure 4. Venn diagram showing the number of proteins shared or unique for four *Pseudomonas* species. Protein sequences of four *Pseudomonas* genomes were compared, and bidirectional best matches that met the following criteria were scored as shared proteins: a p value less than or equal to 10^{-5}, 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

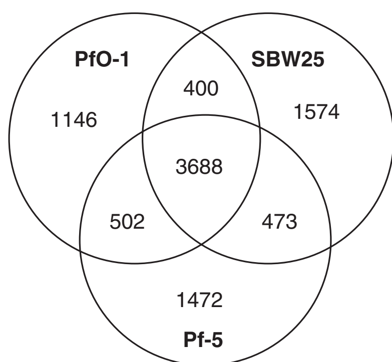

Figure 5. Venn diagram showing the number of proteins shared or unique for three strains of *P. fluorescens*. Sequences of predicted proteins from the genomes of Pf-5, Pf-01 (GenBank accession number NC_007492) and SBW25 (http://www.sanger.ac.uk/Projects/P_fluorescens) were compared. Bidirectional best 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.