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# Four Genes from *Pseudomonas fluorescens* That Encode the Biosynthesis of Pyrrolnitrin

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**Pyrrolnitrin is a secondary metabolite of *Pseudomonas* and *Burkholderia* sp. strains with strong antifungal activity. Production of pyrrolnitrin has been correlated with the ability of some bacteria to control plant diseases caused by fungal pathogens, including the damping-off pathogen *Rhizoctonia solani*. *Pseudomonas fluorescens* BL915 has been reported to produce pyrrolnitrin and to be an effective biocontrol agent for this pathogen. We have isolated a 32-kb genomic DNA fragment from this strain that contains genes involved in the biosynthesis of pyrrolnitrin. Marker-exchange mutagenesis of this DNA with Tn*5* revealed the presence of a 6.2-kb region that contains genes required for the synthesis of pyrrolnitrin. The nucleotide sequence of the 6.2-kb region was determined and found to contain a cluster of four genes that are required for the production of pyrrolnitrin. Deletion mutations in any of the four genes resulted in a pyrrolnitrin-nonproducing phenotype. The putative coding sequences of the four individual genes were cloned by PCR and fused to the *tac* promoter from *Escherichia coli*. In each case, the appropriate *tac* promoter-pyrrolnitrin gene fusion was shown to complement the pyrrolnitrin-negative phenotype of the corresponding deletion mutant. Transfer of the four gene cluster to *E. coli* resulted in the production of pyrrolnitrin by this organism, thereby demonstrating that the four genes are sufficient for the production of this metabolite and represent all of the genes required to encode the pathway for pyrrolnitrin biosynthesis.**

The ability of certain antagonistic bacteria to protect plants from soil-borne fungal pathogens is well established (21, 23, 38, 39, 50). Biological control of plant pathogens, widely known as biocontrol, is strongly correlated with the production by the bacterial antagonists of antifungal factors such as antibiotics, hydrolytic enzymes, and siderophores (4, 21, 23, 41, 48). Production of the antifungal metabolite phenazine by a biocontrol pseudomonad in the rhizosphere of wheat and correlation with biocontrol activity have been demonstrated (42). The ability of biocontrol bacteria to produce pyrrolnitrin [Prn; 3-chloro-4-(2′-nitro-3′-chlorophenyl)-pyrrole] (Fig. 1) has been correlated with biocontrol activity of fungal plant pathogens (19, 20, 22). Prn and its production by *Pseudomonas* species were first described by Arima et al. (3). This highly active metabolite has been used as a clinical antifungal agent for the treatment of skin mycoses (40, 43), and a phenylpyrrole derivative of Prn has been developed as an agricultural fungicide (15, 33).

Previously, we described *Pseudomonas fluorescens* BL915, which is an effective biocontrol agent of *Rhizoctonia solani* and other soil-borne plant pathogens, and we described a DNA fragment from this organism that contains a gene required for the synthesis of Prn (17). In a subsequent report, we demonstrated that, in addition to Prn, strain BL915 also produces other antifungal metabolites, including chitinase, 2-hexyl-5-propyl resorcinol, and hydrogen cyanide (HCN) (14). Furthermore, the gene isolated by Hill et al. (17) was characterized and shown to be a homolog of the *Pseudomonas gacA* gene (14). It encodes a transcriptional activator typical of two-component bacterial regulatory systems. This gene, with the *lemA* gene homolog, was shown to coordinately regulate the synthesis of all known antifungal metabolites produced by the strain, including Prn. Laville et al. (28) reported the involvement of a global regulatory gene (*gacA*) in the regulation of the coordinate synthesis of multiple antifungal metabolites and biocontrol activity in a *P. fluorescens* strain. Similar two-component global regulatory systems for the regulation of antifungal factors are thought to be common in biocontrol bacteria. This has made it difficult to identify the structural genes for individual antifungal factors by mutagenesis, since regulatory mutants affected in the global regulation of all antifungal factors are commonly found (18). For example, one report described the identification of a Prn-nonproducing *Pseudomonas* mutant (35) which upon further investigation was found to be mutant in *rpoS*, which has a role in the regulation of secondary metabolite biosynthetic genes, including those for Prn (37). A screening system designed to identify genes that are regulated by such regulatory systems was recently described and used to identify a mutant of *P. fluorescens* BL914 that is deficient in the production of Prn but still produces chitinase and HCN (26). We used this mutant to isolate a genetic region from *P. fluorescens* BL915 that contains a cluster of four open reading frames (ORFs) which are each specifically required for the synthesis of Prn. Transfer of these genes and induction of their expression in *Escherichia coli* resulted in the production of Prn in this organism, indicating that the four genes encode the complete pathway for the biosynthesis of Prn. This report constitutes the first description of genes that encode enzymes directly involved in the biosynthesis of Prn.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and bacterial culture conditions.** The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown at 37°C in liquid Luria-Bertani (LB [7]) medium or LB medium supplemented with agar (15 g/liter). *P. fluorescens* strains were cultured at 28°C in LB medium or *Pseudomonas* minimal medium (30), supplemented with agar when required. When necessary, growth media were supplemented with antibi-

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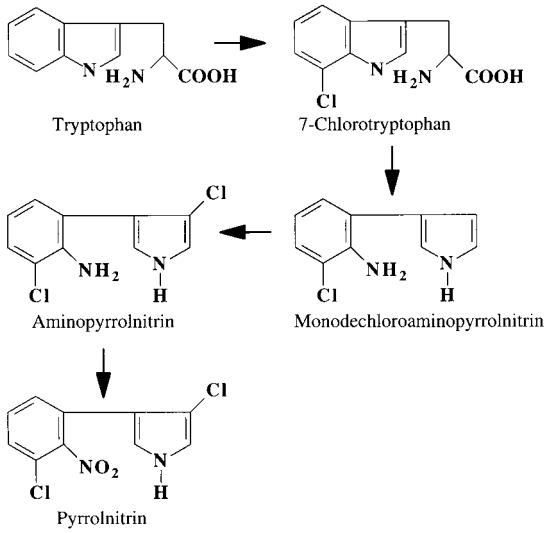


FIG. 1. Proposed biosynthetic pathway for the synthesis of Prn according to van Pée et al. (47).

otics as follows: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; and kanamycin, 50 μg/ml.

