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The Genomisotopic Approach: A Systematic Method to Isolate Products of Orphan Biosynthetic Gene Clusters

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SUMMARY

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. Here, we describe a "genomisotopic approach," which employs a combination of genomic sequence analysis and isotope-guided fractionation to identify unknown compounds synthesized from orphan gene clusters containing nonribosomal peptide synthetases. Analysis of the *Pseudomonas fluorescens* Pf-5 genome led to the identification of an orphan gene cluster predicted to code for the biosynthesis of a lipopeptide natural product. Application of the genomisotopic approach to isolate the product of this gene cluster resulted in the discovery of orfamide A, founder of a group of bioactive cyclic lipopeptides.

INTRODUCTION

Orphan (cryptic) gene clusters, containing biosynthetic loci for which the corresponding metabolite is unknown, are found in the genomes of many microorganisms. The products of these biosynthetic loci represent a rich source of hitherto unexplored and possibly bioactive compounds, but, to date, only a small fraction of this potential has been examined. Strategies employed to purify and characterize the products of orphan gene clusters include heterologous expression of orphan gene clusters [1, 2], metabolic profiling coupled with mutagenesis of orphan genes [3, 4], and assay-guided fractionation [5–7].

In this paper, we propose a straightforward genomisotopic approach, which combines the power of genomics and isotope-guided fractionation to isolate the products of orphan gene clusters containing nonribosomal peptide synthetases (NRPSs). We demonstrate the power of this approach with the discovery of a group of cyclic lipopeptides (CLPs) produced by the bacterium *Pseudomonas fluorescens* Pf-5. This gram-negative bacterium inhabits the root surfaces (rhizosphere) of many plants and functions as a biological control agent, suppressing plant diseases caused by soil-borne plant pathogens [8, 9]. Strain Pf-5 produces at least six secondary metabolites: the antibiotics pyrrolnitrin [8], pyoluteorin [9], and 2,4-diacetylphloroglucinol [10], two siderophores (a pyoverdine and pyochelin or a related compound), and hydrogen cyanide [11]. Bioinformatic analyses of the genomic sequence of Pf-5 [12] identified three orphan, natural product gene clusters in addition to the known secondary metabolite gene clusters. One of these orphan gene clusters contains NRPSs postulated to synthesize a CLP [12], composed of a fatty acid tail linked to a peptide that is cyclized to form a lactone ring [13, 14]. CLPs are synthesized by NRPSs via a thiotemplate mechanism [15–17], and signature sequences within the adenylation domains of the NRPSs are known to specify the amino acid composition of the peptide chain [17, 18]. Because the amino acid composition of the product can be predicted from the nucleotide sequence of NRPSs [18, 19], we explored the hypothesis that an isotopically labeled amino acid, predicted to be a precursor from bioinformatic analysis, would be incorporated and therefore enable identification of the natural product. As a proof of principle, we employed the genomisotopic approach in parallel with traditional assay-guided fractionation to isolate the CLP, termed orfamide A, from cultures of Pf-5 (Figure 1). Orfamide A is, to our knowledge, the first member of a new subclass of lipopeptides produced by *Pseudomonas* spp. and was shown to have a role in motility of the producing bacterium and to exhibit antimicrobial activity.

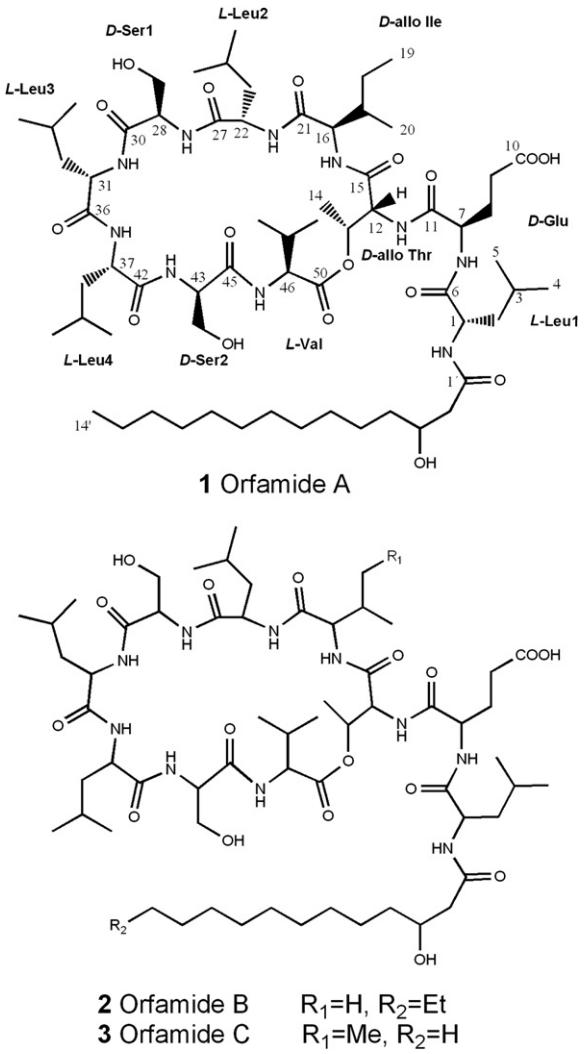

Figure 1. Chemical Structures of the Orfamides
Orfamides A, **1**; B, **2**; and C, **3**.

RESULTS

Analysis of the Orphan Gene Cluster Encoding for the Biosynthesis of Orfamide A

The orphan gene cluster includes three large ORFs termed *ofa*A, *ofa*B, and *ofa*C, whose deduced amino acid sequences are similar to NRPSs. The amino acid sequences most similar to OfaA, OfaB, and OfaC are NRPSs that synthesize CLPs, including arthrofactin (Arf) [15], syringomycin [16], and syringopeptin [17]. Together, OfaA, OfaB, and OfaC comprise ten modules, each of which contain a condensation (C), adenylation (A), and thiolation (T) domain characteristic of NRPS elongation modules (Figure 2). A typical initiation module, which lacks a C domain [20], is not present in OfaA. The lack of a typical initiation module has been reported in other NRPSs that encode for the biosynthesis of lipopeptides [15–17]. Each of the ten C domains in the Ofa NRPS contains the conserved motif HHxxxD involved in amide bond formation [21]. No epimerization (E) domains are found in OfaA, OfaB, or OfaC, but six of the ten C domains contain conserved dual C/E sequences in the N-terminal region (see Supplemental Data available with this article online), as recently described for other NRPSs, including those for Arf biosynthesis [22]. The modules having these domains were not strictly associated with D-amino acids in the resulting peptide, however, suggesting a complexity in the relationship between the sequence and final product that is not fully understood. Each C domain is followed by an A domain, which catalyzes substrate recognition and activation. Each A domain contains the conserved core sequences described by Schwarzer et al. [21] (see Supplemental Data). The A domains are followed by T domains, which function in the transport of the activated amino acid and elongation intermediates between catalytic centers.

