REVIEW PAPER

Genomic analysis of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5

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Abstract The complete genomic sequences of several *Pseudomonas* spp. that inhabit the rhizosphere are now available, providing a new opportunity to advance knowledge of plant growth-promoting rhizobacteria (PGPR) through genomics. Among these is the biological control bacterium *Pseudomonas fluorescens* Pf-5. Nearly 6% of the 7.07 Mb genome of Pf-5 is devoted to the biosynthesis of secondary metabolites, including antibiotics toxic to soilborne fungi and Oomycetes that infect plant roots, and two siderophores involved in iron acquisition. Three orphan gene clusters, for which the encoded natural product was unknown, also were identified in the genome of Pf-5. The product synthesized from one of the orphan gene clusters was identified recently using a new 'genomisotopic approach', which employs a combination of genomic sequence analysis and isotope guided fractionation. Application of the genomisotopic approach to one orphan gene cluster in Pf-5 resulted in the discovery of orfamide A, founder of a new group of bioactive cyclic lipopeptides with a putative role in biological control of plant disease.

Abbreviations

CLP	cyclic lipopeptide
DAPG	2,4-diacetylphloroglucinol
GI approach	genomisotopic approach
HCN	hydrogen cyanide
NRPS	non-ribosomal peptide synthetase
PGPR	Plant growth-promoting rhizobacteria
PKS	polyketide synthase
Mcf	'makes caterpillars floppy'

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Introduction

Plant growth-promoting rhizobacteria (PGPR) are a diverse group of organisms that share two characteristics: the capacity to colonize the rhizosphere and to have a positive influence on the growth of plants whose rhizospheres they inhabit. Knowledge of these rhizobacteria and their interactions with plants and other components of microbial communities associated with roots has advanced tremendously during the past two decades, and molecular approaches have been important tools employed to build this

knowledge. With the recent availability of genomic sequence data for rhizosphere bacteria, the field of genomics can now contribute to advancing knowledge of PGPR and their effects on plant health and productivity. Complete genomic sequences for five species of *Pseudomonas*, including several strains that colonize the rhizosphere, are available to date (December 2006). This review will focus on the rhizosphere bacterium *Pseudomonas fluorescens* Pf-5, whose complete genome was sequenced recently (Paulsen et al. 2005). Here, we provide a brief summary of the biology of Pf-5 and its genome, before focusing on gene clusters for antifungal metabolites and the characterization of Orfamide A, a newly discovered cyclic lipopeptide (CLP) whose structure was predicted from the genomic sequence data and solved using a new genomisotopic approach. We also refer the reader to recent articles providing further analysis of the genomic sequence of Pf-5 (Loper et al. 2007; Mavrodi et al. 2007).

Pseudomonas fluorescens strain Pf-5

Strain Pf-5, which was isolated from the soil in Texas, USA, was first described for its capacity to suppress soilborne diseases of cotton caused by *Rhizoctonia solani* (Howell and Stipanovic 1979) and *Pythium ultimum* (Howell and Stipanovic 1980). Pf-5 has since been shown to suppress these pathogens on other plant hosts including cucumber (Kraus and Loper 1992) and pea (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* (Pfender et al. 1993). The biocontrol agent also suppresses two diseases of turf grass: dollar spot caused by *Sclerotinia homoeocarpa* and a leaf spot caused by *Dreschlera poae* (Rodriguez and Pfender 1997). These are widespread, destructive diseases affecting golf courses, home lawns, and amenity turf areas. Pf-5 also suppresses Fusarium root and crown rot of tomato, caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Sharifi-Tehrani et al. 1998) and seed piece decay of potato caused by the bacterial pathogen *Erwinia carotovora* (Xu and Gross 1986).

Secondary metabolite production by Pf-5

Pf-5 produces the antibiotics pyrrolnitrin (Howell and Stipanovic 1979), pyoluteorin (Howell and Stipanovic 1980; Kraus and Loper 1995), and 2,4-diacetylphloroglucinol (Nowak-Thompson et al. 1994); it also produces hydrogen cyanide (Kraus and Loper 1992) and two siderophores: a pyoverdine of unconfirmed structure and pyochelin (or a related compound). The spectrum of antibiotics produced by Pf-5 is remarkably similar to that produced by the well-characterized biological control strain *P. fluorescens* CHA0, which was isolated from roots of tobacco grown in a soil near Payerne, Switzerland (Stutz et al. 1986; Haas and Keel 2003; Haas and Défago 2005). Many other biological control strains of rhizobacteria produce a subset of metabolites produced by Pf-5 and CHA0 (Raaijmakers et al. 2002). Pf-5 does not produce the phenazine antibiotics that are produced by certain biological control strains of *Pseudomonas* spp. (Mavrodi et al. 2006).

The genomic sequence of Pf-5

In this section, we provide a brief overview of the genome of Pf-5, focusing on aspects of the genomic sequence of particular significance to the ecology of this rhizosphere bacterium and its interactions with the plant and other plant-associated microorganisms. Data presented in the following paragraphs are summarized from Paulsen et al. (2005).

