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NOTES

Functions Encoded by Pyrrolnitrin Biosynthetic Genes from *Pseudomonas fluorescens*

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Pyrrolnitrin is a secondary metabolite derived from tryptophan and has strong antifungal activity. Recently we described four genes, *prnABCD*, from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. In the work presented here, we describe the function of each *prn* gene product. The four genes encode proteins identical in size and serology to proteins present in wild-type *Pseudomonas fluorescens*, but absent from a mutant from which the entire *prn* gene region had been deleted. The *prnA* gene product catalyzes the chlorination of L-tryptophan to form 7-chloro-L-tryptophan. The *prnB* gene product catalyzes a ring rearrangement and decarboxylation to convert 7-chloro-L-tryptophan to monodechloroaminopyrrolnitrin. The *prnC* gene product chlorinates monodechloroaminopyrrolnitrin at the 3 position to form aminopyrrolnitrin. The *prnD* gene product catalyzes the oxidation of the amino group of aminopyrrolnitrin to a nitro group to form pyrrolnitrin. The organization of the *prn* genes in the operon is identical to the order of the reactions in the biosynthetic pathway.

The antibiotic pyrrolnitrin [3-chloro-4-(2′-nitro-3′-chlorophenyl)pyrrole] (PRN) is produced by many pseudomonads and has broad-spectrum antifungal activity (1, 5, 12–14, 17). PRN has been implicated as an important mechanism of biological control of fungal plant pathogens by several *Pseudomonas* strains (12–14), including *P. fluorescens* BL915, from which the *prn* genes were isolated (10).

Tryptophan was identified as the precursor for PRN, based on the feeding of cultures with isotopically labeled and substituted tryptophan (2, 7, 8, 17, 25). Biosynthetic pathways were proposed as early as 1967 (7) and have been refined on the basis of tracer studies and the isolation of intermediates (Fig. 1) (2, 8, 17, 19, 23, 25). Recently, Hammer et al. (9) described the cloning and characterization of a 5.8-kb DNA region which encodes the PRN biosynthetic pathway. This DNA region confers the ability to produce PRN when expressed heterologously in *Escherichia coli* and contains four genes, *prnABCD*, each of which is required for PRN production. In the research described here, we used mutants in which each of the four genes was disrupted and strains which overexpress the individual genes to elucidate the function of each gene product in PRN biosynthesis.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *Pseudomonas* strains were cultured in Luria-Bertani medium at 28°C. Antibiotics, when used, were added at the following concentrations: tetracycline, 30 μg/ml; and kanamycin, 50 μg/ml. The expression vector pPEH14 consists of the P_{tac} promoter and *rrnB* ribosomal terminator from pKK223-3 (Pharmacia, Uppsala, Sweden) cloned into the *Bgl*II site of the broad-host-range plasmid pRK290 (4). P_{tac} is a strong constitutive promoter in *Pseudomonas* (unpublished data). The PRN biosynthetic genes are the coding regions described by Hammer et al. (9). Each coding region was cloned from the translation initiation codon to the stop codon by PCR with restriction sites added to the ends to facilitate cloning. For *prnB*, the native GTG initiation codon was changed to ATG. The clones were sequenced after PCR.

Chemical standards. 7-Cl-D,L-tryptophan (7-CT) was synthesized as described by van Pée et al. (24). Monodechloroaminopyrrolnitrin (MDA) was extracted from cultures of *P. aureofaciens* and verified as described by van Pée et al. (23). Aminopyrrolnitrin (APRN) was prepared from PRN by reduction with sodium dithionite (22). PRN was synthesized according to the method of Gosteli (6).

Western analysis. To produce antigen, each *prn* gene was subcloned into a pET3 vector and transformed into *E. coli* BL21(De3) (Novagen, Inc., Madison, Wis.). Inclusion bodies were purified from induced cultures with protocols from Novagen. Inclusion body protein (100 μg) was run on a preparative Laemmli polyacrylamide electrophoresis gel, blotted to nitrocellulose filters, and stained with Ponceau S. The major band was excised, solubilized in dimethyl sulfoxide, and used by Duncroft, Inc. (Lovettsville, Va.), to immunize goats and produce antiserum against each PRN protein.