**Marker-exchange Tn*5* mutagenesis.** Tn*5* mutagenesis of DNA cloned in plasmids was accomplished using λ::Tn*5* bacteriophage (5). λ::Tn*5* phage, with temperature-sensitive replication, were used to transfect *E. coli* HB101 (9) containing target plasmids at 37°C. Tn*5* insertion mutants were selected on LB agar containing kanamycin. Plasmid DNA was prepared, en masse, from the population of Tn*5* insertion mutants and used to transform *E. coli* HB101 to isolate plasmids with Tn*5* insertions. The location of the Tn*5* insertion in each plasmid was mapped by using restriction endonucleases, and those with insertions in the plasmid vector were discarded. Each Tn*5* insertion with flanking DNA was cloned as an *Eco*RI fragment into pBR322 (8) and mobilized into *P. fluorescens* BL915 by triparental mating (12). In each case, mutants in which the Tn*5* had been exchanged through homologous recombination between the plasmid and chromosome were isolated based on resistance to kanamycin. These were verified by positive Southern hybridization results with Tn*5* DNA and negative hybridization with pBR322 DNA.

**DNA sequencing.** DNA sequencing was accomplished by using a model 373A automated DNA sequencer and Taq DyeDeoxy Terminator Cycle Sequencing kits (Applied Biosystems, Inc.). DNA sequence data were assembled and edited by using Inherit software (Applied Biosystems, Inc.). The sequence was analyzed for the presence of ORFs by using the FRAMES program (16) and incorporating the codon preference for BL915 determined from the sequence of the *uvrC* gene homolog derived from this strain (14).

**Construction of *prn* gene disruption mutants.** Individual gene deletion mutations were created independently in each ORF, in vitro, by restriction digestion to remove small DNA fragments internal to the ORF coding sequence followed by religation. The aminoglycoside 3′-phosphotransferase (APH) gene cassette from pUC4K (Pharmacia), conferring resistance to kanamycin, was cloned in place of the deleted regions to facilitate subsequent introduction of the disrupted ORFs into the chromosome of BL915 by homologous gene replacement as described above for the Tn*5* mutants. The individual deletion constructs were generated as follows:

**ΔORF1.** Plasmid pPRN1.77E was digested with *Mlu*I to liberate a 78-bp fragment from within the coding sequence of ORF1 (Fig. 2C). The vector-containing DNA fragment was recovered and blunt ended with T4 DNA polymerase, and the APH gene cassette was ligated into the plasmid. The *Eco*RI fragment containing the gene disruption was excised from the pBluescript II vector (Stratagene) and ligated into the *Eco*RI site of pBR322.

**ΔORF2.** Plasmids pPRN1.24E and pPRN1.01E, with contiguous *Eco*RI fragments containing ORF2, were digested with *Eco*RI and *Xho*I (Fig. 2C). The 0.69-kb *Eco*RI/*Xho*I fragment from pPRN1.01E and the 1.09-kb *Xho*I/*Eco*RI fragment from pPRN1.24 E were recovered and ligated together into the *Eco*RI site of pBR322. The resulting plasmid was linearized with *Xho*I, and the APH gene cassette was ligated into this site.

**ΔORF3.** Plasmid pPRN2.5S was digested with *Pst*I to liberate a 350-bp fragment from within the coding sequence of ORF3 (Fig. 2C). The vector-containing fragment was recovered, and the APH gene cassette was ligated into the *Pst*I site. The *Sph*I fragment containing the gene disruption was excised from the vector and ligated into the *Sph*I site of pBR322.

**ΔORF4.** Plasmid pPRN2.18 E/N was digested with *Aat*II to liberate a 156-bp fragment from within the coding sequence of ORF4 (Fig. 2C). The vector-containing fragment was recovered and blunt ended, and the APH gene cassette was ligated into the plasmid. The *Eco*RI fragment containing the gene disruption was excised from the vector and ligated into the *Eco*RI site of pBR322.

**ΔORF1-4.** The 16-kb *Kpn*I fragment (Fig. 2A) that includes all four ORFs was cloned into a modified version of the plasmid vector pKK223-3MCS. Plasmid pKK223-3MCS was derived from plasmid pKK223-3 (Pharmacia) by ligating a short DNA fragment containing *Xba*I, *Xho*I, *Kpn*I, and *Not*I restriction sites (in that order) between the *Pst*I and *Hin*dIII cloning sites of pKK223-3. The *Eco*RI, *Not*I, and *Bam*HI sites of pKK223-3MCS were individually removed by filling in the ends of each restricted site with T4 DNA ligase and religation of the ends. The 16-kb *Kpn*I fragment was subsequently ligated into the sole *Kpn*I site of the modified pKK223-3MCS to create plasmid pPRN16K. Plasmid pPRN16K was digested with *Eco*RI and *Not*I to remove the fragments containing ORF1 to

