Pseudobactin Biogenesis in the Plant Growth-Promoting Rhizobacterium *Pseudomonas* Strain B10: Identification and Functional Analysis of the L-Ornithine *N*^5^-Oxygenase (*psb*A) Gene

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PseudobactinB₁₀, the fluorescent siderophore produced by the rhizobacterium *Pseudomonas* strain B10, contains the hydroxamate ligand D-*N*^5^-hydroxyornithine (D-*N*^5^-OH-Orn). We cloned the l-Orn *N*^5^-oxygenase (*psb*A) gene from a genomic library of *Pseudomonas* strain B10 and demonstrated that PsbA is involved in the conversion of L-Orn to its *N*^5^-OH derivative. PsbA shows significant similarity to microbial *ω*-amino acid hydroxylases containing flavin adenine dinucleotide and NADP cofactor-binding sites and the FATGY signature of the putative substrate recognition pocket. The *psb*A gene is monocistronic, and its transcription is negatively controlled by iron. A site-specific *psb*A mutant of *Pseudomonas* strain B10 was biochemically complemented with the precursor L-*N*^5^-OH-Orn, suggesting that l-Orn is hydroxylated before conversion to the D isomer. The l-Orn *N*^5^-hydroxylase-defective mutants of *Pseudomonas* strain B10 and *Pseudomonas aeruginosa* PAO1 were much less effective than the parental strains in suppressing the growth of the phytopathogen *Erwinia carotovora* in iron-poor medium. The extent of in vitro inhibition of *E. carotovora* was strictly iron dependent and directly correlated with the amount of released siderophores. These data strengthen the role of fluorescent siderophores in biocontrol of deleterious rhizomicroorganisms.

Pseudobactins (synonym, pyoverdines), the fluorescent siderophores produced by group I pseudomonads, have been proposed to play a crucial role in the biological control of phytopathogenic microorganisms of the rhizosphere (reviewed in reference 26). Pseudobactins are thought to form stable complexes with soil iron, rendering this essential element nutritionally unavailable to deleterious rhizomicroorganisms. Pseudobactins are the most complex structures among microbial siderophores. They consist of a conserved dihydroxyquinoline derivative (the fluorescent chromophore) linked to a variable peptide which confers strict uptake specificities (8). As a general rule, the peptide backbone contains one or two hydroxamate ligands, in the form of D- or L-*N*^5^-OH-ornithine (D/L-*N*^5^-OH-Orn), which participate in Fe(III) coordination with the quinoline hydroxyls of the chromophore.

Pioneer studies with the rhizobacterium *Pseudomonas* strain B10 provided early evidence for the involvement of pseudobactinB₁₀ in the disease-suppressing ability of the producing strain (14, 15, 16, 38, 41). PseudobactinB₁₀ consists of a linear hexapeptide (L-Lys-D-threo-β-OH-Asp-L-Ala-D-α-thr-L-Ala-D-*N*^5^-OH-Orn) linked to the chromophore via an amide bond (39). In addition to pseudobactinB₁₀, *Pseudomonas* strain B10 produces pseudobactin A, a nonfluorescent hydroxamate-like siderophore and a likely intermediate in pseudobactin biogenesis (40).

While the siderophore-mediated disease suppression correlates well with the elevated chemical stability and affinity for iron (*K*_f = 10^12) of pseudobactins (1, 5), the possibility exists that secondary iron-binding compounds from the same bacterium, namely, multiple pseudobactin forms (1, 40), ferribactins (21, 29), and salicylate-based siderophores (31, 43), may contribute to scavenge the limited iron pool in the rhizosphere.