Pseudomonas fluorescens Pf-5 inhabits root and seed surfaces, and its genome has a complement of genes specifying broad metabolic capacity, including the utilization of a variety of organic acids, sugars, and amino acids found in seed or root exudates. In this respect, it is very similar to *P. putida* KT2440, which also inhabits the rhizosphere (dos Santos et al. 2004). Pf-5 has genes for the metabolism of plant-derived carbohydrates such as maltose, sucrose, trehalose and xylose, and for more complex plant-derived molecules such as the aromatic compounds vanillate, benzoate and hydroxybenzoate, as well as long chain fatty acids and hydrocarbons, of which many plant oils are comprised. Present in its genome are several extracellular hydrolytic enzymes, including chitinases, proteases and lipases, which are involved in the degradation of polymers commonly

found in soil. For iron acquisition, the genome specifies the biosynthesis of two siderophores, as well as 45 predicted TonB-dependent outer membrane proteins, many of which are likely to serve as receptors for a diverse collection of ferric siderophores. *Pseudomonas* spp. are known to utilize siderophores produced by other microorganisms as sources of iron. In natural habitats on root surfaces, these bacteria can acquire iron by uptake of exogenous siderophores, obviating the need to rely on siderophore production alone (Loper and Henkels 1999). Determining the roles of these outer membrane receptors in the ecology of Pf-5 in the soil will be an enlightening subject for future inquiry.

Bacteria inhabiting the rhizosphere are likely to be exposed to a variety of toxic metabolites produced by the plant or other microorganisms. The genome of Pf-5 contains an expanded set of membrane efflux systems, which typically confer protection against a range of toxic metabolites. Among those are genes with predicted roles in the efflux of secondary metabolites produced by Pf-5 (Abbas et al. 2004; Brodhagen et al. 2005; Huang et al. 2006), resistance to fusaric acid and copper (Mellano and Cooksey 1998), and the phytotoxin tabtoxin. Consistent with the proposed importance of oxidative stress tolerance as a determinant of rhizosphere fitness, the genome encodes multiple peroxidases, catalases, and superoxide dismutases.

As expected of an organism living in a complex and rapidly changing environment, Pf-5 has an extensive array of regulatory genes. These include 68 predicted histidine kinases and 113 predicted response regulators (Kiil et al. 2005b). Pf-5 has 27 genes encoding sigma factors in the extracytoplasmic factor (ECF) class, the most among all of the *Proteobacteria* whose genomes have been sequenced (Kiil et al. 2005a). These sigma factors typically 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 (Helmann 2002). In Pf-5, 18 of the 27 ECF sigma factor genes are adjacent to genes encoding predicted TonB-dependent outer-membrane proteins, indicating a possible role in iron acquisition (Mavrodi et al. 2007). The numerous genes with putative roles in transcriptional regulation indicate that exceedingly complex regulatory networks exist in this environmental bacterium.

The genome of Pf-5 is notable for the absence of virulence factors found in pathogenic *Pseudomonas* spp. Lacking are genes for the synthesis of the phytotoxins tabtoxin, syringomycin, syringotoxin, syringopeptin, or coronatine. Also absent are genes for exoenzymes associated with degradation of plant cell walls or cell wall components, such as amylases or cellulases. There is no evidence for a type III secretion system although genes for such systems have been reported in many other strains of *P. fluorescens* (Mazurier et al. 2004; Preston et al. 2001; Rezzonico et al. 2005).

Pf-5 has several genes that specify exported factors with possible roles in biological control. The genome contains a homolog of *chiC* (Folders et al. 2001), which encodes chitinase, an enzyme that degrades chitin, an important fungal cell wall component. Chitinase production by other microorganisms has been implicated in biological control (Harman et al. 2004), and Pf-5 produces the enzyme (Donald Kobayashi, personal communication) but its role in disease suppression by Pf-5 has not been established. As typical for the genus, Pf-5 produces an extracellular alkaline protease(s) and has two homologs of the exoprotease gene *aprA*, which contributes to root knot nematode suppression by *P. fluorescens* CHA0 (Siddiqui et al. 2005). The genome also has two homologs of *llpA*, which encodes a bacteriocin related to LlpA (Parret et al. 2005). Intriguingly, a homolog of *mcf* (for makes caterpillars floppy) is also 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 (Daborn et al. 2002). If injected into the hemocoel, Pf-5 kills caterpillars of tobacco hornworms (*Manduca sexta*) whereas an *mcf* mutant of Pf-5 is less virulent (M. Pechy-Tarr, D. Bruck, M. Maurhofer, M. Henkels, K. Donahue, J. Loper, and C. Keel, unpublished data). The genomic sequence data provides direction for new inquiries highlighting the diverse biological activities of this fascinating soil bacterium.

Antibiotic biosynthesis gene clusters

Antibiotics produced by rhizosphere bacteria play an important role in biological control, and several excellent reviews provide perspectives of the recent

literature on this subject (Haas and Keel 2003; Raaijmakers et al. 2002; Mavrodi et al. 2006; Weller et al. 2002). Well-characterized antibiotics that contribute to biological control include pyoluteorin, 2,4-diacetylphloroglucinol, pyrrolnitrin, and hydrogen cyanide, all of which are produced by *P. fluorescens* Pf-5. Nearly 6% of the Pf-5 genome is devoted