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Characteristics[a] | Reference or source |
|---|---|---|
| *P. fluorescens* | | |
| BL915 | $Prn^+$ $Cht^+$ $HCN^+$ $Res^+$ | 17 |
| BL914 | $Prn^-$ $Cht^-$ $HCN^-$ $Res^-$ | 17 |
| BL915ΔORF1 | Deletion in ORF1 of BL915, $Prn^-$ $Km^r$ | This study |
| BL915ΔORF2 | Deletion in ORF2 of BL915, $Prn^-$ $Km^r$ | This study |
| BL915ΔORF3 | Deletion in ORF3 of BL915, $Prn^-$ $Km^r$ | This study |
| BL915ΔORF4 | Deletion in ORF4 of BL915, $Prn^-$ $Km^r$ | This study |
| BL915ΔORF1-4 | Deletion of ORF1 to ORF4 of BL915, $Prn^-$ $Km^r$ | This study |
| BL915APH | BL915, insertion of APH gene 5′ to ORF1 to ORF4 | This study |
| Plasmids | | |
| pCIB189 | pBR322 with *Not*I site at *Eco*RI | This study |
| pCIB169 | pCIB119 with genomic DNA of BL915 containing ORF1 to ORF4 | This study |
| pPRN14N | 14-kb *Not*I fragment from pCIB169 cloned into pCIB189 | This study |
| pPRN18N | 18-kb *Not*I fragment from pCIB169 cloned into pCIB189 | This study |
| pPRN16K | 16-kb *Kpn*I fragment from pCIB169 cloned into pKK223-3MCS | This study |
| pPRN1.77E | 1.77-kb *Eco*RI fragment from pPRN18N cloned into pBluescript II SK | This study |
| pPRN1.24E | 1.24-kb *Eco*RI fragment from pPRN18N cloned into pBluescript II SK | This study |
| pPRN1.01E | 1.01-kb *Eco*RI fragment from pPRN18N cloned into pBluescript II SK | This study |
| pPRN2.5S | 2.5-kb *Sph*I fragment from pPRN18N cloned into pBluescript II SK | This study |
| pPRN2.18E/N | 2.18-kb *Eco*RI/*Not*I fragment from pPRN18N cloned into pBluescript II SK | This study |
| pPRN5.8X/N | 5.8-kb *Xba*I/*Not*I fragment from pPRN18N cloned behind the *tac* promoter in pKK223-3MCS | This study |

[a] Prn, Cht, HCN, and Res represent pyrrolnitrin, chitinase, hydrogen cyanide, and 2-hexyl-5-propyl resorcinol, respectively.

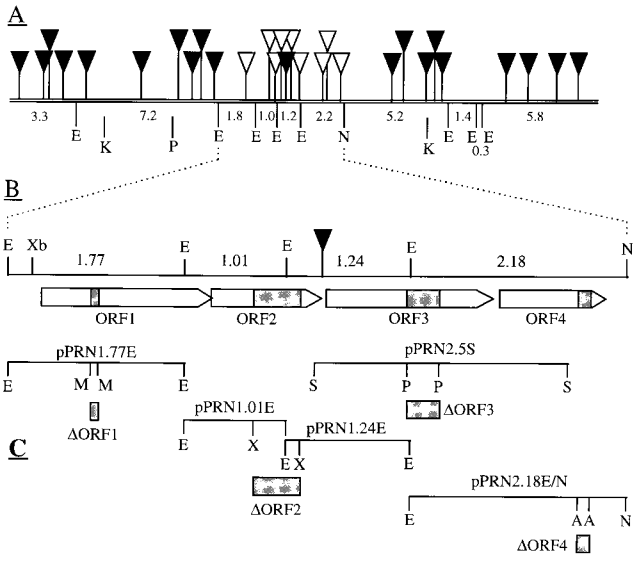


FIG. 2. (A) Restriction map of the cloned DNA in plasmid pCIB169 and the positions of Tn*5* insertions. Tn*5* insertions are shown as filled and open triangles to indicate those with no effect on Prn synthesis and those resulting in no Prn synthesis, respectively. The four Prn ORFs and their directions of transcription (open arrows) and the positions of the deletions constructed in each (filled boxes) are shown in panel B. (C) Strategy used to construct the individual ORF deletions (see Materials and Methods). Restriction sites: A, *Aat*II; E, *Eco*RI; K, *Kpn*I; M, *Mlu*I; N, *Not*I; P, *Pst*I; S, *Sph*I; X, *Xho*I; Xb, *Xba*I. The single *Pst*I site shown in panel A is the site of the insertion of the APH gene fragment in BL915APH and is not the only *Pst*I site in pCIB169.

ORF4, including the 1.8-, 1.0-, and 1.2-kb *Eco*RI fragments and the 2.2-kb *Eco*RI/*Not*I fragment. *Bam*HI linkers were added to the *Eco*RI and *Not*I ends prior to religation, thus leaving a *Bam*HI restriction site at the location of the deleted fragments. The APH gene was cloned as a *Bam*HI fragment into this unique *Bam*HI site.

**APH.** As a control for possible effects due to the insertion of the APH kanamycin resistance marker into the chromosome of strain BL915, the APH gene cassette was inserted 5′ to the *prn* gene region. A 2.5-kb *Sph*I fragment derived from the 7.2-kb *Eco*RI fragment located to the left of the region containing the four ORFs (Fig. 2A) was restricted at a unique *Pst*I site within the 2.5-kb *Sph*I fragment, and the APH gene cassette was ligated into this site. The fragment containing the APH gene was ligated into the *Sph*I site of pBR322.

**Homologous gene replacement.** The individual deletion mutation constructs described above were mobilized into strain BL915 as described previously (17). Perfect gene replacement events were distinguished from single crossover events leading to chromosomal integration of the whole plasmid as described previously (17) and were verified by Southern hybridization (data not presented). Perfect gene replacements were confirmed by the following criteria: (i) resistance to kanamycin and susceptibility to tetracycline, (ii) no hybridization of chromosomal DNA to plasmid vector DNA, (iii) probing with radiolabeled DNA of pPRN14N or pPRN18N and demonstration of the expected shift in the size of the affected chromosomal fragments due to the deletion and insertion of the APH gene, and (iv) hybridization to the APH gene fragment of the DNA fragments whose size was changed. Deletion mutants thus confirmed were designated BL915ΔORF1, BL915ΔORF2, BL915ΔORF3, BL915ΔORF4, and BL915ΔORF1-4, representing mutants of strain BL915 with deletions of ORF1, ORF2, ORF3, and ORF4, and a deletion of ORF1 to ORF4, respectively. The strain modified by insertion of the APH gene 5′ to the ORF1-ORF4 region was designated BL915APH.

**Detection of antifungal metabolites.** HCN production by bacterial cultures grown in LB broth in microtiter plates was detected with indicator paper tightly covering the wells as described by Voisard et al. (49). Chitinase activity was detected by using the substrate 4-methylumbelliferyl β-D-*N*,*N*′-diacetylchitobioside as described by Gaffney et al. (14). Prn and 2-hexyl-5-propyl resorcinol produced by bacterial cultures were detected by thin-layer chromotography (TLC) essentially as described by van Pée et al. (46).

