Analysis of the pmsCEAB Gene Cluster Involved in Biosynthesis of Salicylic Acid and the Siderophore Pseudomonine in the Biocontrol Strain Pseudomonas fluorescens WCS374

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Mutants of Pseudomonas fluorescens WCS374 defective in biosynthesis of the fluorescent siderophore pseudobactin still display siderophore activity, indicating the production of a second siderophore. A recombinant cosmid clone (pMB374-07) of a WCS374 gene library harboring loci necessary for the biosynthesis of salicylic acid (SA) and this second siderophore pseudomonine was isolated. The salicylate biosynthesis region of WCS374 was localized in a 5-kb EcoRI fragment of pMB374-07. The SA and pseudomonine biosynthesis region was identified by transfer of cosmid pMB374-07 to a pseudobactin-deficient strain of P. putida. Sequence analysis of the 5-kb subclone revealed the presence of four open reading frames (ORFs). Products of two ORFs (pmsC and pmsB) showed homologies with chorismate-utilizing enzymes; a third ORF (pmsE) encoded a protein with strong similarity with enzymes involved in the biosynthesis of siderophores in other bacterial species. The region also contained a putative histidine decarboxylase gene (pmsX). A putative promoter region and two predicted iron boxes were localized upstream of pmsC. We determined by reverse transcriptase-mediated PCR that the pmsCEAB genes are cotranscribed and that expression is iron regulated. In vivo expression of SA genes was achieved in P. putida and Escherichia coli cells. In E. coli, deletion affecting the first ORF (pmsC) diminished SA production, whereas deletion of pmsB abolished it completely. The pmsB gene induced low levels of SA production in E. coli when expressed under control of the lacZ promoter. Several lines of evidence indicate that SA and pseudomonine biosynthesis are related. Moreover, we isolated a Tn5 mutant (374-05) that is simultaneously impaired in SA and pseudomonine production.

Despite its abundance in Earth’s crust, iron is largely unavailable for microbial assimilation. Soil microorganisms have developed a mechanism that ensures iron availability in an environment that provides iron (Fe3+) at only about 10−18 M. This strategy involves the secretion of low-molecular-weight iron-chelating compounds called siderophores (45) that show a considerable structural diversity and can be classified according to their main chelating groups (26). Bacteria produce a large variety of siderophores under iron-limiting conditions: hydroxamates, phenol-catecholates, and carboxylates. Moreover, some bacteria produce several types of siderophores, as described for strains of Enterobacter cloacae (37) and Pseudomonas spp. (2, 11, 43, 61). Siderophores produced by certain strains of fluorescent Pseudomonas spp. have been linked to suppression of soil-borne plant diseases. It has been suggested that siderophores are antagonistic by means of sequestering iron from the environment, restricting growth of the pathogen (5, 34, 36). Pseudomonas siderophores have also been implicated in inducing systemic resistance (ISR) in plants (33, 40), that is, an enhancement of the defense capacity of the plant against a broad spectrum of pathogens, triggered by nonpathogenic plant growth-promoting rhizobacteria (63).