to secondary metabolism, with loci for nine different secondary metabolites distributed around the genome (Fig. 1). Two of the gene clusters are involved in siderophore biosynthesis and two contain biosynthetic loci for which the corresponding metabolite is unknown. The following discussion will focus on the five gene clusters that contain biosynthetic loci
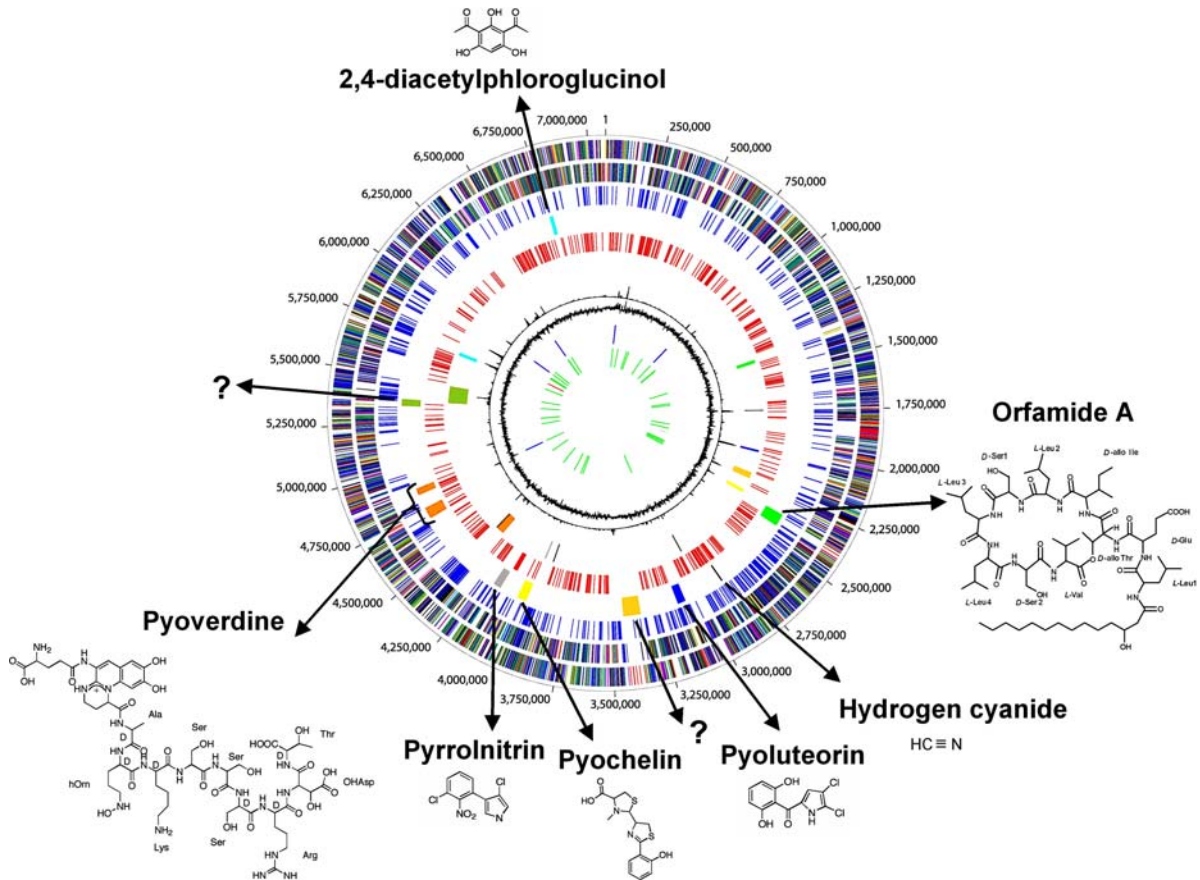

Fig. 1 Circular representation of the genome of *Pseudomonas fluorescens* Pf-5. The outer scale designates coordinates in base pairs (bp), with the origin of replication at 1 bp. The first circle (*outermost circle*) shows predicted coding regions on the plus strand colour-coded by role categories: violet, 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 grey, 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; grey, unknown function; salmon, other categories; blue, hypothetical proteins. The second circle shows predicted coding regions on the minus strand colour-coded by role categories. The third circle shows the set of 656 *P. fluorescens* Pf-5 genes not found in the genomes of *P. aeruginosa* PAO1, *P. syringae* pv. tomato DC3000, and *P. putida* KT2440. The fourth circle shows nine secondary metabolite gene clusters, with the structures and names of the corresponding metabolite indicated with lines. Two orphan gene clusters, whose metabolic products are unknown, are designated with a question mark. The fifth circle shows 1,052 copies of a 34 bp REP repeat element. The sixth circle shows a mobile island in olive and seven phage regions. The seventh circle shows trinucleotide composition. The eighth circle shows percentage G + C in relation to the mean G + C in a 2,000-bp window. The ninth circle shows rRNA operons in blue. The tenth circle (*innermost circle*) shows tRNA genes in green. Adapted from Paulsen et al. (2005)
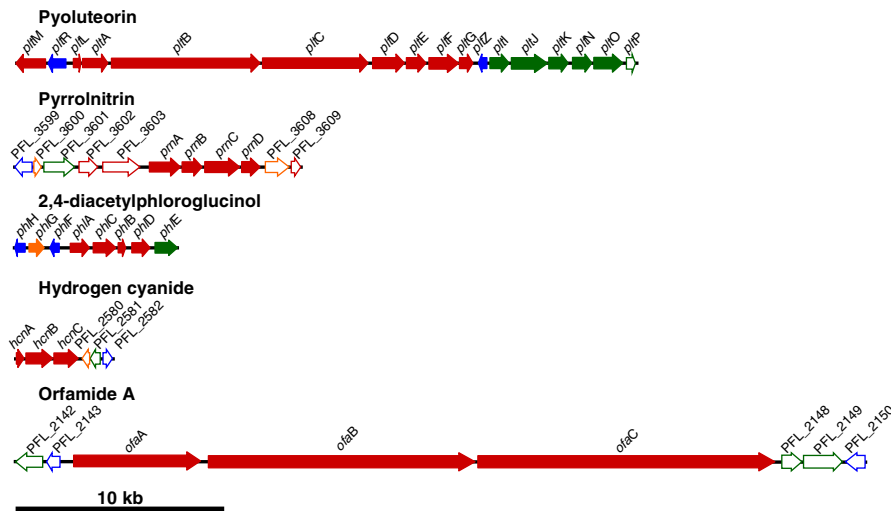
Fig. 2 Antibiotic biosynthetic gene clusters in the genome of *P. fluorescens* Pf-5. Solid arrows denote genes for which there is experimental evidence for involvement in antibiotic production by *Pseudomonas* spp. Red, structural genes for antibiotic biosynthesis; blue, regulation; green, transport; orange, acces-sory genes. Open arrows denote genes flanking antibiotic biosynthetic gene clusters and are shown to provide context within the genome of Pf-5. Colours of open arrows correspond to the same deduced functions defined for solid arrows. Adapted from Paulsen et al. (2005)