**Synthesis of DNA fragments containing putative ORFs.** PCR primers were designed and synthesized by Integrated DNA Technologies (Coralville, Iowa) to amplify each putative ORF in the *prn* gene region. In each case, DNAs beginning 5′ to the putative translation initiation codon and including potential ribosome binding sites (RBS) and ending a few bases 3′ to the first in-frame stop codon were amplified (Table 2). The PCR-amplified fragments were cloned in the proper orientation behind the *tac* promoter from pKK223-3 (Pharmacia) in the mobilizable, broad-host-range plasmid pRK290 (12). Plasmids containing the different ORF constructs were mobilized into the respective BL915ΔORF deletion mutant by triparental matings (12). Transconjugants were selected by plating on *Pseudomonas* minimal medium agar supplemented with 30 mg of tetracycline per liter.

**Expression of the four ORFs in *E. coli*.** The 5.8-kb DNA fragment derived from pCIB169 and extending from the *Xba*I site located approximately 100 bp 5′ to the start of ORF1 to the *Not*I site 3′ to the end of ORF4 (Fig. 2) was cloned into plasmid pKK223-3MCS. The resulting plasmid, pPRN5.8X/N, contains the four ORFs properly juxtaposed with the *tac* promoter adjacent to the cloning site to affect expression of the ORFs from this promoter. Plasmid pPRN5.8X/N was transformed into *E. coli* XL1-BLUE (Stratagene, La Jolla, Calif.), and expression from the *tac* promoter was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to the growth medium to a final concentration of 2.5 mM. The production of Prn by the *E. coli* cells was assessed by TLC 24 h after induction with IPTG.

**Nucleotide sequence accession number.** The nucleotide sequence of the *prn* gene region reported here has been assigned GenBank accession no. U74493.

TABLE 2. Characteristics of DNA fragments representing potential coding sequences of the four ORFs that were used to identify translation initiation sites of *prn* genes

| Fragment | Position[a] | | | | No. of amino acids in the encoded protein | Prn production[d] |
|---|---|---|---|---|---|---|
| | Start of amplified fragment | Putative start codon[b] | Stop codon[c] | End of amplified segment | | |
| ORF1-A | 294 | 357 | 2039 | 2056 | 560 | + |
| ORF1-B | 396 | 423 | 2039 | 2056 | 538 | + |
| ORF1-C | 438 | 477 | 2039 | 2056 | 520 | − |
| ORF2-A | 2026 | 2039 | 3124 | 3167 | 361 | + |
| ORF2-B | 2145 | 2162 | 3124 | 3167 | 320 | − |
| ORF2-C | 2215 | 2249 | 3124 | 3167 | 291 | − |
| ORF2-D | 2440 | 2480 | 3124 | 3167 | 214 | − |
| ORF3-A | 3131 | 3167 | 4870 | 4905 | 567 | + |
| ORF3-B | 3208 | 3236 | 4870 | 4905 | 544 | − |
| ORF3-C | 3330 | 3356 | 4870 | 4905 | 504 | − |
| ORF4-A | 4852 | 4895 | 5986 | 6123 | 363 | + |
| ORF4-B | 4968 | 4991 | 5986 | 6123 | 331 | − |
| ORF4-C | 5015 | 5087 | 5986 | 6123 | 299 | − |

[a] All position numbers refer to the sequence in GenBank accession no. U74493.
[b] The first base of the putative start codon.
[c] The last base of the stop codon.
[d] Determined by TLC after introduction into the corresponding BL915 deletion mutant.

## RESULTS

**Cloning of the *prn* gene region.** *P. fluorescens* BL914 is a wild-type strain that does not produce Prn, chitinase, or HCN under normal culturing conditions, and it provides no biocontrol activity (17). However, this strain produced Prn, chitinase, and HCN and controlled damping-off pathogens upon the introduction of the plasmid-borne *gacA* homolog from *P. fluorescens* BL915 (14, 17). Lam et al. (26) used TnCIB116, a Tn*5* transposon containing the promoterless *lacZY* genes, as a promoter probe to identify genes in strain BL914 that are regulated by *gacA* and *lemA*. One TnCIB116 insertion mutant of a *gacA* mutant BL914 strain from this screen was shown not to produce Prn when the *gacA* gene from strain BL915 was present in *trans*. This mutant BL914 strain produced chitinase and HCN when the *gacA* gene was introduced on a plasmid, indicating that the Prn$^-$ phenotype was not due to a defect of the global regulatory system that regulates the production of antifungal compounds in this strain. Genomic DNA from the Prn$^-$ BL914::TnCIB116 mutant was digested with *Xho*I and cloned into plasmid pSP72 (Promega). A clone carrying an *Xho*I fragment containing the portion of TnCIB116 with the kanamycin resistance gene and approximately 1 kb of flanking DNA from the chromosome of strain BL914 was identified by selection for kanamycin resistance. The approximately 1 kb of flanking DNA derived from the chromosome of the Prn$^-$ BL914 mutant was shown to hybridize to genomic DNA from strains BL914 and BL915 by Southern hybridization (data not shown). A cosmid clone, pCIB169, containing approximately 32 kb of genomic DNA from strain BL915, was isolated from a genomic library of DNA from strain BL915 (17) based on hybridization to the 1 kb of BL914 chromosomal DNA flanking the TnCIB116 insertion. Transfer of pCIB169 to the BL914::TnCIB116 Prn$^-$ mutant resulted in the restoration of its ability to produce Prn (data not shown), indicating that it contains an analogous gene(s) from strain BL915 that is specifically required for Prn synthesis.

