JOURNAL OF BACTERIOLOGY, Apr. 1999, p. 2166–2174 Vol. 181, No. 7
0021-9193/99/$04.00+0

Characterization of the Pyoluteorin Biosynthetic Gene Cluster of *Pseudomonas fluorescens* Pf-5

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Received 27 October 1998/Accepted 19 January 1999

Ten genes (*plt*) required for the biosynthesis of pyoluteorin, an antifungal compound composed of a bichlorinated pyrrole linked to a resorcinol moiety, were identified within a 24-kb genomic region of *Pseudomonas fluorescens* Pf-5. The deduced amino acid sequences of eight *plt* genes were similar to the amino acid sequences of genes with known biosynthetic functions, including type I polyketide synthases (*pltB*, *pltC*), an acyl coenzyme A (acyl-CoA) dehydrogenase (*pltE*), an acyl-CoA synthetase (*pltF*), a thioesterase (*pltG*), and three halogenases (*pltA*, *pltD*, and *pltM*). Insertions of the transposon Tn*5* or Tn*3*-nice or a kanamycin resistance gene in each of these genes abolished pyoluteorin production by Pf-5. The presumed functions of the eight *plt* products are consistent with biochemical transformations involved in pyoluteorin biosynthesis from proline and acetate precursors. Isotope labeling studies demonstrated that proline is the primary precursor to the dichloropyrrole moiety of pyoluteorin. The deduced amino acid sequence of the product of another *plt* gene, *pltR*, is similar to those of members of the LysR family of transcriptional activators. *pltR* and *pltM* are transcribed divergently from the *pltLABCDEFG* gene cluster, and a sequence with the characteristics of a LysR binding site was identified within the 486-bp intergenic region separating *pltRM* from *pltLABCDEFG*. Transcription of the pyoluteorin biosynthesis genes *pltB*, *pltE*, and *pltF*, assessed with transcriptional fusions to an ice nucleation reporter gene, was significantly greater in Pf-5 than in a *pltR* mutant of Pf-5. Therefore, PltR is proposed to be a transcriptional activator of linked pyoluteorin biosynthesis genes.

Certain strains of *Pseudomonas fluorescens* produce secondary metabolites that are toxic to plant-pathogenic fungi. It is not surprising, therefore, that the production of antifungal compounds enhances the ability of these bacteria to suppress a variety of plant diseases and in some instances contributes to the ecological competence of the producing strain within the rhizosphere (16, 54). The mechanisms that regulate antifungal metabolite production in *P. fluorescens* include global regulatory factors that simultaneously affect multiple biosynthetic pathways (9, 18, 31, 46) and pathway-specific regulators that control linked biosynthetic genes (4, 41).

Pyoluteorin is an antibiotic that inhibits Oomycete fungi, including the plant pathogen *Pythium ultimum*, and suppresses plant diseases caused by this fungus (25). Pyoluteorin is composed of a resorcinol ring, derived through polyketide biosynthesis (10, 40), which is linked to a bichlorinated pyrrole moiety whose biosynthesis remains uncharacterized. Because halogenation can increase the pharmacological effects of many compounds (38), considerable effort has been directed toward the isolation and characterization of haloperoxidases, enzymes that are capable of forming carbon-halogen bonds in the presence of halide ions and hydrogen peroxide (56). It has yet to be demonstrated, however, that any of the haloperoxidases thus far characterized are responsible for the in vivo halogenation of known natural products. Like halogenation, little is known about pyrrole formation in secondary metabolic pathways. Whereas the pyrrole ring within porphobilinogen, a precursor to heme ring systems, originates from 5-aminolevulinic acid (26), the specific incorporation of [1,2-$^{13}C_2$]acetate into the pyoluteorin dichloropyrrole moiety indicates that this functionality is derived directly from a tricarboxylic acid cycle intermediate (10). Therefore, in addition to its ecological importance, pyoluteorin provides an opportunity to understand novel biochemical transformations involved in chlorinated pyrrole synthesis.

A genomic region of *P. fluorescens* Pf-5 that is required for pyoluteorin biosynthesis was identified previously by Tn*5* mutagenesis and cosmid cloning (30). We recently described the DNA sequence analysis of two open reading frames (ORFs) (*pltB* and *pltC*) within this genomic region that encode a type I polyketide synthase (40). PltB and PltC contain several discrete functional domains similar to those required for polyketide and fatty acid biosynthesis. These domains are organized into three distinct modules, two in PltB and one in PltC, that are thought to catalyze the formation of the resorcinol ring within pyoluteorin. By analogy to other type I polyketide synthases, each module is likely to incorporate and modify a single malonate extender unit into the resorcinol moiety of pyoluteorin. The pyoluteorin polyketide synthase is unusual, however, because it does not possess either a loading module or a thioesterase domain responsible for the initiation and the termination, respectively, of polyketide biosynthesis. This paper describes the further characterization of the pyoluteorin biosynthesis region. We report the nucleotide sequence of an additional eight ORFs within the pyoluteorin gene cluster, including seven putative biosynthetic loci and a regulatory gene encoding a transcriptional activator of linked