Siderophore-deficient mutants proved to be valuable tools for determining the roles of individual iron chelators in biocontrol (reference 6 and references therein). However, mutations blocking siderophore biosynthetic pathways can lead to production of intermediates endowed with iron-binding activity (31, 37, 44) or overexpression of a secondary siderophore system(s) (31, 33, 37, 44). Moreover, mutations resulting in the siderophore-deficient phenotype can involve global regulatory genes which control a number of functions relevant to disease suppression besides iron uptake (4, 20, 30, 46). Synthesis of metabolites other than siderophores can also be altered due to intracellular iron depletion (4, 11, 19). Finally, at least for salicylic acid, a siderophore-independent activity in biocontrol has been demonstrated, due to the induction of systemic acquired resistance in plants (9). Because of these problems, we revisited the antagonistic role of pseudobactinB₁₀ by a reverse approach. First, we identified a key enzyme of the biosynthetic pathway of the fluorescent siderophore; then we mutated the cognate gene and compared the antagonistic properties of wild-type and siderophore-deficient strains.

To gain insight into the genetic basis of pseudobactin (pseudobactinB₁₀ and pseudobactin A) biogenesis, we cloned the l-Orn *N*^5^-hydroxylase gene of *Pseudomonas* strain B10. The bacterial strains and plasmids used are listed in Table 1. Eight pLaFR1-derived cosmids from a genomic library of *Pseudomonas* strain B10 (25) were individually screened for the ability to complement the *pvd*A mutation in the nonfluorescent, l-Orn *N*^5^-hydroxylase-deficient *Pseudomonas aeruginosa* mutant PALS124 (*pvd*A). Only pJLM1, carrying a DNA insert of approximately 23 kb (25), was able to restore the fluorescent phenotype in PALS124 grown on KB agar plates.
(13). Subcloning in the shuttle vector pUCP18 and deletion analysis localized the complementing DNA region within the 2.7-kb PstI-SphI insert of pCAΔSh. Plasmid pCAΔSh restored nearly wild-type production of both pyoverdine and hydroxamates in PALS124, as determined by spectrophotometric and chemical analyses of iron-poor (IFKB) culture supernatants (1, 18, 42, 44). As for wild-type Pseudomonas strain B10 and P. aeruginosa PAO1, synthesis of both pyoverdine and hydroxamate (IucD2) (reference 12 and SwissProt accession no. P50155) was under stringent iron control and was undetectable in cultures supplemented with 100 μM FeCl3 (data not shown).

Sequence analysis of the 2.7-kb DNA insert of pCAΔSh made it possible to identify an open reading frame of 1,335 bp. The putative gene, designated psba for pseudobactin gene A, is predicted to encode a 49.8-kDa protein showing significant homology to microbial ω-amino acid hydroxylases. The PsBa protein shows the highest similarity to the L-Orn N2-oxygenase (PsBaL1; 76% identity) of P. aeruginosa PAO1 (42) and to the homologous enzyme (PsBa2; 49% identity) from Burkholderia cepacia (33). Significant similarity (ca. 40% identity) was also found with the sidI gene product, the L-Orn N2-oxygenase from Ustilago maydis (23), and with a putative hydroxylase (Hydro) from Aureobasidium pullulans (SwissProt accession no. P31590). Less similarity (ca. 30% identity) was found with the lysine Nα-hydroxylases (IucD1 and Shigella flexneri (IucD2) (reference 12 and SwissProt accession no. AAD4479), respectively, with the putrescine hydroxylases (AlcA) of Bordetella bronchiseptica (10), and with RhbE, a still-uncategorized enzyme likely to be involved in hydroxamate biosynthesis in Sinorhizobium meliloti (SwissProt accession no. AAD09416).