**Identification of the *prn* gene region in pCIB169.** A restriction map of pCIB169 was established (Fig. 2A) and indicated the presence of a unique *Not*I site near the center of the cloned DNA. Cosmid pCIB119 (27), the vector for pCIB169, has two *Not*I sites that flank the multiple cloning site and the cloned DNA of pCIB169. To facilitate Tn*5* mutagenesis of the DNA cloned in pCIB169, two *Not*I fragments of approximately 14 and 18 kb, representing the cloned DNA from pCIB169, were cloned into plasmid pCIB189, a derivative of pBR322 in which the *Eco*RI site was converted to a *Not*I site. The resulting plasmids, pPRN14N and pPRN18N, containing the 14- and 18-kb *Not*I fragments of pCIB169, respectively, were independently subjected to Tn*5* mutagenesis. Thirty independent Tn*5* insertions were identified and mapped (Fig. 2A). Each Tn*5* insertion was introduced into the chromosome of strain BL915 by homologous replacement of the corresponding wild-type gene region. The location of the Tn*5* element in the chromosome of each mutant was verified by Southern hybridization (data not shown). The ability of each of the 30 BL915::Tn*5* insertion mutants to produce Prn was assessed by TLC. This analysis revealed that nine individual Tn*5* insertions clustered in the center of the cloned DNA in pCIB169 were unable to synthesize Prn (Fig. 2A). These results indicated that contiguous DNA fragments, including *Eco*RI fragments of 1.8, 1.0, and 1.2 kb and an *Eco*RI/*Not*I fragment of 2.2 kb, contained genetic information required for the synthesis of Prn. One insertion located in the 1.2-kb *Eco*RI fragment had no effect on Prn synthesis (Fig. 2B).

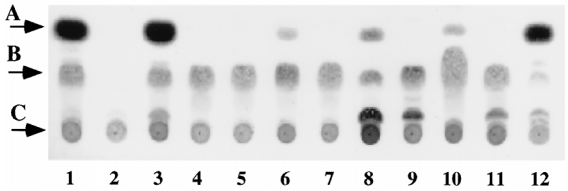


FIG. 3. Thin-layer chromatogram of culture extracts derived from cultures of *P. fluorescens* BL915 (lane 1), BL915 *gacA* mutant (lane 2) (17), BL915APH (lane 3), BL915ΔORF1-4 (lane 4), BL915ΔORF1 and BL915ΔORF1 containing *tac*–ORF1-B on a plasmid (lanes 5 and 6), BL915ΔORF2 and BL915ΔORF2 containing *tac*–ORF2-A on a plasmid (lanes 7 and 8), BL915ΔORF3 and BL915ΔORF3 containing *tac*–ORF3-A on a plasmid (lanes 9 and 10), and BL915ΔORF4 and BL915ΔORF4 containing *tac*–ORF4-A on a plasmid (lanes 11 and 12). Arrows on the left indicate Prn (A), 2-hexyl-5-propyl resorcinol (B), and the sample origin (C).

**Analysis of the nucleotide sequence of the *prn* gene region.** The nucleotide sequence encompassing the 1.8-, 1.0-, and 1.2-kb *Eco*RI fragments and the adjacent 2.2-kb *Eco*RI/*Not*I fragment was determined and analyzed for the presence of ORFs by using the program FRAMES (16). Incorporated in the search for ORFs was the codon preference for this organism determined from the sequence of the *uvrC* gene homolog that is located near the *gacA* gene in the chromosome of strain BL915 (14). The results indicated the presence of four putative ORFs located entirely within the 6.2-kb sequence. By definition of the FRAMES analysis, all of the ORFs begin with an ATG translation initiation codon. These ORFs are organized colinearly (Fig. 2), and compared to regions outside the ORFs or in alternate reading frames within them, they contain very few rare codons relative to the codon preference obtained for this strain from the *uvrC* gene homolog. Further analysis of the sequence identified two stem-loop structures suggestive of ρ-independent transcription termination structures. One of these is located 5′ to ORF1 and the other is located 3′ to the end of ORF4, but none were found within or between the coding regions of the four putative ORFs. Northern analysis of RNA isolated from 24-h cultures of strain BL915 with radiolabeled DNA of ORF4 indicated that ORF4 is contained on an approximately 5.5-kb transcript (data not shown). A transcript of 5.5 kb is approximately the size expected of one containing the four ORFs. These results strongly suggest that ORF1 to ORF4 are contained within a single transcriptional unit.

The Tn*5* insertion shown in Fig. 2 that is located in the midst of the region shown to be involved in Prn synthesis, but which resulted in a wild-type Prn phenotype, was cloned with flanking DNA from the BL915 chromosome. By using primers specific to the ends of the Tn*5* transposon and directed toward the outside of the element, the sequence of the DNA around the insertion site was determined. Comparison of this sequence with that of the entire 6.2-kb *prn* gene region demonstrated that this transposon is located precisely in the noncoding region between ORF2 and ORF3.

**Analysis of ORF-specific deletion mutants.** Mutants of strain BL915 containing chromosomal deletions in the individual ORFs and the ORF1-4 deletion mutant, BL915ΔORF1-4, produced chitinase, HCN, and 2-hexyl-5-propyl resorcinol, the production of which is regulated by LemA and GacA. However, none of the *prn* gene deletion mutants produced detectible amounts of Prn (Fig. 3). The phenotype of strain BL915APH in regard to the production of Prn, 2-hexyl-5-propyl resorcinol, chitinase, and HCN was not different from that of the wild-type strain.

**Identification of translation initiation sites of the ORFs.** The four ORFs identified in the analysis of the nucleotide sequence of the *prn* gene region represent the largest possible coding regions starting with ATG translation initiation codons. Examination of the deduced amino acid sequence of these ORFs revealed the presence of alternative, in-frame ATG translation initiation sites in all of the ORFs. To identify the minimum functional coding region for each ORF, the potential coding regions beginning with each of the possible ATG initiation codons for each of the ORFs were amplified by PCR and cloned. Each amplified ORF fragment included the region immediately upstream of the ATG start codon that would contain the associated RBS. The cloned coding regions were subsequently fused to the *tac* promoter lacking an indigenous RBS in order to provide constitutive expression in *Pseudomonas* and were cloned into the mobilizable, broad-host-plasmid vector pRK290. The resulting plasmids containing the *tac* promoter-ORF fusions were transferred by triparental mating into the corresponding BL915ΔORF deletion mutant. Functional complementation of the deletion mutations was determined by assessing each complemented mutant for its ability to produce Prn.