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
P. fluorescens		
Pf-5	Rhizosphere isolate; Plt$^+$, Amp^r	25
JL4365	*pltE*::Tn*3-nice* derivative of Pf-5; Plt$^-$, Ina$^+$, Km^r	30
JL4389	*plt*B::Tn*3-nice* derivative of Pf-5; Plt$^-$, Ina$^+$, Km^r	30
JL4390	*pltF*::Tn*3-nice* derivative of Pf-5; Plt$^-$, Ina$^+$, Km^r	30
JL4562	*pltM*::*aphI* derivative of Pf-5, constructed by marker exchange with pJEL6049; Plt$^-$, Tc^r, Km^r	This study
JL4563	*pltR*::*aacC1* derivative of Pf-5; Plt$^-$, Gm^r	This study
JL4564	*pltR*::*aacC1*, *pltE*::Tn*3-nice* derivative of Pf-5, constructed by marker exchange mutagenesis of JL4365 with pJEL6051; Plt$^-$, Gm^r, Km^r	This study
JL4565	Pf-5 with *pltR*::*aacC1*, *plt*B::Tn*3-nice* derivative of Pf-5, constructed by marker exchange mutagenesis of JL4389 with pJEL6051; Plt$^-$, Gm^r, Km^r	This study
JL4566	*pltR*::*aacC1*, *pltF*::Tn*3-nice* derivative of Pf-5, constructed by marker exchange mutagenesis of JL4390 with pJEL6051; Plt$^-$, Gm^r, Km^r	This study
E. coli		
DH5α	F$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96*, *relA1*, 80*dlacZ*, M15, λ$^-$	44
S17-1	Res$^-$, Mod$^+$, *recA*, Tra$^+$	50
Plasmids		
pLAFR3	IncP1 replicon; *cos*, Mob$^+$, Tc^r	51
pME3087	ColE1 replicon; Mob$^+$, Tc^r	59
pMGm	ColE1 replicon, source of *aacC1* cassette; Amp^r, Gm^r	36
pRK415	IncP1 replicon; Mob$^+$, Tc^r	27
pUC4K	ColE1 replicon, source of *aphI* cassette; Amp^r, Km^r	58
pUC18, pUC19	ColE1 replicon; Amp^r	44
pUC18Δ EcoRI	pUC18 with the *Eco*RI site removed from the polylinker; Amp^r	8
pJEL1938	29 kb of genomic DNA from Pf-5 containing part of the *plt* region cloned into pLAFR3; Tc^r	30
pJEL1939	ca. 24 kb of genomic DNA from Pf-5 containing part of *plt* region cloned into pLAFR3; Tc^r	30
pJEL5981	2.2-kb *Kpn*I-*Hin*dIII fragment from pJEL1938 containing *pltR* of Pf-5 cloned in pUC18Δ*Eco*RI; Amp^r	This study
pJEL6041	2.7-kb *Eco*RI fragment from pJEL1939 containing *pltM* cloned into pUC19; Amp^r	This study
pJEL6043	*pltR*::*aacC1*, constructed by cloning a 2.0-kb *Bam*HI fragment containing *aacC1* from pMGm into the *Eco*RI site internal to the *pltR* gene in pJEL5981; Amp^r, Gm^r	This study
pJEL6047	*pltM*::*aphI*, constructed by cloning an *Acc*I fragment containing *aphI* from pUC4K into the *Nsp*V site internal to *pltM* in pJEL6041; Amp^r, Gm^r	This study
pJEL6049	*pltM*::*aphI*, constructed by cloning 4-kb *Eco*RI fragment containing *pltM*::*aphI* from pJEL6047 into pME3087; Tc^r, Gm^r	This study
pJEL6051	*pltR*::*aacC1*, constructed by cloning the 4.4-kb *Hin*dIII-*Kpn*I fragment containing *pltR*::*aacC1* from pJEL6043 into pRK415; Tc^r, Gm^r	This study

pyoluteorin biosynthesis genes. In addition, we describe isotopic labeling studies that unequivocally demonstrate that proline is the primary precursor to the dichloropyrrole moiety of pyoluteorin. Although pathway intermediates have yet to be identified, the predicted functions of proteins encoded by pyoluteorin biosynthetic genes are consistent with a model specifying the biochemical transformations required for pyoluteorin biosynthesis.

MATERIALS AND METHODS

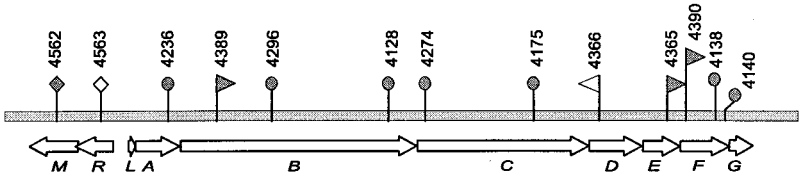
Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was cultured in Luria-Bertani medium (44) at 37°C. *P. fluorescens* was cultured routinely in King's medium B (28) at 27°C. Antibiotic concentrations were as follows unless otherwise specified: 100 μg of ampicillin per ml, 10 μg gentamicin per ml, 50 μg of kanamycin per ml, and 20 μg of tetracycline per ml.

DNA manipulations. Plasmids were isolated from *E. coli* and *P. fluorescens* by an alkali lysis method (44). Genomic DNA was isolated by a cetyltrimethylammonium bromide (CTAB) method with isopropanol precipitation (3). Electrophoresis was done in 0.7% (wt/vol) agarose gels with Tris-phosphate-EDTA buffer (44). Restriction digestions and ligation procedures were performed by standard methods (44).

Nucleotide sequence analysis. Sequence analysis was done on restriction fragments from pJEL1938 and pJEL1939, two members of a *P. fluorescens* Pf-5 genomic library that hybridized to DNA flanking Tn*5* insertions of Plt$^-$ mutants (30) (Fig. 1). Restriction fragments were cloned into pUC19, and sequencing was initiated from primers complementary to pUC19 and subsequently completed by "primer walking" in each direction across the length of the plasmid insert. Automated DNA sequence analysis and primer synthesis were performed by the Center for Gene Research Central Services Laboratory, Oregon State University, and by Macromolecular Resources Sequi-net Division, Colorado State University, by using dideoxynucleotide chain termination (45) on Applied Biosystems model 373A and 377 sequencers. Compilation, manual editing, and analysis of the sequence data were done with the University of Wisconsin Genetics Computer Group programs (19). Open reading frames were identified within the DNA sequence by codon usage analysis with the codon preference frequencies compiled for *P. aeruginosa* (60). The precise locations of Tn*5* and Tn*3-nice* insertions in the *plt* region (30) were determined from restriction fragments containing the transposon and flanking DNA of each Plt$^-$ mutant. Restriction fragments were cloned and DNA flanking transposon insertions was sequenced from primers complementary to a terminal region of Tn*5* (5′-GGTTCCGTTC AGGACGCTAC-3′) or Tn*3-nice* (5′-AGACCATTAAAAGAGGCGTCAGA G-3′).

Deduced peptide sequence analysis. Deduced protein sequences encoded by ORFs in the pyoluteorin gene cluster were compared to those in databases available through the National Center for Biotechnology Information (U.S. National Institutes of Health, Bethesda, Md.) with the BLAST algorithm and standard parameters of Altschul et al. (2). Functional domains and catalytic residues within the pyoluteorin biosynthetic enzymes were identified from protein sequence alignments performed with PILEUP (GCG version 8.0), using standard parameters.