The ten T domains in OfaA, OfaB, or OfaC (see Supplemental Data) have a highly conserved core sequence motif (DHFFE[L/Q]GGHSL[L/S]A), which includes the invariant serine residue (underlined) involved in binding of the 4′-phosphopantetheine cofactor [21]. This sequence motif is identical to that found in the T domains of the Arf NRPSs [15]. Similarly, two distinct sequence motifs are found downstream of the core motif in the Ofa T domains, as described previously in the T domains of the Arf NRPSs [15]. The C-terminal regions of NRPSs typically contain a thioesterase domain required for liberation of the peptide from the enzyme complex. Two thioesterase domains, each containing the conserved GXSXG sequence motif, were found in the C terminus of OfaC. Similarly, two thioesterase domains are present in the C termini of NRPSs for the CLPs Arf [15], syringomycin [16], and syringopeptin [17] in other *Pseudomonas* species. Therefore, analysis of the DNA sequences of *ofa*A, *ofa*B, and *ofa*C provided three lines of evidence that the product synthesized by the NRPS is a lipopeptide: (1) the predicted amino acid sequence is most similar to NRPSs for the biosynthesis of lipopeptides by other *Pseudomonas* species; (2) OfaA lacks a typical initiation module; and (3) two T domains are present in the C terminus of OfaC.

Bioinformatic analysis of the A domains led to the prediction of their cognate amino acids and, due to the common colinearity between the sequence of specific A domains in NRPSs and the sequence of amino acids in the peptide product [15], also allowed prediction of the amino acid sequence of the resulting peptide (Figure 2). Alignment of the amino acids of the predicted compound with known *Pseudomonas* lipopeptides of the amphisin and viscosin classes [13, 14, 23] suggested that the orphan metabolite is novel, but closely related to the latter group (Table 1). From these data, together with the DNA sequence analysis presented above, we deduced that orfamide A possesses a lipid side chain, most likely connected to Leu1. Genes with putative roles in fatty acid biosynthesis were not found in the *ofa* gene cluster; thus, the chain length of the lipid side chain could not be predicted.

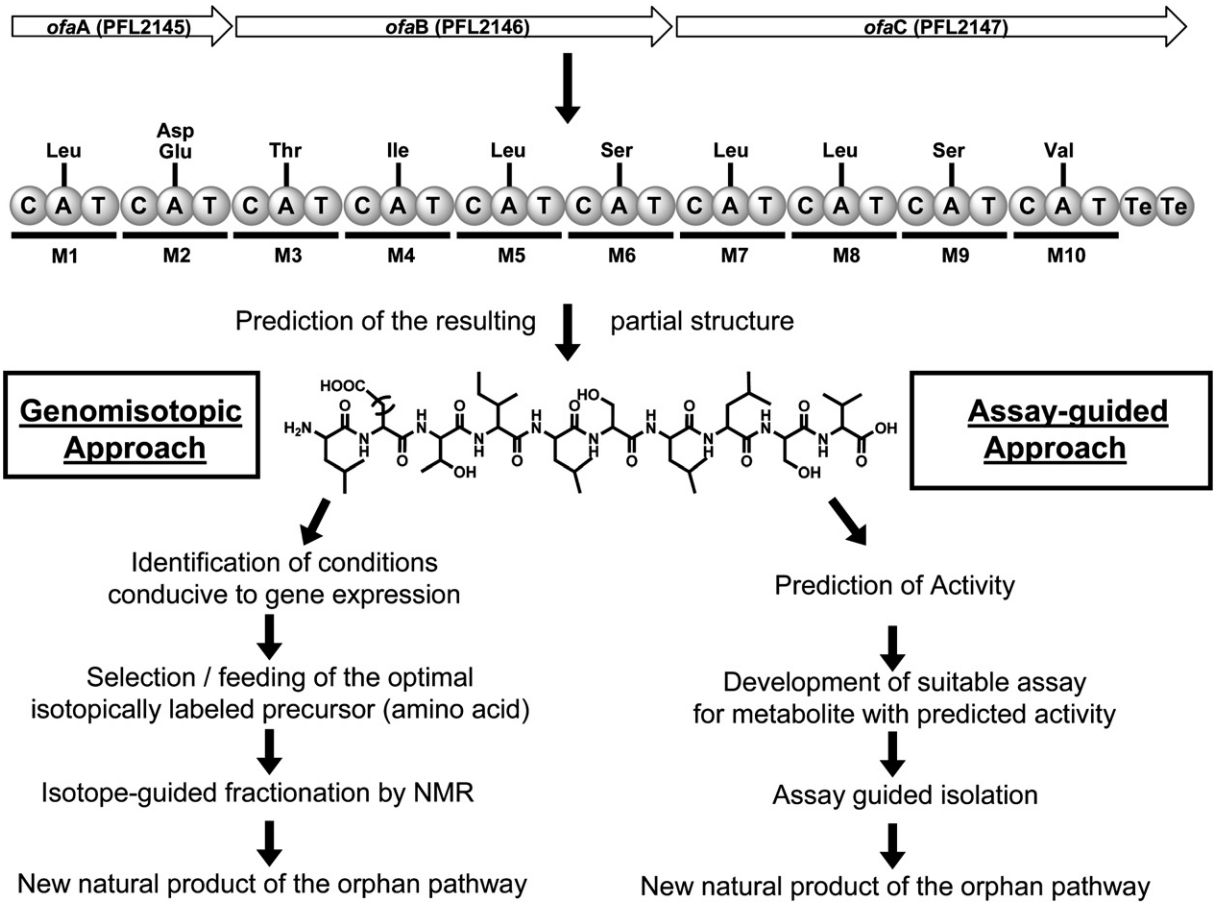

Figure 2. Flowchart of the Two-Pronged Approach Used to Isolate the Orfamides

The orfamide gene cluster contains three genes (*ofa*A, *ofa*B, and *ofa*C) encoding nonribosomal peptide synthetases (NRPSs). The domain organization of the NRPSs (C, condensation domain; A, adenylation domain; T, thiolation domain; Te, thioesterase domain; M, module) and the specific amino acid predicted from the sequence of each A domain is shown. The assay-guided approach relies on the development of an assay that guides natural product isolation. For the genomisotopic approach, the organism of interest is grown under culture conditions favorable to the expression of biosynthetic genes. A labeled amino acid predicted to be present in the metabolic product is added to the culture medium, and the label is used to guide a fractionation scheme for purification of the natural product.

Two-Pronged Approach to Isolate the Product of the Orphan Pathway, Orfamide A

The genomisotopic approach was evaluated in parallel with a traditional assay-guided approach for isolation of orfamide A. Because the structure of the predicted orphan compound resembles CLPs, the assay-guided arm of the study relied on the surfactant activity of CLPs. Initially, we identified a defined culture medium (MBDgly) in which *P. fluorescens* Pf-5 produced a biosurfactant and expressed the orfamide biosynthetic gene *ofa*B, assessed with RT-PCR (see Supplemental Data).