Cultures of *P. fluorescens* BL915 were grown for 48 h in Luria-Bertani medium with the appropriate antibiotics. The cells were pelleted and resuspended in a small volume of Tris-EDTA. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western analysis were performed as described by Sambrook et al. (21). The primary antiserum (goat anti-PRN

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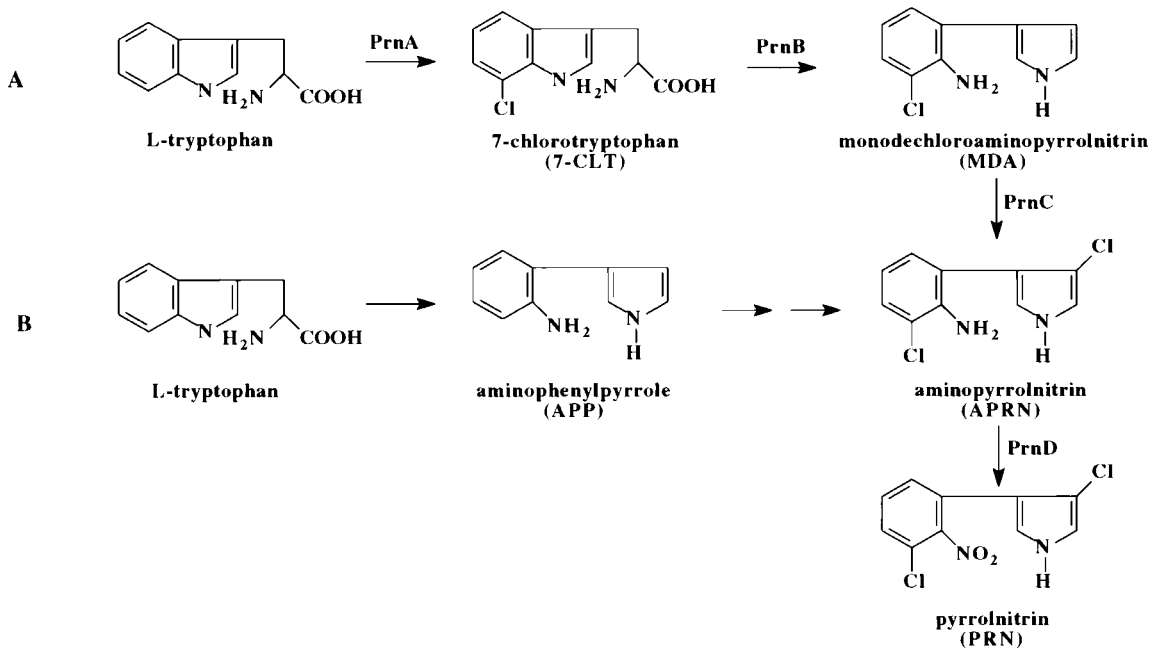

FIG. 1. Biosynthetic pathways for PRN as proposed by van Pée et al. (23) (A) and by Chang et al. (2) (B). The reactions catalyzed by the PRN biosynthetic enzymes encoded by the *prnABCD* genes are indicated above the appropriate reaction arrows.

protein) was diluted 1/1,000, and the secondary antibody (rabbit anti-goat immunoglobulin G conjugated to peroxidase; Pierce, Rockford, Ill.) was diluted 1/3,000. Bands were visualized with an enhanced chemiluminescence kit (Amersham, Arlington Heights, Ill.). This Western analysis demonstrated that each antibody recognized a single protein band from wild-type BL915, and these bands were not present in BL915ΔORF1–4 (Fig. 2). The molecular weights of the recognized proteins were consistent with the sizes predicted from the gene sequences. Each *prn* gene was expressed on a plasmid in BL915ΔORF1–4. In each case, the protein product of the cloned gene reacted only with the expected antibody and was identical in size to the band detected by that antibody in wild-type BL915 (Fig. 2).

Intermediate analysis and feeding experiments. To determine which biosynthetic intermediates were produced by the *prn* gene deletion mutants, 2-day-old cultures were extracted with an equal volume of ethyl acetate. The organic phase was dried under vacuum, and the residue was dissolved in a small volume of methanol. Thin-layer chromatography (TLC) was performed on silica-coated plates with toluene or hexane-ethyl acetate (2:1) as the mobile phase. PRN, APRN, MDA, and aminophenylpyrrole (APP) were visualized with van Urk's reagent as described previously (22).

To further clarify which biosynthetic step was blocked in each deletion mutant, intermediate feeding experiments were conducted. Cultures (10 ml) were incubated at 28°C for 48 h. Biosynthetic intermediates were dissolved in a small volume of methanol and added to 4 ml of culture at the following final concentrations: 7-CT, 2.5 μg/ml; MDA, 25 μg/ml; APRN, 12.5 μg/ml. The cultures were incubated for an additional 4 h at 28°C and then extracted with ethyl acetate and analyzed by TLC and liquid chromatography-mass spectrometry as described above.