PltA, PltD, and PltM were aligned with Cts4 of *Streptomyces aureofaciens* (11) and PrnC of *P. fluorescens* (20). The 5′ regions of *pltA*, *pltD*, and *pltM* were similar to nucleotides upstream of the assigned translational start site of *cts4*, and codon preference analysis of the chlortetracycline biosynthesis gene cluster suggested that the translational start site of *cts4* was likely to be located 306 nucleotides (nt) upstream of that assigned (39). Sequence alignments to *pltA*, *pltD*, *pltM* (39), and *prnC* (20) indicated that the *cts4* sequence contained a frame shift error between bases 2856 and 2859. Therefore, an additional 102 amino acid (aa)



ORF	Size (nt)	Deduced function	Possible function within pathway
pltA	1,347	halogenase	pyrrole chlorination
pltB	7,374	polyketide synthase	resorcinol synthesis
pltC	5,322	polyketide synthase	resorcinol synthesis
pltD	1,632	halogenase	pyrrole chlorination
pltE	1,140	acyl-CoA dehydrogenase	pyrrole synthesis
pltF	1,494	acyl-CoA synthetase	precursor activation
pltG	780	thioesterase	termination of resorcinol synthesis
pltL	264	unknown	unknown
pltM	1,506	halogenase	pyrrole chlorination
pltR	1,029	transcriptional regulator	pathway-specific regulator

FIG. 1. Pyoluteorin biosynthesis gene cluster of *P. fluorescens* Pf-5. ORFs (*pltA-G*, *pltR*, and *pltM*) identified in the gene cluster are indicated by large arrows. ↟, Tn*5* insertion site. ⚑ and ◁, Tn*3-nice* insertion site (the flag indicates the direction of transcription for the *inaZ* gene). Shaded and open flags represent the presence and absence, respectively, of ice nucleation activity in Pf-5 derivatives containing Tn*3-nice* insertions. ↟, *aphI* insertion site. ♀, *aacCI* insertion site. Strain numbers of Pf-5 derivatives are designated above the corresponding insertions.

residues encoded by nucleotides 2634 to 2858 and 2860 to 2940 of the reported *cts4* locus were added to the N terminus of Cts4 for our analysis.

Domains within PltR were identified by comparison with a LysR profile sequence by using standard parameters for the ProfileGap algorithm. The LysR profile was compiled by using ProfileMake (GCG version 8.0) from the following representative LysR-type regulators: PtxR (GenBank accession no. U35068) and TrpI (X51868) of *P. aeruginosa*, TcbR of an unidentified *Pseudomonas* species (M80212), RcbR of *Chromatium vinosum* (M64032), NahR (J04233) and CatR (U12557) of *P. putida*, LysR (J01614), LeuO (J03862), and IlvY (M14492) of *E. coli*, and GltC of *Bacillus subtilis* (M28509). The presence of a helix-turn-helix motif was confirmed with the program Helix-turn-helix version 1.0.5 (21a). The putative PltR-binding promoter sequence was identified manually by using the search routine in the sequence editor SeqEd (GCG version 8.0).

Insertional inactivation of *pltM* and *pltR*. The 2-kb *Bam*HI fragment containing *aacC1*, which confers gentamicin resistance, was isolated from pMGm and inserted into the *Eco*RI site internal to *pltR*, which had been cloned previously into the vector pUC18Δ*Eco*RI as a 2.2-kb *Hin*dIII-*Kpn*I fragment. The 4.4-kb *Hin*dIII-*Kpn*I fragment containing *pltR*::*aacC1* was then cloned into pRK415 to construct pJEL6051.

pltM was mutagenized by insertion of an *aphI* cassette from pUC4K, which causes nonpolar mutations in *P. fluorescens* (29). The 2.7-kb *Eco*RI fragment containing *pltM* was cloned from the cosmid pJEL1939 into pUC19. The 1.2-kb *Acc*I fragment containing *aphI* was isolated from pUC4K and cloned into the *Nsp*V site internal to *pltM*. The 4.5-kb *Eco*RI fragment containing *pltM*::*aphI* was then cloned into the suicide vector pME3087 to construct pJEL6049.

The *pltR*::*aacC1* and *pltM*::*aphI* fusions were introduced into the genome of Pf-5 or derivatives by marker exchange mutagenesis, exploiting the instability of pRK415 or the ColE1 replicon of pME3087, which does not replicate in *Pseudomonas* spp. pJEL6049 and pJEL6051 were mobilized from *E. coli* S17-1 to Pf-5 in conjugation experiments done as described previously (30), selecting for colonies that were resistant to ampicillin, to counterselect against the *E. coli* donor, and either kanamycin or 40 μg of gentamicin per ml, to select for mutants containing genomic integrations. Selected colonies were scored for growth on King's medium B containing 200 μg of tetracycline per ml, to determine if the plasmid vector was still present in the cell. Putative *pltR*::*aacCI* mutants (i.e., gentamicin-resistant, tetracycline-sensitive colonies selected following the introduction of pJEL6051 into Pf-5, JL4365, JL4389, or JL4390) were confirmed as such by Southern blot analysis. Putative *pltM*::*aphI* mutants (i.e., kanamycin-resistant, tetracycline-sensitive colonies selected following the introduction of pJEL6049 into Pf-5) were confirmed as such by PCR with primers flanking the *Nsp*V site internal to *pltM*.

Transcription of *plt* genes assessed with an ice nucleation reporter gene. The transposon Tn*3-nice* contains a promoterless *inaZ* gene that, when inserted into a gene in the appropriate orientation, generates a transcriptional fusion that confers ice nucleation activity (INA) on its bacterial host (30, 33). The effect of *pltR* on the transcription of the pyoluteorin biosynthesis genes was determined by comparing INA expressed by derivatives of Pf-5 containing insertions of Tn*3-nice* in genomic *plt* genes (JL4365, JL4389, and JL4390) (Fig. 1) to INA expressed by near-isogenic strains with *pltR*::*aacCI* mutations (JL4564, JL4565, and JL4566, respectively). INA was quantified by a droplet-freezing assay at −5°C as described previously (34) from cultures grown for 2 days with shaking at 20°C in nutrient broth (Difco Laboratories, Detroit, Mich.) amended with 2% glycerol. Data were analyzed with the general linear models program of Statistical Analysis Systems (SAS Institute Inc., Cary, N.C.). Treatments were replicated three times, the experiment was repeated, and the results of a representative experiment are presented.