To optimize the specificity and sensitivity of the genomisotopic-guided approach, an amino acid that is incorporated exclusively into the cryptic metabolite is preferred. Four amino acids (Leu, Ile, and Glu/Asp), which are present in the predicted structure of orfamide A (Figure 2) and are absent from the other known Pf-5 metabolites, were added to MBDgly and evaluated for toxicity to Pf-5. None diminished growth or biosurfactant production at the intended feeding levels. Because the binding pockets of the A domains for Glu or Asp showed only moderate levels of sequence identity (70% and 80%, respectively) to known A domains, the Glu/Asp set was excluded from further consideration. Of the two remaining amino acids, Leu was selected over Ile because its predicted incorporation into orfamide A at four sites was expected to give a more robust NMR resonance signal. ^{15}N-labeled Leu was selected because its incorporation can be detected readily by ^{1}H-^{15}N HMBC NMR spectroscopy due, in part, to the low natural abundance of ^{15}N.

Structure Elucidation of Orfamide A (1)

Both the genomisotopic approach and the assay-guided approach led to the isolation of orfamide A (**1**), which was isolated as a yellowish glass from cultures of *P. fluorescens* Pf-5 at a yield of 5.2–8.4 mg/l. The molecular formula of **1** was deduced as $C_{64}H_{114}N_{10}O_{17}$ by HR ESI-TOF-MS and NMR data. The ^{1}H NMR spectrum of orfamide A (**1**)

Table 1. Amino Acid Sequence Alignments of Orfamide A with CLPs in the Viscosin and Amphisin Classes, which Contain 9 and 11 Amino Acids, Respectively

	Amino Acid Sequence									3-OH Fatty Acid
Viscosin	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	D-Val	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-10
Viscosinamide	**L-Leu**	**D-Gln**	**D-*allo*- Thr**	D-Val	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-10
WLIP	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	D-Val	**D-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-10
Massetolide A	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	**D-*allo*- Ile**	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-10
Massetolide B	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	**D-*allo*- Ile**	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-11
Massetolide C	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	**D-*allo*- Ile**	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-12
Massetolide D	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	**D-*allo*- Ile**	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Leu	C-10
Massetolide E	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	D-Val	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	**L-Val**	C-10
Massetolide F	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	D-Val	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Leu	C-10
Massetolide G	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	D-Val	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-11
Massetolide H	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	D-Val	**L-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-12
Pseudophomin A	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	**D-Ile**	**D-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-10
Pseudophomin B	**L-Leu**	**D-Glu**	**D-*allo*- Thr**	**D-Ile**	**D-Leu**	**D-Ser**	**L-Leu**	**D-Ser**	L-Ile	C-12
Predicted peptide (**1**)	**Leu**	**Asp/Glu**	**Thr**	**Ile**	**Leu**	**Ser**	**Leu**	**Leu**	**Ser**	**Val**	?
Amphisin	**D-Leu**	**D-Asp**	**D-*allo*- Thr**	D-Leu	**D-Leu**	**D-Ser**	**L-Leu**	D-Gln	L-Leu	L-Ile	L-Asp	C-10
Tensin	**D-Leu**	**D-Asp**	**D-*allo*- Thr**	D-Leu	**D-Leu**	**D-Ser**	**L-Leu**	D-Gln	L-Leu	L-Ile	L-Glu	C-10
Lokisin	**D-Leu**	**D-Asp**	**D-*allo*- Thr**	D-Leu	**D-Leu**	**D-Ser**	**L-Leu**	D-Ser	L-Leu	L-Ile	L-Asp	C-10
Pholipeptin	**D-Leu**	**L-Asp**	L-Thr	D-Leu	**D-Leu**	**D-Ser**	**D-Leu**	D-Ser	D-Leu	L-Ile	D-Asp	C-10
Arthrofactin	**D-Leu**	**D-Asp**	D-Thr	D-Leu	**D-Leu**	**D-Ser**	**L-Leu**	D-Ser	L-Ile	L-Ile	L-Asp	C-10

Amino acid residues shown in bold are conserved between the predicted peptide structure of orfamide A and the known CLPs.

exhibited three clusters of resonances typical of peptides: exchangeable downfield amide signals at δ 7.55–9.43; α proton resonances observed between δ 3.89 and 4.35; and side chain protons at δ 0.88–2.34. Additional evidence for the peptidic nature of **1** includes the presence of numerous carbonyl resonances (δ 170.9–180.0) in the ^{13}C NMR spectrum and the presence of peptide bonds, illustrated by characteristic IR bands at 3300, 1650, and 1540 cm^{-1}.

Standard amino acid analysis of the acid hydrolysate of **1** indicated the presence of the proteinogenic amino acids Thr, Ser, Glx, Val, Leu, and Met in the molar ratio of 1:2:1:1:4:1. Extensive NMR analysis of **1**, including ^{1}H-^{1}H-COSY, 1D- and 2D-TOCSY, HSQC-TOCSY, and HMBC NMR spectra, confirmed the presence of nine of ten amino acids and revealed that Glx was glutamic acid (Glu). The Met residue was revised to be a stereoisomer of Ile, which was not recognized as such by amino acid analysis due to its nonstandard chiral character. Data in support for this revision involved NMR and MS/MS data along with results of the absolute configuration analysis.

The remaining unassigned 14 carbon and 2 oxygen atoms not accounted for by amino acids consisted of 1 methyl (δ 14.3), 1 carbinol methine (δ 69.8), 1 carbonyl carbon (δ 174.9), and 11 aliphatic methylene carbons (δ 44.6, 38.5, and 9 × 30.6), suggesting a linear hydroxy-tetradecanoic acid. Correlations in the ^{1}H-^{1}H-COSY delineated a connected spin system for protons at δ 2.42 (H_2-2′), δ 4.08 (H-3′), and δ 1.49 (H_2-4′). The observed long-range ^{1}H-^{13}C coupling between H_2-2′ and C-1′ subsequently identified the fatty acid fragment as 3-hydroxy myristic acid (HMA).

Sequencing of the amino acids and the fatty acid in **1** was accomplished by HMBC and ROESY experiments (see Supplemental Data). Further evidence supporting this sequence was developed from ESI-MS/MS experiments. Collisionally induced fragmentation of the parent ion at *m/z* 1296 $(C_{64}H_{114}N_{10}O_{17}+H)^+$ gave ions at *m/z* 1197 (Ser2-Leu4-Leu3-Ser1-Leu2-Ile-Thr-Glu-Leu1-HMA), *m/z* 1110 (Leu4-Leu3-Ser1-Leu2-Ile-Thr-Glu-Leu1-HMA), *m/z* 884 (Ser1-Leu2-Ile-Thr-Glu-Leu1-HMA), *m/z* 797 (Leu2-Ile-Thr-Glu-Leu1-HMA), *m/z* 683 (Ile-Thr-Glu-Leu1-HMA), and *m/z* 340 (Leu1-HMA). Additionally, a second fragmentation pattern, deriving from initial cleavage and elimination of Thr, showed peaks at *m/z* 979 (Leu3-Ser1-Leu2-Ile-Thr[-H_2O]-Glu-Leu1-HMA), *m/z* 865 (Ser1-Leu2-Ile-Thr[-H_2O]-Glu-Leu1-HMA), *m/z* 779 (Leu2-Ile-Thr[-H_2O]-Glu-Leu1-HMA), *m/z* 665 (Ile-Thr[-H_2O]-Glu-Leu1-HMA), and *m/z* 552 (Thr[-H_2O]-Glu-Leu1-HMA) and thus provided additional support for the proposed sequence in orfamide A (**1**).