The multiple alignment shown in Fig. 1 highlights three dominant areas of similarity along the whole sequence of ω-amino acid hydroxylases. The conserved N-terminal region (residues 2 to 34 relative to PsBa) is prevalently hydrophobic and contains the putative flavin adenine dinucleotide (FAD)-binding domain. The type 1 signature of the FAD-dependent oxidoreductases (motif GXGXXG; residues 16 to 21 of PsBa) shows a typical replacement of the last glycine with proline; this is a unique feature of hydroxylases involved in siderophore biosynthesis (34). Although such deviation is predicted to distort the α-helical structure within the secondary βββ fingerprint of flavin-binding proteins (45), recent studies suggest that the deviated sequence is still compatible with the binding of FAD and association with the cytoplasmic membrane (35, 36). The existence of an additional dinucleotide (NADP)-binding site in the central portion of all the members of the group (residues 214 to 219 of PsBa) (34). This central signature site is inferred from the identification of a type 3 signature of the FAD-dependent oxidoreductases [GXGXX(G/A)] in the central portion of all the members of the group (residues 214 to 219 of PsBa) (34). The third signature region is located in the C-terminal half, corresponding to the L/FATGY motif (34). This element constitutes the core of a wider similarity region extending for 14 prevalently hydrophobic residues, starting with an aspartate and ending with a proline residue [D(X)2(L/F)ATGY(X)4(H/P)]. Although no specific function has been conclusively assigned to this element, its conservation

![FIG. 1. Multiple sequence alignment of microbial ω-amino acid hydroxylases. The deduced sequence of PsBa was used for BLAST database searches (National Center for Biotechnology Information), and the best-matching sequences were aligned by the BESTFIT and PILEUP programs (Genetics Computer Group [University of Wisconsin, Madison] package, version 10.0). IucD1, E. coli l-lysine Nα-hydroxylase; IucD2, S. flexneri lysine Nα-hydroxylase; AlcA, B. bronchiseptica putrescine hydroxylase; RhbE, S. mellioti hydroxylase; PsBaL1, P. aeruginosa PAO1 l-Orn N2-oxygenase; PsBa, Pseudomonas strain B10 l-Orn N2-oxygenase; PsBa2, B. cepacia l-Orn N2-oxygenase; Hydro, A. pullulans putative hydroxylase; SidI, U. maydis l-Orn N2-oxygenase. Residues common to the nine hydroxylases are shaded in dark gray and printed in white. Identical residues are shaded in medium gray, and similar residues are in light gray. Cysteine residues are printed in white and highlighted in black. Dominant areas of similarity are boxed. The two dinucleotide-binding domains were recognized by the MOTIF program (http://www.motif.genome.ad.jp). Putative transmembrane regions were localized by the PSORT (http://psort.nibb.ac.jp), TopPred2, and DAS (http://www.biocmi.csu.edu) programs. Secondary-structure predictions were inferred by the PredictProtein program (http://www.embl-heidelberg.de).](http://jb.asm.org/Downloadedfromhttp://jb.asm.org/)
strain B10 was generated by gene disruption. The chlor- from *Pseudomonas* left. (B) Northern blot analysis of the *psbA* grown in IFKB medium. Reference molecular mass markers are shown on the left. (Fig. 2B). The length of the efficient cells, while no signal was detected with iron-rich cells in all ω-amino acid hydroxylases is consistent with the proposed role in substrate binding, with the (L/F)ATGY core providing the hydrophobic pocket and the highly conserved aspartate residue acting as proton-abstracting base to render the ω-amino group more reactive to flavin-dependent hydroxylation (34).

To elucidate whether regulation of *psbA* expression occurs at the transcriptional level, Northern blot analysis of *Pseudomonas* strain B10 cell lysates from iron-poor (IFKB) and iron-rich (IFKB plus 100 μM FeCl₃) cultures was performed (18). Given the extensive similarity between PsbA and PvdA, an anti-PvdA mouse polyclonal serum (L. Putignani, A. Ciervo, and P. Visca, unpublished data) was used to detect the PsbA protein. The results show that anti-PvdA antibodies specifically recognize a 50-kDa protein band which is expressed under low-iron conditions but is undetectable in iron-poor culture lysates probed with the anti-PvdA antisera (data not shown).