Assessment of pyoluteorin production. Triplicate cultures of Pf-5 and derivative strains were shaken for 2 days at 20°C in nutrient broth amended with 2% glycerol. Pyoluteorin was extracted from culture supernatants and analyzed by high-pressure liquid chromatography (Waters Nova-Pak C_{18} reversed-phase column eluted with acetonitrile-methanol-water [30:25:45] at a flow rate of 1.5 ml/min, and photodiode array detection), as described previously (46). Pyoluteorin was detected by UV spectroscopy (λ = 310 nm, retention time of 3.4 min) and quantified with authentic pyoluteorin as a standard. Authentic pyoluteorin was purified from culture supernatants of Pf-5 and structurally characterized by nuclear magnetic resonance spectroscopy (NMR) as described below. The detection limit was 0.02 mg per liter of culture.

Isolation and isotopic labeling of pyoluteorin. For the biosynthetic studies, *P. fluorescens* Pf-5 was grown in a modified King's medium B composed of 2.0% phytone peptone (Becton-Dickinson, Cockeysville, Md.), 0.5% (wt/vol) glycerol, 0.15% K_2HPO_4, and 0.15% $MgSO_4 \cdot 7H_2O$ adjusted to pH 7.0 to 7.2. All cultures were grown at 20°C with stirring at 150 rpm. A 1-ml volume of a 24-h seed culture (40 ml in a 250-ml Erlenmeyer flask) was used to inoculate 100-ml cultures in 1-liter Erylenmeyer flasks.

Culture supernatants were extracted three times with 1/10 volume of ethyl acetate, and the combined organic phases were back-extracted three times with water (1/20 volume). The organic phase was dried over anhydrous $MgSO_4$, and the solvent was removed by rotary evaporation. The crude extract was fractionated by flash chromatography on silica gel (Silica Gel 60, 40 to 63 μm; EM Science, Gibbstown, N.J.) equilibrated and eluted with either 3:1 toluene-acetone or 4:1 $CHCl_3$-acetone. Pyoluteorin was detected as an orange-brown diazosulfanilic acid derivative when the column fractions were analyzed on thin-layer chromatography plates sprayed with a 1:2:3 mixture of 5% $NaNO_2$ and 0.9% sulfanilate in 1 M HCl–20% K_2CO_3 (43). Pyoluteorin was further purified from the combined fractions by recrystallization from hot $CHCl_3$.

The isolated pyoluteorin was identical to an authentic sample and exhibited the following ^1H and ^{13}C NMR resonances. ^1H NMR (300 MHz; d_6-acetone) δ 9.02 (br s, exchangeable), 7.15 (t, 1H, J = 8.2 Hz), 6.80 (s, 1H), 6.47 (d, 2H, J = 8.2 Hz), and 3.00 (br s, exchangeable). ^{13}C NMR (75 MHz; d_6-acetone) δ 183.1, 157.2, 132.5, 130.9, 119.5, 117.3, 113.0, 110.5, and 107.5.

Incorporation of proline into pyoluteorin was demonstrated initially by the following radioisotope feeding study. Equal portions of an aqueous solution containing 9.99 μCi (2.20×10^7 dpm) of L-[U-^{14}C]proline (ICN, Irvine, Calif.) were aseptically transferred to two 1-liter flasks, each containing 100 ml of 16-h-old cultures of the pyoluteorin-overproducing mutant JL4239. At 28 h later, the cultures were combined, the cells were removed by centrifugation, and pyoluteorin was extracted from the culture supernatant. A sample of unlabeled pyoluteorin (20 mg) was added to the crude extract, and pyoluteorin was recovered from the combined samples (yield, 56 mg or 207 μmol). The sample then was recrystallized to a constant specific radioactivity.

To demonstrate the specific incorporation of proline into pyoluteorin, equal portions of an aqueous solution containing 16 mg of L-[1-^{13}C]proline (CIL,

Cambridge, Mass.) were added to each of 10 1-liter flasks containing 100-ml cultures of Pf-5, 13 h after inoculation. The cultures were grown until 48 h after inoculation, at which point the cells were removed from the combined cultures and 47 mg (173 μmol) of pyoluteorin was isolated from the culture supernatant. Isotopic enrichment was determined by comparing the peak height observed for the carbonyl resonance (75 MHz, d_6-acetone; δ 183.0) in the ^{13}C NMR spectra of the enriched sample with the peak height of the same resonance observed in an unenriched sample.

Nucleotide sequence accession number. The accession number of the nucleotide sequence of the pyoluteorin gene cluster from Pf-5 is AF081920.

RESULTS

Identification of coding regions within the pyoluteorin biosynthetic gene cluster. We detected 10 ORFs within the pyoluteorin biosynthesis gene cluster of *P. fluorescens* Pf-5 (Fig. 1). Except for a 486-bp gap between the divergent coding regions of *pltL* and *pltR*, contiguous *plt* genes are separated by less than 50 bp. Putative ribosomal binding sites were identified for *pltLABCDEFG* and *pltR* but not for *pltM*. We presume that *pltM* and *pltR* are translationally coupled, because no ribosomal binding site could be identified for *pltM* and the ATG initiation codon of *pltM* overlaps the 3′ end of *pltR* by 4 bp. Whereas other genes within the pyoluteorin cluster exhibit a G+C bias of the third codon position in the range of 75 to 85%, the G+C bias of the first 500 nt of the *pltR* coding region is approximately 35 to 45%. Consequently, this region has a higher frequency of rare codons than do the other genes within the pyoluteorin gene cluster. An alternative start codon and ribosomal binding site for PltR exist approximately 60 nt upstream of the identified translation initiation site, but it is unlikely that this is the site of translation initiation, because of the presence of an in-frame stop codon 12 nt downstream. No alternative start sites are present within 100 nt downstream of the identified PltR initiation site.