MDA, APRN, and PRN were not detected in cultures of BL915ΔORF1 (Fig. 3), indicating that this mutant is blocked at an early step in PRN biosynthesis. BL915ΔORF1 was able to produce PRN when 7-CT, MDA, or APRN was supplied ex-

TABLE 1. Bacterial strains and plasmids used in this study

P. fluorescens strain or plasmid	Characteristics	Source or reference
Strains		
BL915	Wild type	10
BL915ΔORF1	Deletion in *prnA* of BL915, Prn$^-$, Km^r	9
BL915ΔORF2	Deletion in *prnB* of BL915, Prn$^-$, Km^r	9
BL915ΔORF3	Deletion in *prnC* of BL915, Prn$^-$, Km^r	9
BL915ΔORF4	Deletion in *prnD* of BL915, Prn$^-$, Km^r	9
BL915ΔORF1–4	Deletion in *prnABCD* of BL915, Prn$^-$, Km^r	9
Plasmids		
pPEH14(*prnA*)	pRK290 carrying P_{tac} functionally fused to the 1.6-kb *prnA* coding region	This study
pPEH14(*prnB*)	pRK290 carrying P_{tac} functionally fused to the 1.1-kb *prnB* coding region	This study
pPEH14(*prnC*)	pRK290 carrying P_{tac} functionally fused to the 1.7-kb *prnC* coding region	This study
pPEH14(*prnD*)	pRK290 carrying P_{tac} functionally fused to the 1.1-kb *prnD* coding region	This study

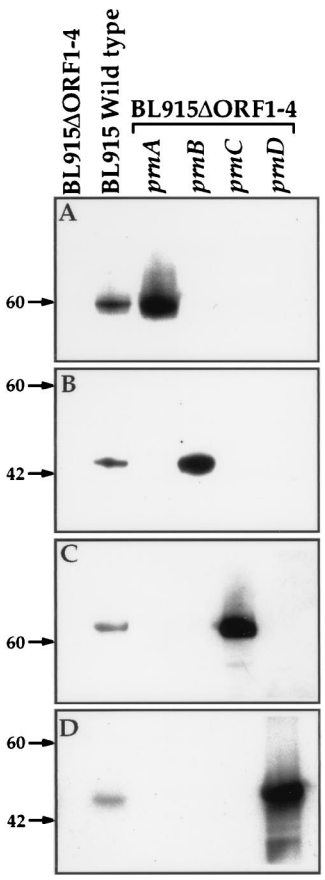

FIG. 2. Western blot analysis of the protein products of *prn* genes cloned from *P. fluorescens* BL915. Individual genes were expressed on plasmids in the host strain BL915ΔORF1–4. BL915 wild-type and BL915ΔORF1–4 controls are included on each blot. Blots A, B, C, and D were probed with antibodies raised against the products of *prnA*, *prnB*, *prnC*, and *prnD*, respectively. Arrows indicate the positions of the 60- and 42-kDa molecular mass markers.

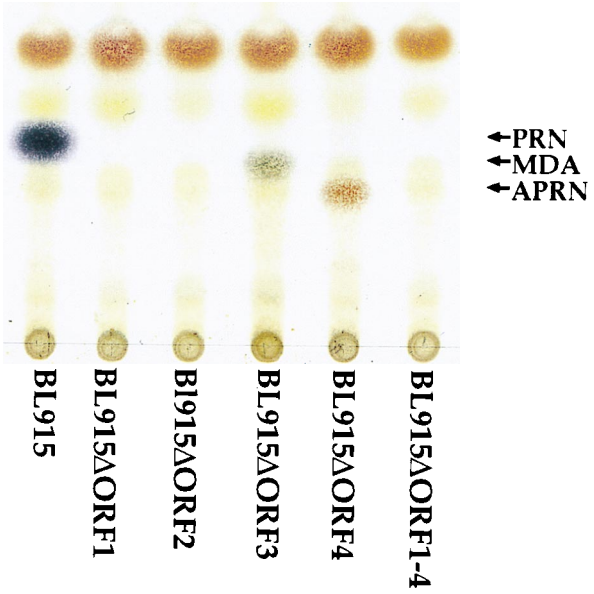

FIG. 3. Accumulation of PRN biosynthetic intermediates in *P. fluorescens* BL915 and *prn* gene deletion mutants derived from it. Extracts from 2-day-old cultures were separated by TLC on silica plates with hexane-ethyl acetate (2:1 [vol/vol]) as the mobile phase. Metabolites were visualized with van Urk's reagent. Arrows indicate the positions of MDA (olive green), APRN (reddish brown), and PRN (purple).

ogenously (Table 2). When *prnA* was expressed in the absence of other *prn* genes (i.e., in BL915ΔORF1–4), 7-chloro-L-tryptophan (7-CLT) accumulated. The identity of 7-CLT was verified by comparison of results of high-performance liquid chromatography and mass spectra with chemically synthesized 7-CT. These results indicate that the *prnA* gene product catalyzes the chlorination of L-tryptophan.