for antibiotics with known structures, one of which has been identified using the Pf-5 genomic sequence data as a starting point.

Pyoluteorin

Pyoluteorin is toxic against the Oomycete *Pythium ultimum* and certain other soilborne pathogens (Howell and Stipanovic 1980; Maurhofer et al. 1995). Pyoluteorin also exhibits some phytotoxicity (Maurhofer et al. 1995; Rao and Reddy 1990). The sequence of the pyoluteorin biosynthetic gene cluster was first described in strain Pf-5 (Nowak-Thompson et al. 1997, 1999). Genes functioning in regulation (*pltZ* and *pltR*) and efflux of pyoluteorin (*pltIJKLMO*) are present in the biosynthetic gene cluster (Fig. 2) (Brodhagen et al. 2005; Huang et al. 2004, 2006; Nowak-Thompson et al. 1999). The organization of the pyoluteorin biosynthesis and efflux gene cluster of *Pseudomonas* sp. M18 (Huang et al. 2006) is identical to that in Pf-5, although the nomenclature used for the efflux genes in M18 is slightly different from that used in Pf-5.

2,4-Diacetylphloroglucinol (DAPG)

DAPG is toxic to a wide range of plant pathogenic fungi and also exhibits antibacterial and anthelminthic

properties (Cronin et al. 1997; Keel et al. 1992). At relatively high concentrations, it is phytotoxic (Keel et al. 1992). Many strains of *P. fluorescens* produce DAPG, and phylogenetic analysis of the DAPG-producing strains places them in at least 22 genotypes (Weller et al. 2007), with Pf-5 classified in the A genotype of DAPG producers. Different genotypes of DAPG producers predominate in different soils, but the D genotype of DAPG producers predominates in the rhizosphere of wheat in soils suppressive to take-all decline, the most well characterized system exhibiting natural biological control (Weller et al. 2002, 2007).

As already demonstrated in many other strains of *P. fluorescens*, genes for DAPG biosynthesis (*phlACB* and *phlD*) (Bangera et al. 1999), efflux (*phlE*) (Abbas et al. 2004), and degradation (*phlG*) (Bottiglieri and Keel 2006) are clustered with those for regulation (*phlF* and *phlH*) (Abbas et al. 2002; Delany et al. 2000; Schnider-Keel et al. 2000) in the genome of Pf-5 (Fig. 2). The organization of the gene cluster is identical and the predicted amino acid sequences of the genes are also highly conserved (between 71 and 99% identity) with homologs in other strains.

Pyrrolnitrin

A diverse range of fungi, representing Basidiomycetes, Deuteromycetes, and Ascomycetes, are sensitive to

pyrrolnitrin (Ligon et al. 2000). Synthetic analogues of pyrrolnitrin have been developed as agricultural fungicides (Ligon et al. 2000), and pyrrolnitrin has also been used as a topical antibiotic for fungal infections of humans. The antibiotic is an inhibitor of fungal respiratory chains (Tripathi and Gottlieb 1969).

Four biosynthetic genes (prnABCD) are highly conserved among strains of P. fluorescens that produce pyrrolnitrin (Hammer et al. 1997, 1999; Kirner et al. 1998). Expression of prnABCD in heterologous bacteria results in pyrrolnitrin production (Hammer et al. 1997), indicating that these genes are sufficient for biosynthesis of the antibiotic from primary precursors. The predicted amino acid sequences of the four genes in the Pf-5 genome (Fig. 2) are very similar (between 90 and 99% identity) to those of homologs in P. fluorescens strains CHA0 (Baehler et al. 2005) and BL915 (Hammer et al. 1999). The amino acid sequences of the four genes are also very similar to homologs in pyrrolnitrin-producing strains of Burkholderia spp. such as B. cepacia strain AMMD (87–95% identity) (accession # NC_008391). Flanking the four known biosynthetic genes in the Pf-5 genome are other genes that could possibly have a role in regulation, efflux, or production of pyrrolnitrin (Fig. 2). Especially notable is PFL_3609, a member of the flavin reductase family, which could provide the reduced $FADH_2$ required for enzymatic activity of the halogenating enzymes PrnA and PrnC (Dong et al. 2005). Other genes of interest include PFL_3601, a transporter in the major facilitator subfamily (MFS), which commonly function in efflux; and PFL_3599, a transcriptional regulator in the LysR family. Because pyrrolnitrin is produced by heterologous bacteria such as Escherichia coli harbouring the prnABCD operon alone (Hammer et al. 1997), the flanking genes are not strictly required for pyrrolnitrin biosynthesis. The potential roles of these genes in pyrrolnitrin production by Pf-5 are subjects for future exploration.