Deduced protein sequences of pyoluteorin biosynthesis genes. Putative functions for each of the translated proteins encoded within the pyoluteorin gene cluster initially were assigned from database search results. Subsequently, motifs within the deduced amino acid sequence of each ORF (except *pltE* and *pltL*) were identified by sequence alignments with proteins of known function.

(i) PltA, PltD, PltM. The deduced amino acid sequences of PltA, PltD, and PltM are similar to the halogenating enzymes required for chlortetracycline biosynthesis by *Streptomyces aureofaciens* (Cts4) (11) and for pyrrolnitrin biosynthesis by *P. fluorescens* BL915 (PrnC) (20). Protein sequence alignment detected considerable similarity among the N-terminal and central regions for each of these five halogenases (Fig. 2). PltA and PltM contain the characteristic motif sequence $GxGx_2(G/A)x_3(G/A)x_6G$, which is believed to form the β-α-β structure required for NADH cofactor binding (49), whereas PltD does not contain this sequence motif.

(ii) PltE. The deduced amino acid sequence of PltE is similar to many flavin-dependent acyl coenzyme A (acyl-CoA) dehydrogenases, which catalyze the α,β-dehydrogenation of acyl-CoA thioesters involved in fatty acid and amino acid degradation. A butyryl-CoA dehydrogenase of *Megasphaera elsdenii*, whose three-dimensional structure has been determined (14), is the most thoroughly characterized acyl-CoA dehydrogenase to which PltE has sequence similarity. Sequence comparison of PltE to the butyryl-CoA dehydrogenase from *M. elsdenii* identified a 291-aa sequence overlap that contains 95 identical (32%) and 160 similar (54%) amino acid residues. The identified active-site Glu residue of the *M. elsdenii* butyryl-CoA dehydrogenase, located 17 residues from the C terminus, is conspicuously absent from PltE. Instead, PltE possesses a Glu residue at aa 243 that aligns with the catalytic Glu residues identified within the human isovaleryl-CoA (35) and long-chain acyl-CoA (13) dehydrogenases. It is thought that the location of this Glu residue determines, in part, the substrate specificity of these enzymes (32, 37). Therefore, it is possible that the identified Glu residue within PltE may function as a catalytic base. A Ser residue (shown in bold type) at position 132 in PltE lies within the consensus sequence TEPxAG**S**D, which is conserved in several short- and medium-chain CoA dehydrogenases. The X-ray crystal structure of the *M. elsdenii* butyryl-CoA dehydrogenase shows that this Ser residue forms a hydrogen bond to the pantetheine moiety of the substrate (14). Moreover, the Thr residue within this same consensus sequence is involved in binding of the flavin adenine dinucleotide cofactor required for catalytic function. Therefore, it is very likely that PltE catalyzes a dehydrogenation within the pyoluteorin pathway. PltE also has 175 identical (46%) and 227 similar (60%) amino acid residues of a total of 377 aa residues within three identified high-scoring segment pairs of RedW from *Streptomyces coelicolor* (AL021530), an acyl-CoA dehydrogenase involved in the biosynthesis of the proline-derived secondary metabolite undecylprodigiosin (5).

(iii) PltF. The deduced protein sequence of PltF is similar to several peptide synthetases including GrsB from *Bacillus brevis* (X61658), SnbC from *Streptomyces pristinaespiralis* (X98690), and PvdD from *P. aeruginosa* (U07359). PltF also contains core sequence motifs present in all adenylate-forming enzymes. Within PltF, the core C, D, and G sequences possess the most striking identity to conserved sequence motifs found within the peptide synthetases (Fig. 3) (42). Core sequences C and G are involved in adenylate formation and bind AMP and ATP, respectively, whereas core sequence F is an ATPase motif that binds ATP (42). A single Gly residue that is essential for amino acid activation by the gramicidin S synthase and that is invariant among other amino acid-adenylating enzymes (55) is also present within the core G sequence of PltF. PltF does not contain the core sequence motif involved in 4′-phosphopantetheine cofactor binding, whose absence is a defining characteristic for coumarate-CoA ligases and acetyl-CoA synthetases (12, 42). Nevertheless, the region of PltF surrounding core sequences E (involved in adenine binding [42]) and G appeared to be more highly conserved among the peptide synthetases than among the CoA synthetases or ligases (data not shown). In addition, PltF contains core sequence H (involved in adenine binding [42]), which is not found in the coumarate-CoA ligase and acetyl-CoA synthetase subfamily of adenylating enzymes (12).

(iv) PltG. The deduced protein sequence of PltG is similar to several thioesterases involved in secondary-metabolite production. PltG contains the motif GxSxG, which is found approximately 100 aa residues from the N terminus of all known thioesterases, and a second diagnostic motif, GxH, near the carboxy terminus. These motifs contain the catalytic Ser and His residues, respectively, that are essential for thioesterase activity (52, 61). In PltG, these motifs are separated by 131 aa residues, which is similar to the spatial arrangement observed in known thioesterases. Consensus sequences of amino acid residues surrounding the GxSxG and GxH motifs within rat thioesterase II (Y00311), GrsT of *B. brevis* (X15577), CmaT of *P. syringae* (B55543), and the thioesterase domain of the chicken fatty acid synthase (J04485) were identified as F^1xGHSF^2GAxIA and PGxHFF, respectively. PltG differs from the former motif by F^2-to-M and I-to-L substitutions and from the latter motif by a P-to-A substitution.

(v) PltL. Of the sequences currently in the National Center for Biotechnology Information databases, PltL shows the greatest similarity (30% identity and 56% similarity over a

FIG. 2. Multiple sequence alignment for PltA, PltD, PltM, Cts4, and PrnC. Residues in capital letters signify conserved substitutions. Invariant residues are indicated in bold capital letters. The consensus sequence was derived by using a plurality of four. Residues involved in forming the secondary structure required for NAD cofactor binding are indicated by asterisks within the boxed region. For this analysis, an additional 102 aa residues were added to the N terminus of the reported Cts4 sequence.