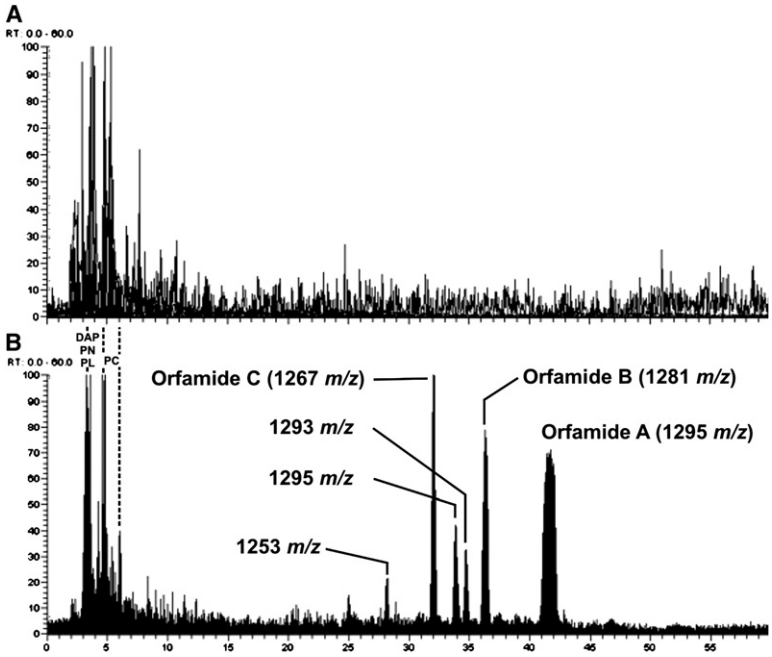

Figure 3. Secondary Metabolite Profiles of *P. fluorescens* Pf-5 and an Orfamide-Deficient Mutant of Pf-5

(A and B) Profiles of (B) wild-type strain Pf-5 and the (A) orfamide-deficient mutant were obtained by LC-MS (overlaid selected mass ranges). Compounds identified in the panels are DAP = 2,4-diacetylphloroglucinol with an m/z = 210–212 and t_R = 3.3 min, PN = pyrrolnitrin with an m/z = 256–258 and t_R = 3.6 min, PL = pyoluteorin with an m/z = 270–272 and t_R = 3.0 min, PC = pyochelin with an m/z = 324–326 and t_R = 4.8 and 5.9 min due to the formation of stereoisomers, and, finally, orfamides with an m/z = 1250–1300 and t_R = 28.0, 32.0, 33.8, 34.8, 36.5, and 41.9 min.

The configurations of the amino acids in **1** were determined by chiral GC-MS and Marfey's analysis. The acid hydrolysate of **1** was subjected to chiral GC-MS to yield L-Val, L-Leu, D-Glu, D-Ser, and D-*allo*-Thr. A modified Marfey's method was employed to resolve the configuration of the remaining Ile residue. Analysis of the derivatized orfamide A hydrolysate in comparison with all four stereoisomers of Ile showed it to be of D-*allo* configuration. The configuration of the hydroxyl group in the lipid side chain was not determined; however, since all β-hydroxy acids so far obtained from related lipopeptides possess an *S* configuration at this center, we assume that the HMA residue in orfamide A also has a 3′*S* configuration.

Characterization of an Ofa$^-$ Mutant of Pf-5

To unambiguously determine if the *ofa* gene cluster coded for orfamide A biosynthesis, two Ofa$^-$ mutants, which were deficient in biosurfactant production, were derived independently by allelic exchange mutagenesis of *ofa*A. Secondary metabolite production by one Ofa$^-$ mutant was evaluated further. Both Pf-5 and the Ofa$^-$ mutant produced pyoluteorin, pyochelin, pyrrolnitrin, and 2,4-diacetylphloroglucinol, but the Ofa$^-$ mutant did not produce orfamides A, B, or C or three related compounds, which were similar to the orfamides in mass range and polarity and were found in small amounts in the culture supernatants (Figure 3). Therefore, *ofa*A was required for orfamide production, but it was not required for the production of other known secondary metabolites. Due to the established role of CLPs in swarming motility and biofilm formation of other *Pseudomonas* sp. [14, 15, 24], Pf-5 and the Ofa$^-$ mutant were compared for these characteristics. Both Pf-5 and the Ofa$^-$ mutant attached to polystyrene surfaces, an important characteristic related to biofilm formation. In contrast, swarming motility of the Ofa$^-$ mutant was dramatically reduced from wild-type levels (Figure 4).

Biological Activity of Orfamide A

Pure **1** lysed zoospores produced by the oomycete causing "sudden oak death" (*Phytophthora ramorum*),

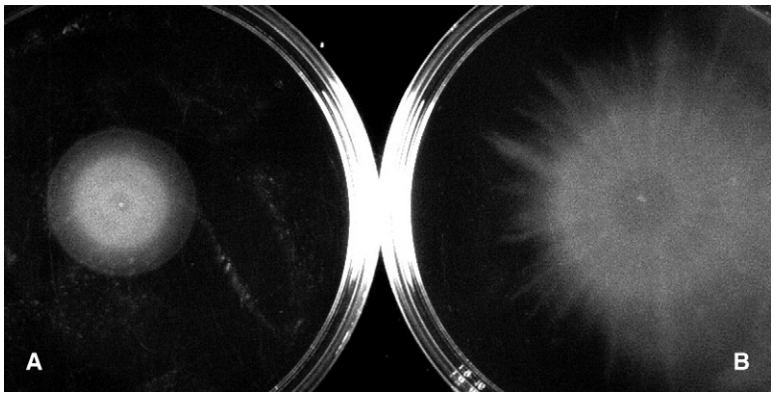

Figure 4. Swarming Motility of *P. fluorescens* Pf-5 and an Orfamide-Deficient Mutant of Pf-5

(A) Orfamide-deficient mutant.
(B) Wild-type *P. fluorescens* Pf-5.

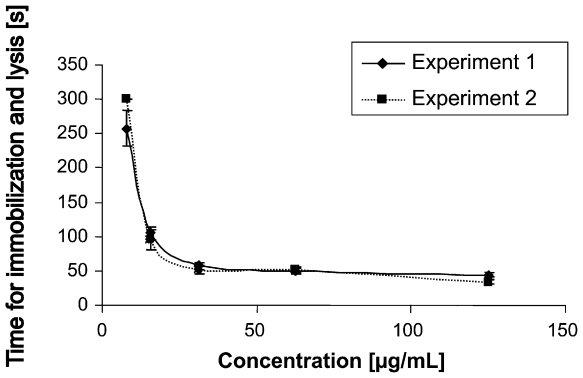

Figure 5. Effect of Orfamide A on the Viability of Zoospores of the Oomycete *Phytophthora ramorum*

Zoospores incubated in the presence of increasing concentrations of orfamide A lysed after decreasing times of exposure. Zoospores treated with orfamide A at greater than 25 μg/ml lysed in less than 60 s. The experiment was done twice. Error bars show the standard error of the mean.

a disease currently ravaging the oak woodlands in California [25]. Zoospores were rendered immotile upon exposure to orfamide A, and subsequent lysis occurred in a dose-dependent manner (e.g., within 60 s at 20 μg/ml, Figure 5). In contrast, orfamide A had no detectable effect on the growth or morphology of hyphae of *Pythium ultimum* or *Rhizoctonia solani*, plant pathogens that are suppressed by Pf-5 and are sensitive to other secondary metabolites produced by this bacterium [8–11]. In antifungal assays, orfamide A showed moderate activity (minimum inhibitory concentration [MIC] = 6.25 μg/ml) against an amphotericin B-resistant strain of *Candida albicans*, but no toxicity against the amphotericin B-sensitive parental strain.