Hydrogen cyanide

HCN is an effective inhibitor of cytochrome c oxidase (Knowles 1976) and other metalloenzymes (Blumer and Haas 2000). Its production by PGPR is implicated in biological control of black root rot of tobacco (Voisard et al. 1989), root rot of tomato, and Pythium damping-off of cucumber (Ramette et al. 2003). Cyanogenesis by Pseudomonas spp. is the product of

an HCN synthase, encoded by three biosynthetic genes (hcnABC) (Laville et al. 1998). These three genes are sufficient for HCN biosynthesis from glycine, the primary precursor (Blumer and Haas 2000).

The hcnABC operon in the Pf-5 genome (Fig. 2) is virtually identical in sequence (99–100% identity at the amino acid level) and organization to the corresponding HCN biosynthetic genes in P. fluorescens CHA0 (Laville et al. 1998), which conforms to an earlier comparison of hcnBC among cyanogenic Pseudomonas spp. (Ramette et al. 2003). Immediately downstream of the hcnABC operon is PFL_2580, a member of the glutathione S-transferase (GST) family, a large and diverse group of proteins that function in detoxification of a wide range of endogenous and xenobiotic compounds. Homologs of PFL_2580 are found in the genomes of the cyanogenic species P. aeruginosa and Pseudomonas entomophila, but they are not linked to hcnABC in those species. Multiple mechanisms contribute to HCN resistance in P. aeruginosa. These include rhdA, which encodes a rhodonase that detoxifies HCN (Cipollone et al. 2007); the cioAB gene pair, which encode a cyanide insensitive terminal oxidase (Cooper et al. 2003); and the excretion of α-ketoglutarate, which detoxifies cyanide (Blumer and Haas 2000). As in P. aeruginosa, homologs of cioAB and rhdA are not linked to the hncABC biosynthetic locus in the Pf-5 genome. Two other ORFs neighbouring hcnABC are PFL_2581, a member of the MFS superfamily of membrane transporters, and PFL_2582, a transcriptional regulator. Further study is needed to ascertain the potential roles of these genes in hydrogen cyanide production by Pf-5.

Orphan gene clusters

An exciting outcome of the Pf-5 genomic sequencing project was the identification of three orphan gene clusters. These three orphan gene clusters contain sequences that are characteristic of polyketide synthases (PKSs) or non-ribosomal peptide synthetases (NRPSs), enzymes that catalyze the formation of secondary metabolites through a non-ribosomal mechanism of biosynthesis (Wenzel and Müller 2005). Although the structures of compounds that are generated non-ribosomally are diverse, their biosynthetic enzymes share a modular

architecture of catalytic domains and an assembly line mechanism of product synthesis. Furthermore, the catalytic domains are encoded by highly conserved sequences that can be used to identify novel biosynthetic gene clusters containing PKSs or NRPSs. The three orphan gene clusters were found by searching the Pf-5 genomic sequence data for the conserved sequences of PKSs and NRPSs. To date, our efforts to identify metabolites synthesized from the orphan gene clusters of Pf-5 have focused on one cluster containing a NRPS.

NRPSs are large, multifunctional enzymes that are organized into modules, each of which synthesizes the addition of an amino acid to a peptide, which is then transferred to downstream modules for the next step in the synthesis of an elongating peptide chain (Finking and Marahiel 2004). Each elongation module contains at least three types of domains: (i) a condensation (C) domain that catalyzes peptide bond formation between amino acids in the growing peptide chain; (ii) an adenylation (A) domain, which is responsible for recognition and activation of a specific amino acid; and (iii) a thiolation (T) domain, which binds the substrate as it moves from module to module along the NRPS. Finally, the elongation process is terminated by the release of the final product through hydrolysis or cyclization, the latter process often catalyzed by a thioesterase (TE).

A remarkable characteristic of natural product biosynthesis via NRPSs is that the number and order of the modules of an NRPS protein generally are colinear to the amino acid sequence of the final peptide product, a relationship termed the 'colinearity rule.' Furthermore, the modules contain characteristic motifs, primarily in the A domains, that select for a specific amino acid to be added to the growing peptide chain (Stachelhaus et al. 1999). These signature motifs can be identified using web-based bioinformatic algorithms (Ansari et al. 2004; Challis et al. 2000) and, because of the colinearity rule, they can be used to predict the order, composition and number of amino acids in the final peptide product.

Below, we summarize the process by which one of the orphan metabolites in the Pf-5 genome was purified and characterized, using the genomic sequence data as a starting point.