78-aa overlap) to the hypothetical protein SC3F7.09 of *Streptomyces coelicolor* (AL021409), an 87-aa peptide encoded by a member of the *red* gene cluster (5). PltL shows less similarity to a 69-aa acyl carrier protein (ACP) involved in fatty acid biosynthesis in *Rhodobacter sphaeroides* (7) and to presumed ACPs in *Borrelia burgdorferi* (AE001170) and *Haemophilus influenzae* (P43709). Although PltL does possess a conserved S residue which is required for 4′-phosphopantetheine binding in the ACP of *R. sphaeroides* (7), flanking amino acid residues deviate significantly from the ADSLD sequence motif present in the ACPs from *R. sphaeroides*, *B. burgdorferi*, and *H. influenzae*. Therefore, we refrain from assigning even a tentative function to PltL.

Insertional inactivation of *pltM*. The pyoluteorin biosynthesis region was defined initially by the location of Tn*5* insertions that abolished pyoluteorin production by Pf-5 (Fig. 1) (30). The pyoluteorin biosynthesis genes *pltLABCDEFG* are located within the defined region, whereas *pltM* falls outside of the region known previously to be required for pyoluteorin biosynthesis. To determine if *pltM* is required for pyoluteorin biosynthesis, we constructed a mutant of Pf-5 by inserting an *aphI* cassette into the genomic *pltM* gene. The resultant *pltM* mutant JL4562 did not produce detectable levels of pyoluteorin (<0.02 mg/liter) in experiments in which parallel cultures of Pf-5 produced 8.2 ± 0.2 mg of pyoluteorin/liter.

Deduced protein sequence of *pltR*. The deduced peptide sequence of PltR exhibits significant similarity to amino acid sequences of more than 20 members of the LysR family of transcriptional regulators (22, 47). For example, the deduced amino acid sequence of PltR was similar to those of GstR of *Rhizobium japonicum* (58% similarity over 293 aa) (AF007569) and *Rhizobium leguminosarum* (52% similarity over 281 aa) (53), CitB of *Klebsiella pneumoniae* (49% similarity over 296 aa) (6), and PtxR of *P. aeruginosa* (49% similarity over 300 aa) (21). The highly conserved N-terminal domain that is characteristic of all LysR-type proteins was readily apparent within PltR (Fig. 4). Further analysis with a calibrated weight matrix (15) predicted that a helix-turn-helix motif exists between residues 20 and 41 within the N-terminal region of PltR. In addition to the N-terminal domain, a coinducer-binding domain and a C-terminal domain were assigned by comparison of the PltR sequence with a 70% consensus sequence for each of the three domains common to LysR-type proteins (Fig. 4) (47). Furthermore, many amino acid residues along the entire length of a profile sequence compiled from 10 LysR-type pro-

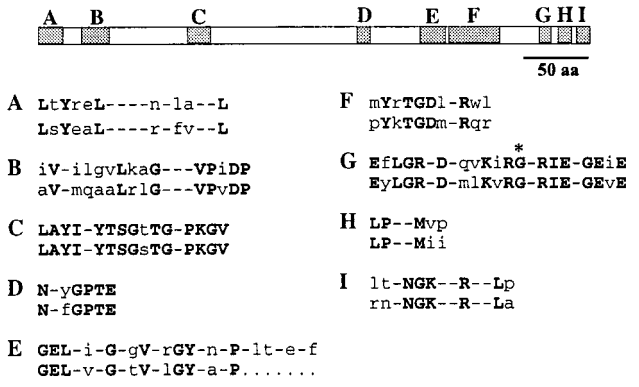


FIG. 3. Organization and sequence motifs identified within PltF. Shaded areas represent the locations of regions present within peptide synthetases that are involved in substrate adenylation. A consensus sequence for each of the regions is aligned above the corresponding region within PltF, and conserved residues are indicated in capital letters. A Gly residue within motif G that is invariant among all aminoacyl-adenylating enzymes is indicated by an asterisk. Motifs B, C, E, F, and G have been previously referred to in the literature as core sequences I, II, III, IV, and V, respectively.

teins representing several phylogenetic groupings (48) were also conserved within PltR.

Identification of a putative PltR-binding promoter sequence. An inverted repeat sequence (TGTAA-N_7-TTACA), which conforms to an Ebright box motif (TNTNA-N_7-TNANA) conserved among many promoters regulated by LysR-type proteins (17, 47), is centered 45 nt 5′ to the putative translational start codon of *pltR*. In addition, sequences flanking the identified Ebright box are particularly A+T rich, an attribute of some LysR-regulated promoters (reference 57 and references therein).

Influence of *pltR* on pyoluteorin production and transcription of pyoluteorin biosynthesis genes. *pltR* mutants, constructed by introducing a gentamicin resistance cartridge into *pltR* (Fig. 1), did not produce detectable concentrations of pyoluteorin, whereas parallel cultures of Pf-5 produced 4.3 mg of pyoluteorin per liter. Disruption of *pltR* in strains containing Tn*3-nice* insertions in *pltB*, *pltE*, and *pltF* allowed us to assess the influence of *pltR* on the transcription of pyoluteorin biosynthesis genes. INA expressed from *plt-inaZ* fusions in *pltR* mutants was 7 orders of magnitude lower than in analogous strains with a wild-type *pltR* gene (Table 2). Because INA is related to the square root of InaZ protein content in *Pseudomonas* spp. (33), these data indicate that *pltR*$^+$ strains had approximately 5,000 times more InaZ than did near-isogenic *pltR* mutants.

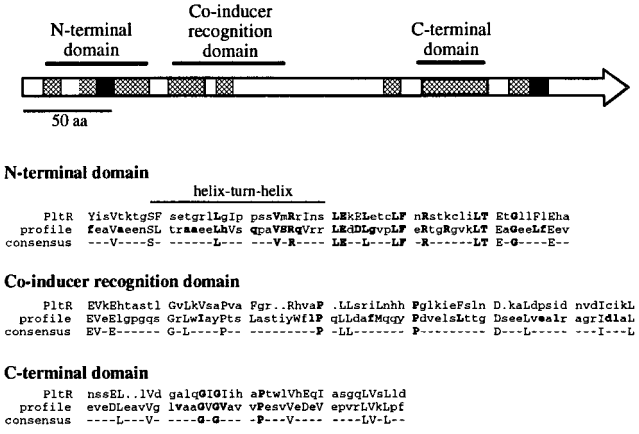


FIG. 4. Regions of PltR exhibiting homology to a LysR profile sequence, obtained by aligning the amino acid sequences of 10 LysR proteins. Solid and cross-hatched regions indicate sequence similarity of >50% and 30 to 49%, respectively, between PltR and the LysR profile sequence. Open regions indicate <29% sequence similarity. Sequence similarity was calculated from a ProfileGap alignment of the two sequences with a window of 10 aa residues. Sequence alignments identify conserved residues (capital letters) within PltR and the LysR profile sequences spanning the functional domains described by Schell (47). Bold type indicates residues that are conserved among the 10 representative LysR-type proteins comprising the profile sequence. These residues were identified in a Pileup alignment with a plurality of 7. The helix-turn-helix motif was identified by the method of Dodd and Egan (15).