Examination of the ORF1 coding region revealed the presence of two additional in-frame ATG methionine codons located in the N-terminal portion of the deduced amino acid sequence of ORF1. Each of the three potential ORF1 coding sequences was amplified, cloned, and fused to the *tac* promoter as described above. After introduction of the plasmids containing the three different versions of ORF1 into the BL915ΔORF1 mutant, it was determined that ORF1-A and ORF1-B complemented the Prn$^-$ phenotype of the BL915ΔORF1 mutant, while ORF1-C did not (Table 2 and Fig. 3). Therefore, ORF1-B is the shortest functional ORF1 coding region and is the only potential ORF1 coding region of the three that is preceded by a typical RBS. Based on these results, we conclude that ORF1-B represents the true coding sequence of this gene. In the case of ORF2, there are two potential in-frame ATG translation initiation codons, and DNA fragments beginning with each were constructed (ORF2-C and -D), but neither complemented the Prn$^-$ phenotype of mutant BL915ΔORF2 (Table 2). Further examination of the region upstream of the ORF2 coding sequence revealed the presence of two in-frame GTG codons that could serve as alternate translation initiation codons. The first GTG codon is preceded by a typical RBS, whereas the second such codon, as well as the shorter coding sequences that have ATG initiation codons, lack good RBSs. ORF2-specific DNA fragments incorporating the GTG translation start codons were constructed in the manner described, and each was introduced into mutant BL915ΔORF2. The longer version of ORF2, ORF2-A, with a GTG start codon was shown to complement the Prn$^-$ phenotype of mutant BL915ΔORF2 (Table 2 and Fig. 3), while the shorter fragment, ORF2-B, did not (Table 2). These results suggest that the functional ORF2 coding region begins with the first GTG translation start codon. This GTG translation initiation codon overlaps one base with the TAG translation stop codon of ORF1, and the putative RBS for ORF2 is in the coding sequence of ORF1, indicating translational coupling of the two genes. Three different potential ORF3 genes were tested in the same manner, and only the longest, ORF3-A, which contains a good RBS upstream of the ATG start codon, was able to complement mutant BL915ΔORF3 (Table 2 and Fig. 3). A similar result was demonstrated for ORF4 since only the largest of three possible ORFs, ORF4-A, complemented the Prn$^-$ phenotype of mutant BL915ΔORF4 (Table 2 and Fig. 3). These results indicate that ORF1 consists of 1,617 nucleotides that encode a protein having 538 amino acids, ORF2 includes 1,086 nucleotides and encodes a protein consisting of 361 amino acids, ORF3 includes 1,704 nucleotides and encodes a protein with 567 amino acids, and ORF4 consists of 1,092 nucleotides encoding a protein with 363 amino acids.

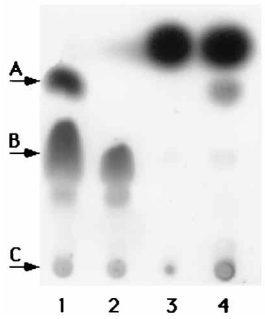


FIG. 4. Thin-layer chromatogram of culture extracts derived from cultures of *P. fluorescens* BL915 (lane 1), BL915ΔORF1-4 (lane 2), and *E. coli* XL1-BLUE containing plasmid pPRN5.8X/N without and with induction of the *tac* promoter by the addition of IPTG (lanes 3 and 4, respectively). Arrows on the left indicate Prn (A), 2-hexyl-5-propyl resorcinol (B), and the sample origin (C).

**Heterologous expression of the four ORFs in *E. coli*.** Plasmid pPRN5.8X/N, containing the cluster of the four ORFs fused to the *tac* promoter, was introduced into *E. coli* XL1-BLUE. Expression from the *tac* promoter was induced by the addition of IPTG to the culture, and 24 h later, the whole culture was assessed for the presence of Prn. Prn was not detected in uninduced XL1-BLUE cells containing pPRN5.8X/N (Fig. 4) or in XL1-BLUE cells lacking the plasmid (data not shown). However, Prn was detected in cells containing plasmid pPRN5.8X/N that were induced with IPTG. To verify the identity of the Prn produced by *E. coli*, the material was purified by high-pressure liquid chromatography (17) and analyzed by $^1$H nuclear magnetic resonance spectroscopy (500 MHz; $CD_3OD$) and by mass spectroscopy. The nuclear magnetic resonance spectrum of the material was identical to spectra previously reported for Prn (36), and the mass spectrum indicated the presence of two chlorine atoms, an even number of nitrogen atoms, a pyrrole group, and a mass consistent with that of Prn. These results clearly demonstrate that *E. coli* harboring plasmid pPRN5.8X/N produces Prn after induction of the *tac* promoter with IPTG. Furthermore, this result demonstrates that the four ORFs identified on the 5.8-kb *Xba*I/*Not*I fragment are sufficient for the heterologous production of Prn in strains not known to make this compound.

**Comparison of the four ORFs with sequences in peptide data banks.** The deduced amino acid sequences of the four ORFs were used to search the peptide sequence data banks by using the BLASTP program (2). ORF1 contains a short region with high similarity to the NAD binding domain of the NADH dehydrogenases from *Haemophilus influenzae* (13) {smallest sum probability $P(N)$ [Sum $P(5)$] = 0.0031} and *E. coli* (52) [Sum $P(3) = 0.5$] and the thioredoxin reductase from *Streptomyces clavuligerus* (1) [Sum $P(3) = 0.16$]. No strong similarities to any protein sequences in the data bank were detected for the protein encoded by ORF2. ORF3 is highly homologous to a protein encoded by the *chl* gene from *Streptomyces aureofaciens*, that is a chlorinating enzyme involved in the synthesis of tetracycline (11) (Fig. 5). From amino acid residue 103 to the end of the ORF3 protein, there is 65% similarity and 42% identity between ORF3 and the *chl* gene product (Fig. 5) [Sum $P(5) = 5.0 \times 10^{-131}$]. In addition, an NAD binding domain similar to that found in ORF1 was found near the N terminus