Sequence analysis of an orphan gene cluster in the genome of Pf-5

One of the three orphan gene clusters in the Pf-5 genome (Fig. 3a) contained three contiguous genes, termed *ofa*A, *ofa*B, and *ofa*C, whose deduced amino acid sequences are similar to NRPSs (Gross et al. 2007; Paulsen et al. 2005). Together, OfaA, OfaB, and OfaC comprise ten modules, each of which contains a condensation (C), adenylation (A), and thiolation (T) domain characteristic of NRPS modules (Fig. 3b). In addition to the ten modules, two thioesterase (TE) domains, required for liberation of the peptide from the enzyme complex, are present in the C-terminus of the predicted amino acid sequence of OfaC. Analysis of the DNA sequence of *ofaA*, *ofaB*, and *ofaC* provided three lines of evidence that the product of the NRPS is a cyclic lipopeptide (CLP), a large class of compounds composed of a fatty acid tail linked to a peptide that is cyclized to form a lactone ring (Nybroe and Sørensen 2004; Raaijmakers et al. 2006): (1) the predicted amino acid sequences of OfaA, OfaB, and OfaC are most related to NRPSs for the biosynthesis of CLPs by other *Pseudomonas* species, (2) the Ofa NRPS lacks a typical initiation module, which is also lacking in the NRPSs for other CLPs (Guenzi et al. 1998; Roongsawang et al. 2003; Scholz-Schroeder et al. 2003); while initiation modules for NRPSs typically lack a C domain, the first module in the NRPS for CLP biosynthesis contains a C domain, which is likely to be involved with bond formation with the lipid starter unit (Roongsawang et al. 2003), (3) two TE domains are present in the C-terminus of the Ofa NRPS, a characteristic of NRPSs for CLPs of *Pseudomonas* spp.

The amino acid sequence of the peptide component of the orphan metabolite was predicted through analysis of signature sequences within the A domains of OfaA, OfaB, and OfaC (Gross et al. 2007; Paulsen et al. 2005). This analysis provided further evidence that the orphan metabolite is a CLP, because the deduced amino acid sequence of peptidyl product is very similar to those of several known CLPs (Gross et al. 2007; Paulsen et al. 2005). Nevertheless, the orphan metabolite from Pf-5 is distinct from previously-described CLPs produced by *Pseudomonas* spp., which are classified into five groups according to the
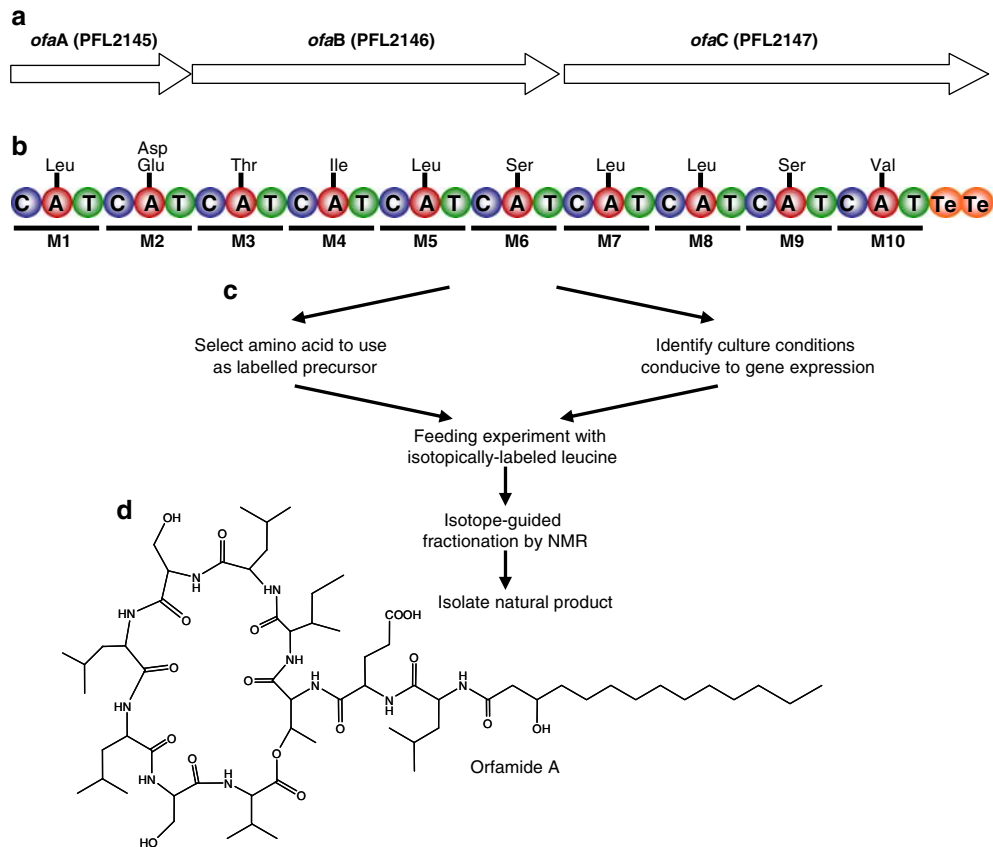
Fig. 3 Schematic diagram depicting the genomisotopic approach for isolation of Orfamide A from cultures of Pf-5. (a) The orfamide A biosynthesis genes, *ofaA*, *ofaB*, and *ofaC*, which encode a non-ribosomal peptide synthetase (NRPS) (b) Ten modules (M1–M10) were identified in the deduced protein sequence of *ofaA*, *ofaB*, and *ofaC*, each with a condensation (C), adenylation (A), and thiolation (T) domain. Two thioesterase (TE) domains were identified in the 3′ end of OfaC. Amino acids incorporated into the final peptide product were predicted by bioinformatic analysis of the A domain of each module. The specified amino acid is shown above the corresponding A domain. (c) Pf-5 was grown under culture conditions favourable to the expression of *ofaA*, *ofaB*, and *ofaC*, as determined using RT-PCR. Isotopically-labelled leucine, an amino acid predicted to be present in the final peptide product, was added to the culture medium and the label was used to guide a fractionation scheme for purification of the natural product. (d) The structure of orfamide A, which was isolated using the genomisotopic approach. Adapted from Gross et al. (2007)

length of the peptidyl and lipid moieties: the viscosin, amphisin, tolaasin syringomycin, and putisolvin groups (Raaijmakers et al. 2006). The size of the peptide portion ranges from nine amino acids (viscosin group) up to 25 amino acids (syringopeptin group), but there are no previously described CLPs from *Pseudomonas* spp. with a 10 amino acid peptide chain.