TABLE 2. Influence of *pltR* on transcription of *plt* biosynthesis genes assessed with an ice nucleation reporter gene (*inaZ*)

Transcriptional fusion[a]	INA [log (ice nuclei per cell)][b]	
	pltR$^+$	*pltR*
pltE::Tn*3-nice*	−1.3 a	−8.8 b
pltB::Tn*3-nice*	−1.6 a	−8.9 b
pltF::Tn*3-nice*	−1.4 a	−8.8 b

[a] *pltE*::Tn*3-nice* derivatives were JL4365 (*pltR*$^+$) and JL4564 (*pltR*), *pltB*::Tn*3-nice* derivatives were JL4389 (*pltR*$^+$) and JL4565 (*pltR*), and *pltF*::Tn*3-nice* derivatives were JL4390 (*pltR*$^+$) and JL4566 (*pltR*).

[b] Values followed by the same letter do not differ significantly ($P = 0.05$) from one another, as determined by Duncan's multiple-range test.

Incorporation of proline into pyoluteorin. It had been suggested previously that the dichloropyrrole ring of pyoluteorin is derived from a tricarboxylic acid cycle intermediate, presumably through proline (10). Initial evidence for the incorporation of proline into pyoluteorin was obtained when L-[U-^{14}C]proline was added to a culture of *P. fluorescens* Pf-5. Following isolation and purification of pyoluteorin from a culture extract that also contained a known quantity of pyoluteorin to serve as an unlabeled carrier, the final specific radioactivity of the pyoluteorin produced in culture was calculated to be $1.18 \times 10^5 \pm 0.01 \times 10^5$ dpm/mg. This corresponds to 19% of the total added radioactivity being present within the pyoluteorin sample. Subsequently, L-[1-^{13}C]proline was added to cultures of Pf-5 cultures and a ^{13}C-enrichment of 5.7% over and above the natural abundance signal of the ^{13}C NMR carbonyl resonance for the purified pyoluteorin sample was observed, confirming the specificity of the proline incorporation.

DISCUSSION

The specific enrichment of the pyoluteorin ^{13}C NMR carbonyl resonance demonstrates that [1-^{13}C] proline was incorporated intact into the dichloropyrrole ring and establishes unequivocally that proline is the primary precursor to the dichloropyrrole ring in the pyoluteorin biosynthetic pathway of *P. fluorescens* Pf-5. Our labeling study corroborates and extends the conclusions of Cuppels et al. (10), who demonstrated that [1,2-$^{13}C_2$]acetate specifically labeled the pyoluteorin dichloropyrrole moiety in a manner consistent with the incorporation of a tricarboxylic acid cycle intermediate into the pyrrole ring of pyoluteorin. Several hypothetical pathways leading from proline to pyoluteorin are possible (Fig. 5). Despite extensive efforts to determine which of these pathways is responsible for pyoluteorin production in *P. fluorescens* Pf-5, we were not successful in demonstrating that any of several tested intermediates were incorporated into pyoluteorin (39). While we can only speculate on the identity of pathway intermediates, DNA sequence analysis of the pyoluteorin gene cluster has

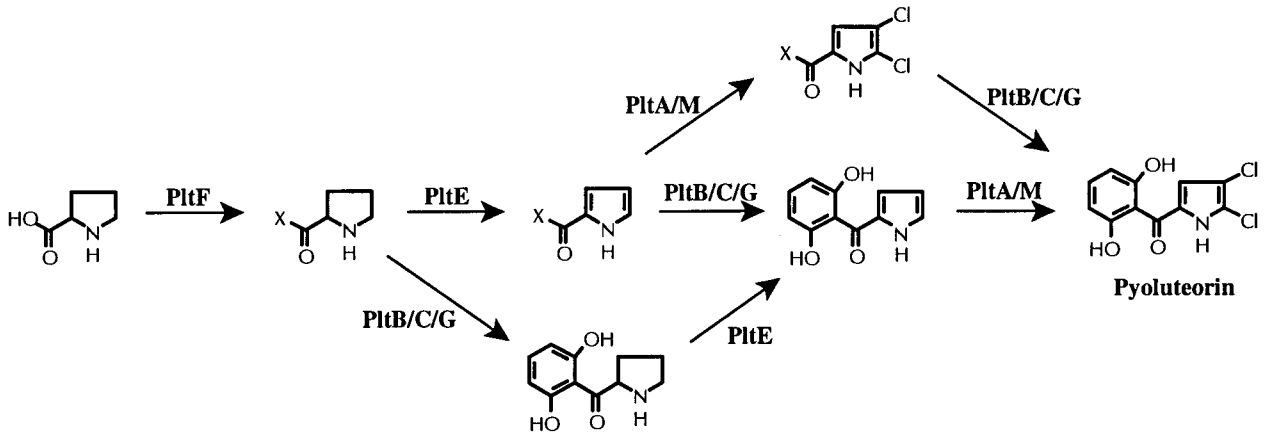


FIG. 5. Hypothetical pyoluteorin biosynthetic pathways. Arrows represent the predicted biochemical transformations catalyzed by the products encoded by the pyoluteorin gene cluster. The designator X indicates covalent attachment of proline either to CoA or to adenosine. PltD was omitted from this scheme based on the assumption that the absence of an NADH-binding region renders it nonfunctional.

identified enzyme activities that logically correlate with the proposed biochemical transformations required for pyoluteorin synthesis (Fig. 5).