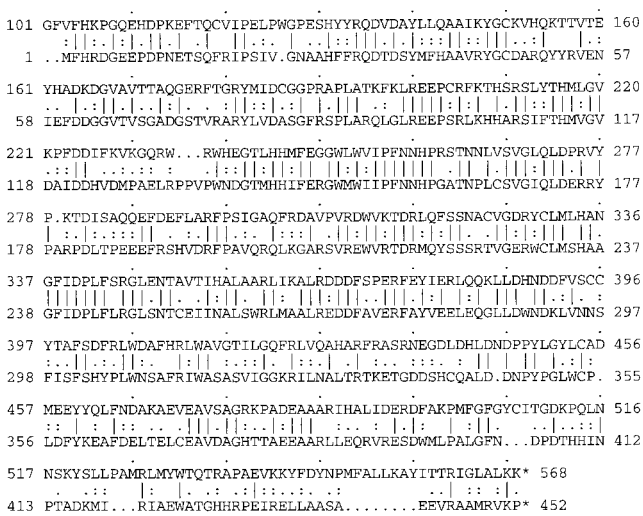

FIG. 5. Alignment of the deduced amino acid sequence of the ORF3 protein (top sequence) and the Chl protein (11) (bottom sequence). |, identical residues; :, conservative replacements; ., neutral replacements. Gaps introduced in the sequences in order to maintain optimal alignment are indicated by ellipses. The residue numbers of the two proteins are indicated at the beginning and end of each line, and the carboxyl terminus of each protein is indicated by an asterisk.

of ORF3. The ORF4 protein has high similarity within a short region to a portion of the 3-chlorobenzoate-3,4-dioxygenase (CbaA) of *Alcaligenes* sp. (31) [Sum $P(2) = 1.3 \times 10^{-13}$], phthalate 4,5-dioxygenase (Pht3) of *Pseudomonas putida* (34) [Sum $P(2) = 3.0 \times 10^{-10}$], and vanillate demethylase (VanA) of *Pseudomonas* sp. (10) [Sum $P(3) = 5.6 \times 10^{-8}$]. This region contains the consensus domain (CxH-15 to 17 amino acids-CxxH) that has been identified as the motif of Rieske iron-sulfur proteins which is involved in the binding of [2Fe-2S] clusters (29). Another motif in ORF4 that is conserved in CbaA, VanA, and Pht3 was found. This is the DxxHxxxxH motif, the proposed binding site of the mononuclear nonheme Fe(II) that is thought to be involved in binding oxygen in oxygenases (31). In ORF4, as in other described oxygenases, this motif is located 90 to 100 residues from the Rieske [2Fe-2S] center.

## DISCUSSION

We have isolated a 32-kb DNA fragment from the chromosome of *P. fluorescens* BL915 that contains a four-gene cluster that is involved in the biosynthesis of the antifungal secondary metabolite Prn. Mapping of this fragment by Tn*5* mutagenesis revealed that a 6.2-kb region in the center of the larger chromosomal fragment contained DNA functionally relevant to the synthesis of Prn. Analysis of the nucleotide sequence of this region revealed the presence of four putative ORFs organized colinearly within the 6.2-kb region. Several characteristics shared by these four ORFs suggest that they represent actual coding sequences. First, all ORFs share the same codon preference common to this bacterium as determined from the sequence of the *uvrC* gene homolog from this strain (14). Second, two stem-loop structures similar to ρ-independent transcription termination signals were identified in the sequence. One is located immediately 5′ to the beginning of ORF1, and the second is located just downstream of the end of ORF4. However, there are no ρ-independent transcription termination signals within or between the four ORFs, and Northern analysis indicates that the four ORFs are located on a single transcriptional unit. Third, individual BL915ΔORF deletion mutants that were created independently in each of the ORFs were defective in Prn production. Mutations in the four ORFs had no effect on production of other antifungal metabolites produced by strain BL915, including chitinase, HCN, and 2-hexyl-5-propyl resorcinol. The synthesis of these compounds is coregulated with the synthesis of Prn (14). Therefore, mutations in the four ORFs specifically affect Prn biosynthesis and do not represent mutations in regulatory genes. Finally, although each ORF contained alternate translation initiation codons, only one in each ORF was preceded by a reasonable RBS. Cloning of the various potential coding sequences representing each ORF and juxtaposition of them with the *tac* promoter demonstrated that the coding regions preceded by plausible RBSs were functional in each case since they were able to complement the corresponding ORF deletion mutant. In the case of ORF1, ORF2, and ORF3, complementation of the corresponding ORF deletion mutant resulted in lower amounts of Prn synthesis than the parent strain, whereas ORF4 complementation resulted wild-type levels of Prn synthesis (Fig. 3). This is most likely due to polar effects from insertion of the APH gene on the transcription of downstream genes in the deletion mutants, which is consistent with the organization of all four ORFs on a single transcriptional unit. Interestingly, it was found that the coding sequence for ORF2 begins with a GTG translation initiation codon which overlaps by one base with the TAG translation stop codon of ORF1, indicating translational coupling of these two ORFs. The strongest evidence that this gene cluster has an involvement in the biosynthesis of Prn comes from the heterologous expression of the genes in *E. coli* and concomitant production of Prn by this organism, which has never before been known to produce this compound. This result strongly indicates that the four genes in this cluster encode the entire pathway for the biosynthesis of Prn.