DISCUSSION

The Genomisotopic Approach

In this investigation, we demonstrated the success of the genomisotopic approach through its application to an orphan biosynthetic gene cluster found in the genome of *P. fluorescens* Pf-5. This approach led to the isolation of the same product, orfamide A (**1**), that was obtained by using a predicted biological activity and assay-guided fractionation approach. The genomisotopic approach, which identifies unknown natural products by feeding isotopically labeled precursors identified through bioinformatic analysis of nucleotide sequence data, differs from traditional isotope-feeding experiments, which map out the biosynthetic units only after the chemical structure of the natural product is known. The genomisotopic approach represents a valuable complement to existing genome-mining strategies based on heterologous expression of orphan gene clusters, metabolic profiling coupled with mutagenesis of orphan genes, and assay-guided fractionation. The genomisotopic approach can be used to identify compounds encoded by large gene clusters, which are difficult to express in their entirety in heterologous hosts, as well as compounds lacking predictable bioactivity or physiochemical properties. Because it requires no previous knowledge of the activity or structure of a compound, the proposed strategy offers a powerful approach for the identification of novel compound classes encoded by orphan gene clusters. The genomisotopic approach was utilized to characterize a peptide in this study, but it could be applied to various other classes of secondary metabolites, including terpenes and shikimates, because the essential precursors to these natural product classes can also be predicted by using bioinformatics.

Selection of the appropriate amino acid is critical for success with the genomisotopic approach, and several selection criteria should be considered. Confidence in the bioinformatic prediction of the incorporated amino acid is of obvious importance. Some amino acids are toxic to certain organisms [26], necessitating the selection of alternative amino acids, a nontoxic precursor, or subtoxic concentrations. Preferably, the selected amino acid will be present only in the product of the orphan pathway, and it will not be incorporated into any other secondary metabolite produced by the organism. Stability of the amino acid is another important characteristic, as degradation can result in dispersal of the label to many other compounds.

Orfamides

The orfamides (**1**–**3**) are, to our knowledge, 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 ten amino acid cyclic peptide. The orfamides are distinct from all known CLPs produced by *Pseudomonas* spp., which fit into four major groups, defined by the length and composition of the peptide chain (ranging from 9 to 25 amino acids) and the lipid side chain (ranging from 6 to 16 carbons) [14, 13]. Although CLPs composed of 10 amino acids are produced by *Bacillus* strains (plipastatin-fengycin group) [27], the orfamides (**1**–**3**) show a different amino acid sequence and cyclization scheme.

The architecture of the *ofa* gene cluster follows the thiotemplate mechanism and is in full agreement with the structure of orfamide A (**1**). Because a mutation in the NRPS-encoding gene *ofa*A eliminated production of all three orfamides by Pf-5, it is clear that all three compounds are products of the same gene cluster. Therefore, the production of orfamide B (**2**) can be attributed to a relaxed substrate specificity of the A domain responsible for the activation of L-Ile. Similar relaxed substrate specificity of the C domain of module 1 could explain the altered fatty acid present in orfamide C (**3**). Neither the respective genes for the biosynthesis of the lipid side chain (e.g., β-ketoacyl-ACP synthetases or acyl-CoA ligases) nor genes involved in an acyltransfer of the 3-hydroxy fatty acid moiety (from the corresponding CoA derivative to the first amino group Leu1) were found up- or downstream of the orfamide operon. In other CLP-producing bacteria, fatty acids for the lipid side chain are synthesized or modified

by pathway-specific enzymes encoded from genes in the biosynthetic gene cluster, as for iturin A biosynthesis by *Bacillus subtilis* [28], or are provided by primary metabolism [29, 30]. Like the *ofa* gene cluster, the large genomic island containing the syringomycin and syringopeptin biosynthetic gene cluster of *P. syringae* lacks genes for fatty acid biosynthesis [31]. Therefore, the hydroxy fatty acids composing the lipid side chains of these CLPs may be produced by primary metabolism (e.g., type II fatty acid synthase systems). Because fluorescent pseudomonads accumulate and utilize 3-hydroxyalkanoates as a carbon and energy source [32], there is support for this hypothesis.

Biological Significance

Due to their properties as surfactants, CLPs have multiple effects on the ecology and physiology of *Pseudomonas* spp., including facilitating bacterial growth on water-insoluble substrates [33] or seed surfaces [34], influencing surface adhesion and swarming motility of bacterial cells [15, 24], and altering biofilm development and stability [24]. Orfamide A appears to share at least some of these properties, as its production has a marked effect on swarming motility of Pf-5 grown on agar surfaces. However, in contrast to the CLPs Arf and putisolvin I and II, orfamide A production did not have a detectable influence on the adhesion of Pf-5 cells to polystyrene surfaces. The processes involved in biofilm formation and development are complex, with adhesion being only one of many aspects. Effects of orfamide A on biofilm formation and other factors influencing its ecological fitness in soil and on plant surfaces are intriguing subjects for future study.

The remarkable structural diversity of CLPs produced by *Pseudomonas* spp. is reflected in the varied antibiotic properties of these compounds, many of which exhibit antifungal, hemolytic, antiviral, phytotoxic, or antibacterial activities [13, 14]. In this study, orfamide A exhibited zoosporicidal activity, observed as a rapid lysis of zoospores of the oomycete *Phytophthora ramorum*. Because zoospores lack a cell wall, they are especially vulnerable to membrane disruption caused CLPs [35]. Membrane disruption can result from nonspecific detergent properties or from the formation of transmembrane pores by CLPs [13]. As the sole freely motile propagule of the oomycetes, zoospores represent a critical stage in the infection cycle of diseases caused by this important group of plant, insect, fish, and animal pathogens. Thus, with the discovery of the orfamides, we have identified another important metabolite class that may contribute to the overall protective effect of *P. fluorescens* Pf-5 for its plant hosts.

In contrast to the toxicity of orfamide A to zoospores, the compound did not inhibit mycelial growth of a fungal or an oomycete plant pathogen in this study. CLPs produced by other strains of *Pseudomonas* spp. are known to inhibit mycelial growth or development of fungal and oomycete plant pathogens (reviewed in [13]). A CLP related to massetolide A, which lyses zoospores of several oomycete pathogens, is also thought to play a critical role in the biological control of root rot caused by the oomycete *Pythium ultimum* [35]. The antifungal effect of orfamide A on the amphotericin B-resistant strain of *Candida albicans* may be due to the fact that such strains have qualitative and quantitative alterations in the lipid composition of their membranes. The antifungal activities of CLPs can be attributed, at least in part, to their interactions with membranes, and orfamide A possesses the suitable size, distribution of polar and lipid moieties, and three-dimensional shape to interact with the altered membrane of the amphotericin B-resistant strains and, consequently, exert a disruptive effect.