The genomisotopic approach

With the increasing number of genomes sequenced and available in the public domain, a large number of orphan gene clusters, for which the encoded natural product is unknown, have been identified. These orphan gene clusters represent a tremendous source of novel and possibly bioactive compounds, and efficient methods to exploit this valuable resource are needed. Recently, a novel approach termed the genomisotopic (GI) approach was developed to meet that need, with the CLP produced by Pf-5 selected as the first orphan metabolite to be isolated using this approach (Gross et al. 2007). The GI approach uses an isotopically-labelled amino acid, predicted to be a precursor of a peptide from bioinformatic analysis of the NRPS nucleotide sequence, to guide fractionation

for the purification of a final peptide product (Fig. 3c). Using a two-pronged approach employing GI and traditional assay-guided fractionation in parallel, novel CLPs termed orfamides were identified from cultures of Pf-5 (Gross et al. 2007). The chemical structure of these molecules conforms to the amino acid sequence predicted bioinformatically from the sequence of A domains of OfaA, OfaB, and OfaC.

Characteristics of the orfamides

The orfamides are founding members of a new class of CLPs characterized by a 3-hydroxy dodecanoic or tetradecanoic (myristic) acid connected to the N-terminus of a 10 amino acid cyclic peptide. Orfamide A (Fig. 3d) is the dominant CLP produced by Pf-5. The amino and fatty acid partial structures of orfamide A were determined by standard amino acid analysis, HR-ESI-TOF-MS and an extensive array of 1D and 2D NMR experiments. The sequence of the residues was accomplished using a combination of MS/MS as well as HMBC and ROESY NMR experiments. Chiral GC-MS and a modified Marfey's analysis were used to establish the absolute configurations of each amino acid (Gross et al. 2007).

Although orfamide A is the dominant CLP produced by Pf-5, related compounds (also called orfamides) are produced in much smaller amounts by the bacterium (Gross et al. 2007). Orfamide B differs from orfamide A by a substitution of valine for D-allo isoleucine at the fourth position on the peptide chain. Orfamide C differs from orfamide A by the substitution of dodecanoic acid for tetradecanoic acid as the lipid moiety. A mutation in the NRPS-encoding gene ofaB eliminates production of all three orfamides by Pf-5 (Gross et al. 2007), indicating that all three compounds are products of the same gene cluster. The relaxed substrate specificity of the A-domain responsible for the activation of L-Ile could explain the production of slight amounts of orfamide B by Pf-5. Similarly, the relaxed substrate specificity of the C domain of module 1 for tetradecanoic acid could explain the altered fatty acid present in orfamide C. In addition to the three characterized orfamides, other compounds with a similar polarity and mass range, which are present in trace amounts in culture supernatants of Pf-5, were not detected in cultures

of the ofaB mutant. It is likely that a range of orfamides are produced in very small concentrations by Pf-5, each of which reflects a relaxed specificity of one or more of the domains in the NRPS responsible for orfamide biosynthesis. Small amounts of derivatives of viscosins or amphisins are produced by other strains of P. fluorescens, which has also been attributed to the relaxed specificities of biosynthetic enzymes (De Souza et al. 2003; Nielsen et al. 2002).

The orfamide biosynthetic gene cluster

The orfamide biosynthesis gene cluster (Fig. 2) includes two putative regulatory genes that encode transcriptional regulators in the LuxR family: PFL_2143 and PFL_2150. Three genes (PFL_2142, PFL_2148, and PFL_2149) with predicted roles in transport have also been described. The predicted peptide sequences of these genes have domains characteristic of the components of an ABC transport system: an outer membrane porin (PFL_2142), a cytoplasmic membrane protein with ATP binding domains (PFL2149), and a membrane fusion protein (PFL_2148). The organization of the nine genes in the proposed orfamide gene cluster (Fig. 2) is identical to that of a CLP biosynthetic gene cluster in P. fluorescens Pf0-1 (Accession no. NC_007492). The predicted amino acid sequences of the genes in the orfamide gene cluster are also very similar (ranging from 62% to 87% identical) to homologs in the Pf0-1 genome. Therefore, it appears that the CLP biosynthesis gene cluster is conserved among the two strains.

Genes for the biosynthesis of the lipid side chain of orfamide have not been identified. Enzymes for fatty acid biosynthesis, such as β-ketoacyl-ACP synthetases or acyl-CoA ligases, are not present in the orfamide gene cluster. Also absent from the gene cluster is an acetyltransferase, purportedly required for the acyltransfer of a 3-hydroxy fatty acid to the first amino acid of the peptide chain. While biosynthetic gene clusters for CLPs can include genes for synthesis of the lipid moiety, as for iturin biosynthesis by Bacillus subtilis (Tsuge et al. 2001), this is not always the case. For example, the large genomic island in P. syringae B728a, which contains the NRPSs for biosynthesis of the CLPs syringomycin and syringopeptin, lacks genes for fatty acid biosyn-

thesis (Feil et al. 2005). Therefore, as in Pf-5, genes for the biosynthesis of the lipid side chains are not co-located with the NRPSs for biosynthesis of these CLPs in the genome of *P. syringae* B728a. It is possible that the lipid moiety is provided from primary metabolism.