Hohaus et al. (11) presented additional evidence of the chlorinating activity of the *prnA* gene product, specifically, the chlorination of L-tryptophan to form 7-CLT by cell extracts from *P. fluorescens* strains which expressed the *prnA* gene, but which did not contain any of the other *prn* genes. To clarify which isomer was produced, Hohaus et al. (11) extracted 7-CLT from the bacteria and oxidized it to the corresponding indole-3-pyruvic acid with amino acid oxidases. Since the isolated 7-CLT was degraded by L-amino acid oxidase, but not by D-amino acid oxidase (11), it must be in the L configuration. The deduced amino acid sequence for *prnA* contains a consensus NAD binding site (9), and, indeed, NADH is a required cofactor for the *prnA* gene product.

Cultures of BL915ΔORF2 produced 7-CLT, but 7-chloro-D-tryptophan (11) and other PRN biosynthetic intermediates were not detected (Fig. 3). BL915ΔORF2 produced PRN when supplied with exogenous MDA or APRN, but not when supplied with 7-CT (Table 2). When *prnB* was expressed in strain BL915ΔORF1–4, exogenously supplied 7-CT was converted to MDA (Fig. 4). These results indicate that the *prnB* gene product catalyzes the rearrangement of the indole ring to a phenylpyrrole and the decarboxylation of 7-CLT to convert 7-CLT to MDA. While it is somewhat surprising that a single enzyme carries out both the ring rearrangement and decarboxylation, Chang et al. (2) postulated a mechanism for such a reaction on a single enzyme some 16 years ago. The *prnB* gene product also catalyzed the production of APP (Fig. 4), presumably by using tryptophan as a substrate.

MDA accumulated in cultures of BL915ΔORF3, but APP, APRN, and PRN were not detected (Fig. 3). BL915ΔORF3 was able to produce PRN when supplied with APRN in the culture medium, but not when supplied with 7-CT or MDA (Table 2). Strain BL915ΔORF1–4 expressing *prnC* converted exogenously supplied MDA to APRN (Fig. 4). These data indicate that the *prnC* gene product catalyzes the chlorination of MDA to form APRN. Cell extracts of the *P. fluorescens* strain which overexpresses the *prnC* gene (but does not contain the other *prn* genes) can also catalyze the chlorination of MDA to form APRN (11).

The *prnC* gene is homologous to the *chl* gene from *Streptomyces aureofaciens*, which encodes a chlorinating enzyme for

TABLE 2. Production of PRN by deletion mutants when supplied with biosynthetic intermediates in the growth medium

Strain	Result with intermediate added to cultures[a]		
	7-CT	MDA	APRN
BL915ΔORF1	+	+	+
BL915ΔORF2	−	+	+
BL915ΔORF3	−	−	+
BL915ΔORF4	−	−	−

[a] +, PRN detected; −, PRN not detected.