SIGNIFICANCE

The genomisotopic approach provides a powerful new method by which to identify and mine the products of orphan biosynthetic gene clusters. This approach relies on bioinformatics to predict precursors of a natural product from the sequence of the biosynthetic gene cluster. A suitable ^{15}N or ^{15}N-^{13}C isotope-labeled precursor is then selected for exogenous feeding of cultures grown under conditions conducive to gene expression, identified by RT-PCR. Natural products produced from this precursor can then be tracked through the isolation process by selective NMR experiments. In the present study, this approach led to the discovery of orfamide A. Orfamide A is, to our knowledge, the first member of a new subclass of lipopeptides produced by *Pseudomonas* spp., which has intriguing physiological and ecological properties, including antimicrobial effects.

EXPERIMENTAL PROCEDURES

General Analytical Procedures

Analytical instrumentation and procedures were essentially as previously published [36] and are provided in the Supplemental Data. NMR spectra were referenced to the residual solvent signal with resonances at $\delta_{H/C}$ 3.31/49.0 (d_3-MeOH). ^{15}N chemical shifts were externally referenced to formamide at 112.0 ppm. Solutions of 15 mg sample/450 μl d_4-MeOH were used for ^{1}H-^{15}N HMBC NMR screening of fractions, if procurable. ^{1}H-^{15}N HMBC experiments were recorded with the standard Bruker pulse program (bmHMBCnolp, 256 × 1024 data points, 100 scans, spectral width of 350 ppm and 12 ppm in f1 and f2, D1 = 2 s, D6 = 63 ms, D16 = 250 μs).

Bioinformatic Analysis of the NRPSs in the Orfamide Biosynthetic Gene Cluster

Catalytic domains present in *ofa*A, *ofa*B, and *ofa*C, three genes predicted to encode NRPSs, were identified by using the web-based software NRPS-PKS [37], and the specificity prediction of the adenylation domains was conducted with the web-based software NRPS predictor [38].

Identification of Culture Conditions Conducive to Expression of an Orfamide Biosynthetic Gene

Reverse transcriptase PCR was used to identify culture conditions in which *P. fluorescens* Pf-5 expressed *ofa*B. Three replicate cultures of Pf-5 were grown in Difco Minimal Broth Davis without dextrose (Becton Dickinson; Sparks, MD) containing 20 mM glycerol (MBDgly) with shaking (150 rpm) at 20°C for 24 hr prior to harvest (OD_{600} of 1.1). RNAProtect (QIAGEN; Valencia, CA) was added to each culture, and RNA was extracted by using the RNA/DNA Midi Kit (QIAGEN),

followed by an on-column DNase treatment (RNeasy Mini Kit with DNase I; QIAGEN). Reverse transcription was done by using SuperScript II (Invitrogen; Carlsbad, CA). Samples processed in parallel without reverse transcriptase were negative controls. cDNA and negative controls from each replicate culture were subjected to PCR amplification by using primers complementary to *ofa*B (PF1260F and PF1260R; see Supplemental Data) and Thermalace (Invitrogen) according to the manufacturer's directions.

Extraction and Isolation of Orfamide A with the Assay-Guided Approach

Cultural Conditions

Starter cultures of Pf-5 were grown in 12.5 ml MBDgly in 50 ml Falcon tubes for 24–48 hr at 21°C in a New Brunswick Scientific C25KC incubator shaker at 150 rpm. Six 2800 ml Fernbach flasks containing 1.25 L MBDgly were inoculated with 1 ml starter culture. Cultures were incubated for 48 hr at 21°C with shaking at 150 rpm in darkness.

Biosurfactant Assay

A modification of the droplet collapse assay [24] was used to test the surfactant activity of bacterial cultures or fractions from solid-phase extraction, which were dried, weighed, and resuspended in sterile, deionized water to a concentration of 1 mg/ml. A 15 μl drop of test material was placed on parafilm (Pechiney Plastic Packaging; Neenah, WI). The flattening of droplets, which also was visualized as an increase in the diameter of the droplet, was observed over 5 min. The relative amount of surfactant activity was estimated with a dilution endpoint method. Controls for each assay consisted of water, noninoculated culture media, and cultures of a *gacA* mutant of Pf-5 (JL4577) [11], which did not produce detectable surfactant activity (data not shown).

Extraction and Isolation

Cell and supernatant fractions of the cultures were separated by centrifugation in a Jouan CR412 centrifuge at 2700 × g for 10 min at room temperature. Portions (0.5 liter) of the supernatant were extracted three times with 0.5 liter ethyl acetate to yield 180.0 mg crude extracellular extract. Cells (7.7 g wet wt) were washed twice with water, resuspended in acetone, and sonicated for 1 min. Unbroken cells and cell fragments were removed by centrifugation at 2700 × g for 10 min at room temperature, and the resulting supernatant was dried down by rotary evaporation to give 142.5 mg crude cellular extract. The EtOAc phase of the extracellular extract was active (at 1:40 dilution) in the droplet collapse assay. Thus, the extracellular crude extract was further fractionated by SPE (Phenomenex Strata C18-E, 2 g) by using stepwise gradient elution with H_2O-MeOH (50:50) to 100% MeOH, yielding three fractions. Fraction C (109.0 mg), which eluted with 100% MeOH, was found to be most active in the droplet collapse assay (1:75 dilution) and was therefore further chromatographed on SPE (Phenomenex Strata C18-E, 2 g) by using gradient elution from H_2O-MeOH (60:40) to 100% MeOH. The most bioactive fraction (1:75), eluting with H_2O-MeOH (10:90), yielded 62.9 mg pure orfamide A (**1**).

Extraction and Isolation of Orfamide A with the Genomisotopic Approach

Evaluation of Precursor Toxicity

Pf-5 was cultured in 5 ml MBDgly at 27°C with agitation at 200 rpm for 48 hr. L-amino acids were added to broth media at a final concentration of 0.37 mM, which was slightly higher than the intended dosage to be used in the ^{15}N feeding studies (0.32 mM). The growth rate of Pf-5 was monitored as an increase in optical density at 600 nm in triplicate cultures. Surfactant activity was assessed with the droplet collapse assay. This experiment was repeated three times.

Culture Conditions

Feeding studies with the isotopically labeled precursor ^{15}N-L-leucine (98%, spectra stable isotopes) were performed under the same culture conditions as described above, except on a 6 liter scale. ^{15}N-L-leucine (41.7 mg/l = 0.32 mM) was added in dH_2O 13 hr after inoculation, which represented the early stage of the exponential phase (12–20 hr).