*Pseudomonas* strain B10CA1 differed from wild-type *Pseudomonas* strain B10 in having impaired synthesis of fluorescent pigment and hydroxamate nitrogen in iron-poor (IFKB) medium. The hypothesis that it is an L-N⁵-OH-Orn auxotroph was confirmed by both genetic and biochemical complementation tests. In fact, B10CA1 produced detectable levels of fluorescent pigment and hydroxamate nitrogen following transformation with pJLM1 (*psbA*) or when fed with 400 μM L-N⁵-OH-Orn (44). Thus, PsbA is responsible for the enzymatic conversion of L-Orn to L-N⁵-OH-Orn, implying the existence of *Pseudomonas* strain B10 of an isomerase capable of turning L- to D-N⁵-OH-Orn during the assembly of the hexapeptide moiety of pseudobactin.

Though mutations of pseudobactin biosynthetic genes were previously reported to result in pleiotropic phenotypes (2), insertional inactivation of the L-Orn N⁵-hydroxylase gene in *P. aeruginosa* PAO1 and *Pseudomonas* strain B10 appeared to cause no secondary effects, except those which can be directly or indirectly predicted for a siderophore biosynthetic mutation (P. Visca and C. Ambrosi, unpublished data). However, unlike *P. aeruginosa* PAAC1 (*pvdA*), *Pseudomonas* strain B10CA1 (*psbA*) showed no reactivity on the CAS agar plates for siderophore detection (32) and failed to grow under severe iron deficiency, i.e., in IFKB supplemented with 600 μM 2,2′-dipyridyl. These phenotypic traits were ascribed to the absence in *Pseudomonas* strain B10 of additional siderophore systems capable of compensating for the defect in pseudobactin synthesis. In fact, thin-layer chromatography analysis of ethyl acetate extracts from IFKB culture supernatants of both wild-type and L-Orn N⁵-hydroxylase-defective mutants (43) did not detect either salicylate or pyochelin in culture extracts of *Pseudomonas* strains B10 and B10CA1, while both compounds were produced by *P. aeruginosa* PAO1 and PAAC1 (data not shown). This observation is consistent with the notion that fluorescent siderophore systems represent the primary strategy for iron assimilation in group I pseudomonads (24), a feature which explains the occurrence of natural strains unable to produce salicylate-based siderophores (22).

*Pseudomonas* strain B10CA1, due to its defect in the biosynthesis of both pseudobactin forms (i.e., pseudobactin₁₀₁₀ and pseudobactin A) and salicylate-based siderophores, was used as an ideal tool to verify the in vitro antagonistic activity of fluorescent siderophores against the soilborne pathogen *Erwinia carotovora* subsp. *carotovora*. This member of the family *Enterobacteriaceae* normally produces the catecholate siderophore chrysobactin, and occasionally aerobactin (3, 28). A previously described antibiosis assay (14) was used to compare the in vitro inhibitory activity of wild-type and L-Orn N⁵-hydroxylase-defective mutants of *Pseudomonas* strain B10 and *P. aeruginosa* PAO1. Inhibition of the test organism by *Pseudomonas* strain B10 occurred only in the iron-poor medium (KB) and not in the iron-rich medium (KB plus 100 μM FeCl₃). Conversely, the pseudobactin-defective mutant B10CA1 did in all ω-amino acid hydroxylases is consistent with the proposed role in substrate binding, with the (L/F)ATGY core providing the hydrophobic pocket and the highly conserved aspartate residue acting as proton-abstracting base to render the ω-amino group more reactive to flavin-dependent hydroxylation (34).

To elucidate whether regulation of *psbA* expression occurs at the transcriptional level, Northern blot analysis of *Pseudomonas* strain B10 cell lysates from iron-poor (IFKB) and iron-rich (IFKB plus 100 μM FeCl₃) conditions (17) and hybridized with a 530-bp SalI DNA probe, internal to the *psbA* coding sequence, which had been labeled with digoxigenin-11-UTP (Boehringer Mannheim). The sizes of RNA standards are shown on the left. (Fig. 2A).