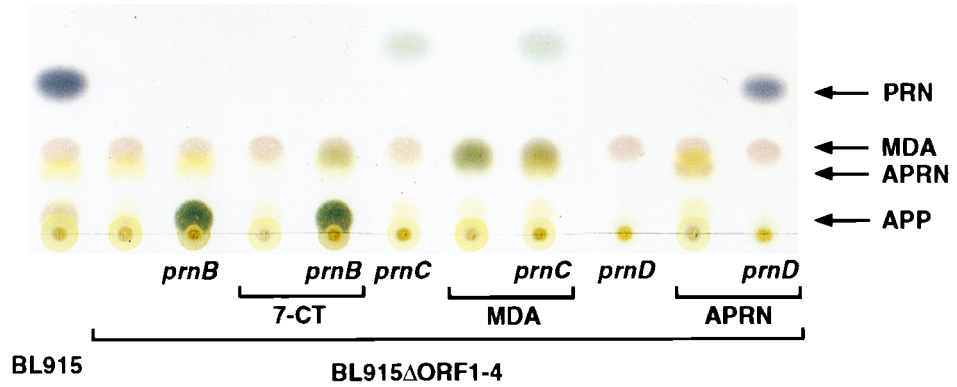

FIG. 4. In vivo conversion of PRN biosynthetic intermediates by the products of single *prn* genes. Individual genes were expressed on plasmids in the host strain BL915ΔORF1–4, and biosynthetic intermediates were added to the culture medium as indicated. Culture extracts were separated by TLC on silica plates with toluene as the mobile phase. Metabolites were visualized with van Urk's reagent. Arrows indicate the positions of APP (dark green), MDA (olive green), APRN (reddish brown), and PRN (purple).

tetracycline biosynthesis (3, 9). Like *prnA*, the *prnC* deduced amino acid sequence contains a consensus NAD binding region (9), and NADH is required for the chlorination of MDA (11). While both *prnA* and *prnC* encode halogenating enzymes, they show no homology to previously cloned haloperoxidases (9) or to each other. Furthermore, in contrast to haloperoxidases (16), the two NADH-dependent halogenating enzymes in the PRN biosynthesis pathway are substrate specific (i.e., the tryptophan halogenase does not catalyze the chlorination of MDA and vice versa) (11).

APRN accumulated in cultures of BL915ΔORF4 (Fig. 3), and this mutant was not able to produce PRN when supplied with any of the known PRN biosynthetic intermediates. Strain BL915ΔORF1–4 expressing *prnD* converted exogenously supplied APRN to PRN (Fig. 4). These results indicate that the *prnD* gene product catalyzes the oxidation of the amino group of APRN to a nitro group forming PRN. In vitro experiments by Kirner and van Pée (15) had suggested that this reaction is catalyzed by a chloroperoxidase; however, gene disruption experiments demonstrated that chloroperoxidases are not involved in PRN biosynthesis in vivo (16). Instead, this oxidation is more likely to be catalyzed by a class IA oxygenase (20), as suggested by the homology of *prnD* with these enzymes (9).

We have shown that each *prn* gene encodes a protein found in the wild-type BL915 strain and have demonstrated in vivo that these four gene products carry out four biochemical steps which convert L-tryptophan to PRN. None of the conversions were observed in strain BL915ΔORF1–4, from which the entire 5.8-kb *prn* gene region has been deleted (Fig. 4). The arrangement of the genes in the operon is identical to the sequence of reactions in the biosynthetic pathway proposed by van Pée et al. (23) (Fig. 1).

Chang et al. (2) proposed an alternate biosynthetic scheme (Fig. 1B) and reported the conversion of exogenously supplied APP to PRN in vivo. Similarly, Zhou et al. (25) reported the conversion of APP to APRN in a cell-free system. These workers concluded that APP is an intermediate in PRN biosynthesis and that ring rearrangement precedes chlorination (Fig. 1B). In the present study, APP accumulated only in strains which overexpressed the *prnB* gene. Furthermore, APP was not detected in cultures of BL915ΔORF1, which contains functional *prnBCD genes* expressed from the native promoter, as would be expected if the ring rearrangement (catalyzed by the *prnB* gene product) occurs before the first chlorination step (catalyzed by the *prnA* gene product). Like Hamill et al. (8) and van Pée et al. (23), we demonstrated that exogenously supplied 7-CT is converted to PRN. These results, together with the finding that the gene product of *prnA* catalyzes the NADH-dependent chlorination of L-tryptophan to 7-CLT (11), support the biosynthetic pathway proposed by van Pée et al. (23) (Fig. 1A) and suggest that APP is a side product or dead-end metabolite. Purification and kinetic characterization of the *prnA* and *prnB* gene products, including investigations of substrate specificity and regioselectivity, will further clarify the roles of 7-CLT and APP in the PRN biosynthetic pathway.

If APP is indeed a dead-end metabolite, it would be advantageous to tightly regulate the amount of *prnB* gene product present in cells, thus minimizing the diversion of substrate into APP. The *prnB* gene begins with GTG (9), which is a two- to threefold-less-efficient initiation codon than ATG (18); however, the *prnB* open reading frame is apparently translationally coupled to the *prnA* open reading frame (9). Coupling increases translational efficiency and is thought to be a mechanism to ensure coordinate expression of the coupled genes (18). In PRN biosynthesis, translational coupling of *prnA* and *prnB* may be a mechanism to regulate the level of *prnB* gene product present in cells and minimize the diversion of tryptophan to APP.

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