We identified a single Tn*5* insertion that is located within the region containing the four ORFs but had no effect on Prn synthesis. Subsequent determination of the DNA sequence flanking this insertion and its comparison to the sequence of the 6.2-kb Prn ORF region indicated that this insertion was located only 30 bp from the translation initiation site of ORF3 in the short noncoding region between ORF2 and ORF3. Although Tn*5* insertions usually cause polar effects on downstream genes within a polycistronic transcriptional unit, it has been reported that some Tn*5* insertions cause weak but constitutive expression of distal genes (6). For example, weak expression of the thiogalactoside transacetylase gene in *E. coli* has been reported for some Tn*5* insertions in this gene. Deletion mapping of these indicated that the promoter activity originated within 186 bp of the end of Tn*5* or that the promoter was hybrid, being composed of both Tn*5* and target DNA sequences (6). It is possible that the $prn^+$ phenotype of this insertion mutant is due to a similar phenomenon that results in some expression of ORF3 and ORF4.

Based on the results of radiotracer studies and isolated metabolites, a hypothetical biochemical pathway for the synthesis of Prn has been proposed by van Pée et al. (47) (Fig. 1). In this pathway, the first step in the biosynthesis of Prn is the chlorination of tryptophan at the 7 position to form 7-chlorotryptophan, followed by rearrangement of the indole ring to a phenylpyrrole ring and decarboxylation to form monodechloroaminopyrrolnitrin. This intermediate is chlorinated a second time to form aminopyrrolnitrin, which, in the last step of the pathway, undergoes oxidation of the amino group to a nitro group to form Prn. A survey of the peptide data banks for peptides with homology to the deduced amino acid sequences of the four ORFs revealed

some interesting similarities, some of which are consistent with the proposed pathway for Prn biosynthesis. The most striking similarity was between the protein encoded by ORF3 and a protein from *S. aureofaciens*, Chl, that catalyzes the chlorination reaction in the synthesis of tetracycline (11). The first residue of the Chl protein aligns with residue 103 of ORF3, and a strong similarity between the two proteins extends from this point to the end of each protein. The major difference between the two proteins is that the ORF3 peptide is larger than Chl by 102 amino acids in the N terminus. This unique region in ORF3 was found to contain a consensus NAD binding domain, which are typically located near the N terminus (29), indicating that the ORF3 peptide may require NADH as a cofactor. Examination of the *chl* gene sequence reported by Dairi et al. (11) indicates the presence of an alternative ATG translation initiation codon with a good RBS located 307 bases 5′ to the translation initiation site cited in the report. Although this start site is not in the same reading frame as that reported for the *chl* gene, removal of one base (e.g., base 2856 in the report) aligns the two reading frames. The protein encoded by the *chl* gene beginning at this new start site would be exactly 102 amino acids larger and contains a consensus NAD binding domain in a position similar to that noted in the ORF3 protein. Furthermore, the additional amino acids share 46% identity and 70% similarity with the analogous region of the ORF3 protein. Based on these results, we strongly suspect that the *chl* gene is in fact longer than reported, and the encoded protein is much more similar to the ORF3 peptide than is evident from the reported sequence. The strong similarity of the ORF3 peptide to the Chl protein which has been demonstrated to be involved in chlorination in the biosynthesis of an antibiotic strongly suggests that this protein catalyzes one of the two chlorination reactions in the synthesis of Prn. The ORF4 peptide was found to have strong similarities to class IA dioxygenases, including a consensus [2Fe-2S] Rieske-type binding domain and a domain that is highly similar to the proposed mononuclear nonheme Fe(II) binding domain also common to terminal dioxygenases (29). The presence of these domains in the ORF4 peptide strongly suggests that this protein is a dioxygenase or catalyzes an oxidation reaction. The last step in the proposed Prn biosynthetic pathway involves the oxidation of the amino group of aminopyrrolnitrin to the nitro group of Prn. Since the ORF4 peptide has strong similarities to known dioxygenases, it is probable that this protein catalyzes the oxidation of the amino group of aminopyrrolnitrin, the final step of Prn synthesis. In the proposed pathway for the synthesis of Prn, there are four intermediates, starting with, and including, L-tryptophan, suggesting four enzymatic steps leading to the synthesis of Prn. We have identified four ORFs in a gene cluster from *P. fluorescens* BL915 that each have a role in the synthesis of Prn. ORF3 and ORF4 appear to encode proteins that are similar to known proteins with functions that are consistent with two of the four biosynthetic steps in Prn synthesis. Furthermore, expression of the four genes in *E. coli* results in the production of Prn by this organism. These results very strongly suggest that the four ORFs in this gene cluster constitute the genes for the entire Prn biosynthetic pathway.

It has been widely accepted that haloperoxidases are involved in the synthesis of halogenated compounds (32), including Prn (44). A chloroperoxidase from the Prn-producing bacterium *Pseudomonas pyrrocinia* was isolated, purified, and shown to catalyze the chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin in vitro (51). This enzyme has also been shown to catalyze, in vitro, the final step in Prn synthesis, the oxidation of aminopyrrolnitrin to Prn (25), strongly suggesting the involvement of this enzyme in Prn biosynthesis. However, recently Kirner et al. (24) reported the cloning of a chloroperoxidase gene from *P. fluorescens* BL914 and demonstrated by disruption of the gene that it has no role in the biosynthesis of Prn. In addition, the chloroperoxidase gene from strain BL914 has homology with chloroperoxidases from other bacteria but no sequence homology to the recently described gene from *S. aureofaciens* that encodes a chlorinating enzyme in the synthesis of tetracycline. Kirner et al. (24) and van Pée (45) have postulated that the chloroperoxidases that have been thought to have a role in Prn synthesis are nonspecific halogenating enzymes with no biological significance to Prn synthesis. None of the four ORFs described in this report were found to share any similarity with the chloroperoxidases thought to have a role in Prn synthesis. These results are consistent with the postulations of Kirner et al. and van Pée that the chloroperoxidases have no biological role in Prn synthesis.

The results presented here present a compelling case for the specific involvement of the four ORFs described herein in the synthesis of Prn. Therefore, we propose that ORF1, ORF2, ORF3, and ORF4 should be assigned the genetic designations *prnA*, *prnB*, *prnC*, and *prnD*, respectively. These are the first genes described that have been shown to be specifically involved in Prn synthesis. The four genes and their corresponding deletion mutants should prove to be useful in future efforts to further elucidate the precise role of each of the genes in the synthesis of Prn.

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