Extraction and Isolation

Cell and supernatant fractions were worked up as described above and resulted in 53.0 mg crude cell extract and 201.4 mg crude EtOAc solubles from the culture supernatant. The occurrence of several correlations around 4 ppm on the ^1H NMR scale and around 110 ppm on the ^{15}N NMR scale in the ^1H-^{15}N HMBC spectra of the latter extract indicated a ^2J long-range coupling between peptidic-bonded nitrogens of amino acids to their corresponding α protons (N-C-H) and was therefore of further interest. This material was subfractionated by SPE (Phenomenex Strata C18-E, 2 g) by using stepwise gradient elution from H_2O-MeOH (50:50) to 100% MeOH to give three fractions, A–C. Fraction C (81.6 mg), eluting with 100% MeOH, showed strong ^2J correlations at about 100 ppm in the ^1H-^{15}N HMBC spectrum as well as several correlations between amide-bonded nitrogens of amino acids around 100 ppm on the ^{15}N scale and their corresponding β protons at 1.5–2.1 ppm on the ^1H NMR scale (^3J, N-C-C-H). Purification of this fraction by RP-HPLC by using a linear gradient of 50:50–90:10 CH_3CN-H_2O (0.05% TFA) over a period of 30 min, followed by isocratic elution at 90:10 for an additional 30 min (Phenomenex Synergi Fusion-RP 80, 250 × 10 mm, 4 μm in combination with a Phenomenex SecurityGuard Fusion-RP 10 × 10 mm precolumn; 2 ml/min flow rate; UV monitoring at 210 nm), yielded pure orfamide A (**1**, 31.2 mg, fraction C4, t_R = 45.1 min), orfamide B (**2**, 3.3 mg, fraction C3, t_R = 41.3 min), and orfamide C (**3**, 0.3 mg, fraction C1, t_R = 37.9 min). In the ^1H-^{15}N HMBC spectra of the pure compounds, orfamides A (**1**) and B (**2**) showed ^2J and ^3J couplings, respectively, in addition to one-bond correlations between peptidic nitrogens and their respective protons. No correlations in the ^1H-^{15}N HMBC spectra were observed for orfamide C (**3**), most likely due to the low concentration of this sample. Fraction C2 (2.6 mg, t_R = 39.8 min) also showed ^1J, ^2J, and ^3J correlations by ^1H-^{15}N HMBC. These were attributed to the presence of a mixture of at least two or three orfamide-related lipopeptides in this fraction.

Orfamide A, 1

Yellowish glass; $[\alpha]_D^{26}$ = +3.8° (*c* 0.5 MeOH); UV(MeOH) λ_{max} 208 nm (ε 14,400); HR ESI-TOF-MS $[M+H]^+$ *m/z* 1295.8494 (calc. for $C_{64}H_{115}N_{10}O_{17}$, 1295.8442, Δ +4.0 ppm). All data were determined with unlabeled orfamide A, which, prior to analysis, was repurified by RP-HPLC by using the same HPLC conditions as described above.

Absolute Stereochemistry of the Peptide Portion of 1

Unlabeled orfamide (1.3 mg) was dissolved in 1 ml 6N HCl and was hydrolyzed in a sealed vial at 100°C for 16 hr. The solvent was removed by using a stream of dry N_2, acetyl chloride (150 μM) in 2-propanol (500 μl) was added, and the solution was heated at 100°C for 45 min to give the isopropyl esters of the various amino acids. After evaporation in a stream of N_2, the residue was treated with pentafluoropropionic anhydride (300 μl) in CH_2Cl_2 (600 μl) at 100°C for 15 min. Excess reagents were again removed by evaporation under a stream of nitrogen, and the resulting mixture of isopropyl esters of N-(pentafluoropropyl)-amino acids was dissolved in CH_2Cl_2 (1 ml) and immediately analyzed by chiral capillary GC-MS by using an Alltech Chirasil Val column (25 m × 0.25 mm; 0.16 μm; program rate: column temperature held at 70°C for 10 min, then 70°C–100°C at 2°C/min, held at 100°C for 10 min, then 100°C–200°C at 15°C/min, held at 200°C for an additional 10 min; flow: 0.6 ml/min; inj. temp. 250°C). Helium was used as the carrier gas.

Standards were prepared and analyzed as described above by using pure amino acids (0.4 mg). Each peak in the chromatographic trace was identified by comparing its retention time with that of the corresponding amino acid standard and by coinjection. Retention times (t_r, min) of derivatized residues in the hydrolysate of **1** matched those of L-Val (10.94), D-*allo*-Thr (18.09), D-Ser (18.55), L-Leu (20.78), and D-Glu (39.22) (see Supplemental Data). However, because the retention times for D-Ile and D-*allo*-Ile standards were identical (13.77), stereochemical analysis of this residue in **1** was precluded by this method. Chiral HPLC analysis of the underivatized hydrolysate of **1** with a Phenomenex Chirex 3126 phase (4.6 × 250; 2 mM $CuSO_4$/MeCN [95:5],

0.9 ml/min, UV detection at 254 nm) suggested the presence of D-*allo*-Ile (49.9 min), but did not match the retention time of the standards (D-Ile, 46.7 min; D-*allo*-Ile, 43.6 min). Coinjection of the D-Ile or D-*allo*-Ile showed in both cases an enhancement of the Ile peak of the hydrolysate. In general, coinjections were also accompanied by strong shifts in retention time, suggesting possible saturation of the chiral selector ligand (D-penicillamine) or disturbance of the ligand exchange by the complex mixture of hydrolysis components. Therefore, Marfey's method [39] and its modifications [40–42] were utilized to determine the configuration of the Ile residue in **1**. Usually, Marfey's is unable to reliably resolve Ile and *allo*-Ile [41, 42]. However, it was recently shown that this method can discriminate between these stereoisomers when 1-fluoro-2,4-dinitrophenyl-5-D-leucine amide (D-FDLA) instead of 1-fluoro-2,4-dinitrophenyl-5-alanine amide (L-FDAA) is used as a derivatizing agent and subsequent RP-HPLC analysis of the resulting derivative is performed (Δt_r = 0.2 min) [43], or when L-FDAA derivatives and a chiral stationary phase HPLC (Δt_r = 0.8 min) are used [44, 45]. Consequently, 1.0 mg of **1** was hydrolyzed as described above and evaporated under N_2. To the residue was added 1 M $NaHCO_3$ (200 μl) and 1% L-FDAA in acetone (100 μl), and the solution was heated at 80°C for 40 min, cooled to room temperature, and neutralized with 2 N HCl (100 μl) and then diluted with CH_3CN (100 μl). Chiral HPLC analysis of a 5 μl aliquot through two different chiral columns in series (Daicel Chiralpak AD-H 4.6 × 250 column, followed by a Daicel Chiracel OD 4.6 × 250 column) with a linear gradient of 5%–25% isopropanol (0.05% TFA) in hexanes over 60 min at 1 ml/min (UV detection at 340 nm) cleanly separated D-*allo*-Ile (38.3 min) and D-Ile (41.1 min). Coinjection of the hydrolysate with the two standards unequivocally established this residue as D-*allo*-Ile.

^{15}N Incorporation Rate

Isotope enrichments in the leucine residues of **1** were calculated from the isotope cluster of the sodium adduct of the molecular ion from HR-ESI-TOF MS. Results were processed as previously described [46] to give 91.6 mole-percent unlabeled and 8.4 mole-percent singly ^{15}N-labeled species. No species with more than one heavy isotope was present. This result was confirmed by IRMS analysis in which a level of 8.34% of ^{15}N enrichment was determined.