Biological significance of the orfamides

Due in part to their biosurfactant properties, CLPs influence the lifestyle of *Pseudomonas* in many ways, facilitating bacterial movement and growth on agar or seed surfaces (Nielsen et al. 2005), influencing surface adhesion and altering biofilm development and stability (Raaijmakers et al. 2006). The amphiphilic properties of CLPs influence the motility of bacterial cells on surfaces, and mutants deficient in CLP production commonly exhibit diminished swarming motility (Kuiper et al. 2004; Roongsawang et al. 2003). Accordingly, an orfamide A deficient mutant of Pf-5 exhibits reduced swarming motility compared to the parental strain (Gross et al. 2007). The surfactant properties of CLPs are also likely to influence adhesion of bacterial cells to surfaces, an important component of biofilm formation. Nevertheless, different CLPs have different reported effects on adhesion and biofilm formation by *Pseudomonas* spp. Biofilm formation was enhanced in a mutant of *P. putida* PCL1445 deficient in the production of the CLP putisolvin. Furthermore, purified putisolvin I broke down established biofilms produced by PCL1445 (Kuiper et al. 2004). A mutant of *Pseudomonas* sp. MIS38 deficient in the production of the CLP arthrofactin produced less stable but more abundant biofilms than the parental strain (Roongsawang et al. 2003). CLP deficient mutants of *P. fluorescens* SWB25 exhibited diminished biofilm formation (de Bruijn et al. 2007). An orfamide-deficient mutant did not differ statistically from Pf-5 in adhesion to polystyrene, a key assay indicative of biofilm formation (Gross et al. 2007). Due perhaps to the diversity of the CLPs and CLP-producing strains, there appears to be no uniform role of these molecules in surface adhesion by *Pseudomonas* spp.

The structural diversity of CLPs is reflected in the diverse biological activities of this class of molecules, members of which act as phytotoxins or anti-microbial agents (Raaijmakers et al. 2006; Nybroe and Sørensen

2004). Certain CLPs isolated from biocontrol strains of *Pseudomonas* spp. inhibit mycelial growth or development of fungal plant pathogens, including the soilborne fungus *Rhizoctonia solani* (Miller et al. 1998; Nielsen et al. 2000, 2002). In contrast, purified orfamide A did not inhibit mycelial growth of *R. solani* on agar surfaces (Gross et al. 2007). Zoospores of Oomycetes, lacking a cell wall, are vulnerable to membrane disruption caused by CLPs (de Souza et al. 2003), effected either through the non-specific detergent properties of CLPs, their capacity to form transmembrane pores (Nybroe and Sørensen 2004), or other mechanisms. Consequently, zoospores of *Phytophthora* spp. lyse within seconds of exposure to CLPs such as a viscosin-like CLP produced by SBW25 (de Bruijn et al. 2007) or orfamide A (Gross et al. 2007). In contrast, the CLP viscosinamide decreased zoospore production and increased zoospore encystment by another Oomycete, *Pythium ultimum* (Thrane et al. 2000). As the only motile propagule of the Oomycetes, zoospores represent a critical stage in the disease cycle, especially for plants grown hydroponically or in water-saturated soils. Surfactants, produced synthetically or by biological control agents, can provide effective management of diseases caused by Oomycetes on plants grown hydroponically (Stanghellini and Miller 1997). Accordingly, a CLP related to massetolide A, which lyses zoospores of several Oomycete pathogens, is also thought to play a critical role in biological control of root rot caused by *P. ultimum* (de Souza et al. 2003). With the discovery of the orfamides, knowledge of the structural diversity of the CLPs has been expanded, as well as their potential effects as factors influencing the interactions of rhizosphere bacteria with other components of the soil microflora and the plant host.

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 revealed pathways for utilization of an extensive array of carbon substrates found in plant root and seed exudates as well as siderophores produced by diverse soil inhabiting microorganisms. The expanded transport 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. At 7.07 Mb, the genome of *P. fluorescens* Pf-5 is larger than those of other *Pseudomonas* spp. sequenced to date, a difference that can be attributed in part to the presence of at least nine gene clusters for secondary metabolite production. Four of these gene clusters specify the biosynthesis of antibiotics with a well established role in biological control, and the organization and sequences of the biosynthetic, transport, and regulatory genes therein are very similar to those described in other strains of *Pseudomonas* spp. The genomic sequence also highlighted three orphan gene clusters, which encode for natural products with unknown structures and biological activities. The structure of one of these orphan metabolites could be predicted bioinformatically from the sequence of a NRPS within the gene cluster. The bioinformatically-predicted structure has since been confirmed, and the product defined as orfamide A, the founding member of a new class of CLPs. Like other CLPs produced by *Pseudomonas* spp., orfamide A functions as a biosurfactant, influencing swarming motility of Pf-5 and lysing zoospores produced by an Oomycete plant pathogen. Recently, the structure of a viscosin-like CLP produced by *P. fluorescens* strain SBW25 was reported, and shown to conform to the structure predicted bioinformatically from the genomic sequence of strain SBW25. While the path from genomic sequence data to chemical discovery has been lightly trod to date, the discovery of orfamide A provides one of the first examples of genome mining from *Pseudomonas* spp. *Pseudomonas* spp. are prolific producers of antibiotics, and the availability of genomic sequences for several *Pseudomonas* spp. now opens the door for discovery of novel natural products with potential roles in the ecology and plant growth-promoting properties of these bacteria.

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