Characteristic domains for the initiation or termination of polyketide assembly are not present in the pyoluteorin polyketide synthase composed of PltB and PltC (40). Typically, starter units used in polyketide assembly are activated as acyl-CoA derivatives and then are transferred to a ketosynthase domain within the polyketide synthase by way of an enzyme-bound 4′-phosphopantotheinyl cofactor (24). The presence of adenylation domains in PltF suggests that this protein activates an early pathway intermediate of pyoluteorin biosynthesis, such as proline, and that the activated intermediate is utilized to initiate polyketide assembly by PltB and PltC. It is not certain from sequence analysis, however, whether PltF is an acyl-CoA synthetase, as indicated by the presence of the core H sequence, or whether it is an AMP-ligase and simply catalyzes adenylate formation. If PltF does not generate an acyl-CoA intermediate, initiation of polyketide assembly may involve an ACP, perhaps PltL, in transferring the starter unit to the ketosynthase domain within module 1 of PltB. This mechanism is analogous to that proposed for initiating nonribosomal peptide biosynthesis by the pristinamycin I peptide synthetase in *S. pristinaespiralis* (12). Initiation of polyketide biosynthesis by using an ACP-bound starter unit may serve to channel amino acid starter units away from nonribosomal peptide pathways competing for activated substrates.

The putative thioesterase encoded by *pltG* is most probably responsible for termination of polyketide assembly. The identified motifs within PltG are predicted to hydrolyze the thioester formed between the fully extended polyketide substrate and the ACP domain of PltC. Considering the favorable transition state for formation of the resorcinol ring following polyketide assembly, it is likely that the required cyclization occurs concurrently with thioester hydrolysis. Nevertheless, we have yet to identify the outer limits of the pyoluteorin biosynthetic gene cluster, and other loci may exist that encode proteins required for cyclization and/or aromatization of the extended polyketide intermediate.

The formation of the pyrrole ring in pyoluteorin formally requires an oxidation of the proline-derived carbon ring, a transformation that most probably involves the catalytic activity of PltE. The overall similarity of PltE to many acyl-CoA dehydrogenases suggests that this protein introduces a double bond adjacent to a thioester linkage within the substrate and implies that PltF is an acyl-CoA synthetase despite the core H sequence motif therein. It is likely, therefore, that PltE catalyzes the formation of a $\Delta^{2,3}$-dehydroproline derivative before assembly of the pyoluteorin resorcinol ring but after the transformation catalyzed by PltF. Aromatization of the resulting pyrroline ring to the pyrrole may occur spontaneously, a reaction with precedence in the formation of pyrrole-2-carboxylate from a pyrroline intermediate of a 4-hydroxyproline catabolic pathway in a *Pseudomonas* sp. (1). The similarity of deduced peptide sequences of *pltE* and *redW*, a gene in the undecylprodigiosin biosynthesis locus of *S. coelicolor*, may reflect a comparable mechanism for the proline-to-pyrrole transformation in the pyoluteorin and undecylprodigiosin biosynthesis pathways.

PltA, PltD, and PltM belong to a new class of halogenase enzymes that chlorinate secondary metabolites (23, 56). Cts4 of the chlortetracycline biosynthetic gene cluster from *Streptomyces aureofaciens* (11) and PrnA and PrnC of the pyrrolnitrin biosynthetic gene cluster from *P. fluorescens* BL915 (20) are the only other previously described halogenases. PltA, PltD, and PltM exhibit sequence similarity only to PrnC, which also shares their proposed function in chlorination of a pyrrole derivative (29). With the exception of PltD, each of the halogenases possesses a putative NADH cofactor-binding site. The lack of a catalytic function for PltD, which could be inferred from the lack of an NADH-binding site, provides a rational explanation for the presence of three halogenase genes within the gene cluster of a dichloro-substituted product. Nevertheless, Tn*5* or *aphI* insertions in *pltA*, *pltD*, and *pltM* abolish pyoluteorin production, indicating a role for each of the genes in production of the antibiotic. Definitive evidence for the involvement of the three halogenases in pyoluteorin production will require the generation of nonpolar mutations in each of these genes.

The similarity of the deduced peptide encoded by *pltR* to other transcriptional activators, the presence of a LysR-type protein binding site (the Ebright box) 5′ to *pltR*, and the loss of pyoluteorin production in *pltR* mutants suggest that PltR is a positive transcriptional activator of linked pyoluteorin biosynthesis genes in *P. fluorescens* Pf-5. This conclusion is supported by studies demonstrating that transcription of *pltB*, *pltE*, and *pltF* genes, assessed from transcriptional fusions to an ice nucleation reporter gene, was lower in *pltR* mutants than in near-

isogenic *pltR*$^+$ derivatives of Pf-5. Previously, we reported the presence of at least two promoters in the *plt* region, based on ice nucleation activities expressed by Pf-5 harboring plasmids with transcriptional fusions of *inaZ* to *pltB* or *pltE* (30), but the specific locations of these promoters are not known. The Ebright box located 5′ to *pltR* was the only unambiguous LysR-type binding site detected in the *plt* gene cluster, but functional sites that lack the characteristic structure could be present. Future studies characterizing promoters within the *plt* region are needed to understand the structural and functional relationships involved in transcriptional regulation of pyoluteorin biosynthesis genes by PltR. *pltR* provides the second example of a gene encoding a positive transcriptional activator linked to loci encoding the biosynthesis of an antifungal metabolite in *Pseudomonas* spp. The other example is the *luxR* homolog *phzR*, which, in concert with the *luxI* homolog *phzI*, regulates the transcription of linked phenazine biosynthesis genes in *Pseudomonas aureofaciens* (41). Due to the positive self-regulation characteristic of the *luxR* and *lysR* homologs, both types of regulators provide a mechanism for amplifying an environmental or physiological signal that controls the expression of antibiotic biosynthesis genes. The *phzI-phzR* gene pair activates the transcription of phenazine biosynthesis genes in response to increased cell density (41), but signals required for the transcription of *pltR* and production of a putative PltR coinducer, which is typically required for optimal activity of LysR regulators (47), are yet to be identified.

ACKNOWLEDGMENTS

We extend our gratitude to Marcella Henkels for assistance in preparing the figures and to Carol Bender, Mary Hagen, Phil Proteau, and Mark Schell for their critical reviews of the manuscript.

Portions of this work were funded by a Tartar fellowship to BNT from the Department of Chemistry, Oregon State University.

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