Allelic Exchange Mutagenesis of *ofa*A to Generate an Ofa$^-$ Mutant of *P. fluorescens* Pf-5

A 1.7 kb PCR product containing 5′ and 3′ truncations of the orfamide biosynthetic gene *ofa*A, obtained with primers 1801_5′ENTR and 1801_3′ENTR, respectively (see Supplemental Data), was cloned into the pENTR/D-TOPO vector (Invitrogen) and integrated into the destination vector, pLVC-D [47], by using the clonase protocol described by Invitrogen. The resultant plasmid (pLVC-D containing 1.7 kb of *ofa*A) was introduced into the mobilizing strain *E. coli* S17.1 [48] by transformation, and was then transferred from S17.1 to Pf-5 via conjugation, selecting for tetracycline resistance (200 μg/ml) conferred by the plasmid. Because pLVC-D is a suicide plasmid in *Pseudomonas* spp., tetracycline-resistant colonies of Pf-5 were expected to have undergone a single-crossover event between the DNA cloned in pLVC-D and the corresponding sequence in the Pf-5 chromosome. Gene disruption and plasmid insertion were confirmed by using PCR with primers specific to the pLVCD vector (L*attB2* and U*attB1*) and genomic DNA sequences up- and downstream from the targeted genes (primers 5′mkr1801 and 3′mkr2, respectively). Two derivatives of Pf-5 with the expected insertion in *ofa*A were tested for surfactant activity, and both were deficient. One of the two mutants (termed Ofa$^-$) was characterized further, as described below.

Characterization of the Ofa$^-$ Mutant

Secondary Metabolite Production

To determine if the Ofa$^-$ mutant was deficient in orfamide production, both Pf-5 and the Ofa$^-$ mutant were grown on a 1 liter scale under the conditions described above. Supernatants were acidified with HCl to pH 2 prior to extraction with EtOAc. The EtOAc-soluble parts of the crude extracellular extracts (mutant, 40.3 mg; wild-type, 43.3 mg) were dissolved in MeOH (5 mg/ml) and profiled (Inj. volume 10 μl) by using the following gradient: linear gradient from 50:50:0.1 to 90:10:0.1 (CH_3CN/H_2O/TFA) in 30 min, isocratic elution at 90:10:0.1 for 10 min, followed by a linear gradient from 90:10:0.1 CH_3CN/H_2O/TFA to 100:0.1 CH_3CN/TFA in 20 min (Waters Symmetry C_{18}, 250 × 4.6 mm, 5 μm; 1.0 ml/min flow rate; TIC and PDA monitoring).

Production of pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol by Pf-5 and the Ofa$^-$ mutant was assessed by using published methods [49]. Briefly, the strains were grown in Difco nutrient broth (Becton Dickinson) containing 1% (w/v) glycerol at 20°C with shaking (200 rpm) for 48 hr. Pyrrolnitrin was extracted from the cell pellet by using acetone, and pyoluteorin and 2,4-diacetylphloroglucinol were extracted from the acidified supernatant (pH 2.0) by using ethyl acetate. Extracts were taken to dryness, dissolved in methanol, and spotted onto a silica gel 60 TLC plate containing a fluorophore (F_{254}) (EM Science; Gibbstown, NJ). Plates were developed in $CHCl_3$:MeOH (9:1), dried, and observed under UV light (254 nm). R_f values of UV-absorbing spots were compared to those of authentic standards for each of the three compounds.

Cell Adhesion

Pf-5 and the Ofa$^-$ mutant were grown overnight in King's Medium B broth (KMB) [50], and 1 μl of the overnight culture was inoculated into 100 μl KMB contained in each of three wells of a polystyrene 96-well plate (Falcon U-bottom, non-tissue culture treated) (BD Biosciences; San Jose, CA). Plates were incubated for 24 and 48 hr at room temperature and were rinsed twice with water to remove nonadhering cells, and 200 μl 0.1% (w/v) crystal violet was added to each well. After incubation for 15 min at room temperature, the crystal violet was removed from wells and the plates were rinsed twice with water. Plates were examined for crystal violet-stained rings of adhering bacterial cells. Crystal violet retention was quantified by adding 200 μl 95% ethanol to wells and reading the absorbance at 595 nm. The experiment was done twice.

Swarming Motility

The method of Kuiper et al. [24] was used to evaluate swarming motility on solidified media. Pf-5 and the Ofa$^-$ mutant were cultured overnight in KMB at 27°C. A 5 μl sample of the culture medium was spotted onto the center of the surface of five replicate plates containing 20-fold diluted KMB solidified with 0.3% (w/v) Bacto-Agar (Becton Dickinson). Plates were incubated at 27°C. The experiment was done twice.

Biological Activity of Orfamide A

Antifungal Assays

Wild-type and amphotericin B-resistant strains of *Candida albicans* were grown overnight in 30°C in RPMI 1640 media (Invitrogen). A hemacytometer was used to make a cell suspension of each strain at a concentration of 1 × 10^4 cells/ml. The indicator Alamar Blue 100× (TREK Diagnostic Systems) was added to the cell suspension; subsequently, cells were added to the microtiter plates. Test materials were added, serially diluted (2-fold), and incubated for 12–15 hr at 37°C. The viability of the fungal strains was quantified using the dye Alamar Blue to monitor the reductive environment of living cells. As a positive control, amphotericin B (0.5 mg/ml) was included; the solvent DMSO served as a negative control.

The plant pathogens *Pythium ultimum* isolate N1 and *Rhizoctonia solani* AG4 were grown on Potato Dextrose Agar (Becton Dickinson) for 48 hr. Agar plugs were removed from colony margins and were placed in the center of petri dishes containing either 25% potato dextrose agar for *R. solani* or simply 2% agar for *P. ultimum*. Orfamide A (100 μg) was dissolved in 10 μl methanol and placed on a 5 mm glass fiber filter disk. The disks were dried and placed on the agar surface 1.5 cm from the plug of inoculum. Plates were incubated at room temperature, radial growth was measured, and hyphal growth and morphology were assessed microscopically.

Zoospore Lysis

A zoospore suspension (40 μl) of *Phytophthora ramorum* Pr-102 (obtained from Niklaus Grunwald, USDA-ARS; Corvallis, OR) was placed

into a depression in a glass slide and was gently mixed with 40 μl orfamide A to a final concentration of 0.0, 7.8, 15.6, 31.3, 62.5, or 125.0 μg/ml. There were three replicate slides for each orfamide A concentration. Immediately after mixing, suspensions were observed under a stereomicroscope, and a timed measurement was taken for immobilization and lysis of the zoospores. The experiment was conducted twice with essentially identical results.

Supplemental Data

General analytical instrumentation and methods as well as tables detailing the bioinformatical analysis; proof of transcription of the *ofa* gene cluster; spectral data for the ^{1}H-^{15}N HMBC-guided isolation, NMR, and MS spectral data; and amino acid analysis of orfamides A–C (**1**–**3**), including structure elucidation for orfamides B (**2**) and C (**3**), are available at http://www.chembiol.com/cgi/content/full/14/1/53/DC1/.

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