![FIG. 2.](image-url)  
**A** Immunodetection of the 50-kDa PsbA protein in *Pseudomonas* strain B10 cell extracts probed with the anti-PvdA mouse antiserum. Lane 1, *Pseudomonas* strain B10 grown in IFKB medium supplemented with 100 μM FeCl₃; lane 2, *Pseudomonas* strain B10 grown in IFKB medium. Reference molecular mass markers are shown on the left. (B) Northern blot analysis of the *psbA* transcript(s). Total RNA was isolated from *Pseudomonas* strain B10 grown in IFKB medium plus 100 μM FeCl₃ (lane 1) or IFKB medium (lane 2). RNA samples (10 μg) were hybridized with a 530-bp SalI DNA probe, internal to the *psbA* coding sequence, which had been labeled with digoxigenin-11-UTP (Boehringer Mannheim). The sizes of RNA standards are shown on the left.
not exert any inhibitory activity independently of the iron concentration of the medium (Fig. 3). The antagonistic activity was more evident for wild-type *P. aeruginosa* PAO1, while it was strongly reduced for the pyoverdine-defective mutant PAAC1 (Fig. 3). UV light inspection of iron-poor plates disclosed that the fluorescent halo around PAO1 was much larger than that around B10. To correlate the inhibitory activity with siderophore production, the relationship between bacterial growth and release of fluorescent pigment was determined for *P. aeruginosa* PAO1 and *Pseudomonas* strain B10. The two strains showed comparable growth kinetics at 28°C but differed in siderophore biosynthetic capability, in that PAO1 produced threefold more fluorescent pigment than B10 after 24 h of growth in IFKB (data not shown). At this growth stage, PAO1 also released approximately 25 μM pyochelin and 50 μM salicylate into the medium, while B10 did not. These results are consistent with a dose-response effect for siderophore-dependent inhibition of *E. carotovora*.

Our experiments demonstrate that the ability of *Pseudomonas* strain B10 to produce pseudobactin is critical for in vitro antagonistic activity against *E. carotovora*. Transfer of Fe(III) from chrysobactin to fluorescent siderophores is expected on the basis of the unfavorable (2:1) iron-binding stoichiometry and the extremely low affinity for iron of chrysobactin compared with pseudobactin or pyoverdine (28). Given this assumption, the inability of chrysobactin to compete with pseudobactin for iron is the only plausible reason for the in vitro antagonistic activity of *Pseudomonas* strain B10. Likewise, the stronger antagonistic activity of *P. aeruginosa* PAO1 correlates well with its greater capability for iron withdrawal. The overexpression of salicylate and pyochelin iron uptake systems could be responsible for the residual antagonistic activity of the *pvdA* mutant under low-iron conditions (Fig. 3). Interestingly, both *P. aeruginosa* PAO1 and its *pvdA* derivative had residual inhibitory effects in iron-rich medium, meaning that additional antimicrobial metabolites, whose production is unaffected or increased by iron availability, may contribute to the in vitro antagonistic activity (4, 11, 26, 30). Thus, an efficient system for iron uptake is expected to be doubly advantageous for the success of fluorescent pseudomonads in biocontrol; not only would it suppress phytopathogens by scavenging the limited amounts of bioavailable iron in the rhizosphere, it would also provide the cell with sufficient iron to turn on the synthesis of secondary metabolites endowed with antimicrobial properties.

**Nucleotide sequence accession number.** The sequence discussed in this paper has been deposited in the EMBL-GenBank database under accession no. AF230494.

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**REFERENCES**


ERRATA

Revised Translation Start Site for secM Defines an Atypical Signal Peptide That Regulates Escherichia coli secA Expression

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Volume 182, no. 19, p. 5592–5595, 2000. Page 5594, Fig. 4B: The first bar in each series is for CG155 and should be white, and the second bar is for CG29 and should be black.

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Volume 182, no. 21, p. 6233–6238, 2000. Page 6235: Fig. 1 should appear as shown on the facing page.