Another compound with siderophore activity that can be produced by microorganisms is salicylic acid (SA; 2-hydroxybenzoic acid), SA acting as an authentic, endogenous siderophore has been found under iron limitation in Mycobacterium spp. (51), Pseudomonas spp. (2, 3, 4, 64), Azospirillum lipoforum (54), and Burkholderia (Pseudomonas) cepacia (61). SA is also the precursor or intermediate in the biosynthesis of microbial siderophores, such as pyochelin in Pseudomonas aeruginosa (14). Mutants defective in SA production are unable to synthesize pyochelin (2). The salicylamide moiety is encountered not only in pyochelin but also in aeruginic acid and pseudomonine produced by P. fluorescens strains (3, 12). SA has also been reported to be incorporated into mycobactin S produced by Mycobacterium smegmatis (27, 50), mycobactin T, isolated from M. tuberculosis (60), parabactin produced by Paracoccus denitrificans (47), and maduraferrin isolated from Acinomadura madurae (31). Finally, vulnibactin, a polyamine-containing siderophore from Vibrio vulnificus, contains two SA residues in its molecule (46).
TABLE 1. Strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>P. fluorescens</strong></td>
<td></td>
<td></td>
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<tr>
<td>WCS374</td>
<td>Wild-type PGPR; Psb+ Pms+ SA+</td>
<td>22</td>
</tr>
<tr>
<td>374-05</td>
<td>WCS374 Tns5 mutant; Psb+ Pms+ SA+</td>
<td>This work</td>
</tr>
<tr>
<td>374-05.1</td>
<td>374-05 Tns5 (Tc+) Psb+ mutant derivative</td>
<td>This work</td>
</tr>
<tr>
<td>374-08</td>
<td>WCS374 Tns5 mutant; Psb+ Pms+ SA+</td>
<td>This work</td>
</tr>
<tr>
<td><strong>P. putida</strong></td>
<td></td>
<td></td>
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<tr>
<td>WCS358</td>
<td>Wild-type PGPR; SA- Psb+</td>
<td>22</td>
</tr>
<tr>
<td>JM218</td>
<td>WCS358 Tns5 mutant; Psb-</td>
<td>39</td>
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<td><strong>E. coli</strong></td>
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<tr>
<td>S17-1</td>
<td>thi pro recA hisdR hsdM RP4-2-Tc::Mu-Km::Tn7 Tp' Sm'</td>
<td>59</td>
</tr>
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<td>DH5es</td>
<td>recA1 endA1 p800 lacZ dam-15</td>
<td>Clontech</td>
</tr>
<tr>
<td>HB101</td>
<td>pro leu thi lacY endA recA hisdR hsdM Sm'</td>
<td>10</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pLAFR1</td>
<td>pRK290 containing λ cos site; Tc- Mob+ Tra-</td>
<td>21</td>
</tr>
<tr>
<td>pLAFR3</td>
<td>pLAFR1 plus multiple cloning site of pUC8</td>
<td>59</td>
</tr>
<tr>
<td>pJQ18</td>
<td>pSUP501I derivative; carries Tns-Mob-Tc</td>
<td>Alfred Pühler</td>
</tr>
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<td>pSUP2011</td>
<td>pBR325 replicon; Ap' Km' Tn5 Mob; Tn5 delivery plasmid</td>
<td>59</td>
</tr>
<tr>
<td>pRK2013</td>
<td>ColE1 replicon, helper plasmid; Km' tra</td>
<td></td>
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<tr>
<td>pGEM-3Z</td>
<td>Cloning and transcription vector; Ap'</td>
<td>Promega</td>
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<tr>
<td>pMB374-07</td>
<td>28-kb fragment from WCS374 cloned in pLAFR1; carries SA and pseudomonine synthesis genes</td>
<td>This work</td>
</tr>
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<td>5-kb HindIII fragment from plasmid pMB374-07 cloned in pLAFR3</td>
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<tr>
<td>p07E34</td>
<td>8.5-kb EcoRI fragment from plasmid PM374-07 cloned in pLAFR3</td>
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<td>p07E3/p07E3R</td>
<td>5-kb EcoRI fragment containing pmsCEAB genes cloned in pLAFR3 in both orientations relative to lacZ promoter</td>
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<tr>
<td>pE3/pE3R</td>
<td>5-kb EcoRI fragment containing pmsCEAB genes cloned in pGEM-3Z in both orientations relative to lacZ promoter</td>
<td>This work</td>
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<td>pGEM-3Z with 0.7-kb EcoRI-PstI fragment containing pmsB gene</td>
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<tr>
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<td>pGEM-3Z with 1.7-kb EcoRI-HindIII fragment containing pmsAB genes</td>
<td>This work</td>
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<tr>
<td>pLAs5al-10</td>
<td>pLAFR3 with 0.7-kb PstI fragment containing pmsB gene</td>
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Besides its siderophore activity, bacterial SA has an important role in inducing resistance in plants against pathogen infection. Thus, SA produced by the rhizobacterium P. aeruginosa 7NSK2 has been demonstrated to induce resistance against the fungal pathogen Botrytis cinerea on beans (17, 18). Likewise, heterologous expression of SA biosynthesis genes in P. fluorescens improves ISR in tobacco plants against tobacco necrosis virus (41). In plants, endogenous SA has a key role in systemic acquired resistance, an enhanced resistance status reached by a plant upon pathogen infection (63).

The route of SA biosynthesis in bacteria is not completely known. It has been suggested that in M. smegmatis, salicylate is derived from shikimate (49). Conversion of chorismate to iso-chorismate and subsequently to salicylate has been obtained from cell extracts of this microorganism (27, 38). Two genes responsible for SA biosynthesis in P. aeruginosa have been identified. The products of pchB and pchA genes have been proposed to catalyze the conversion of chorismate to salicylate via iso-chorismate (56). P. fluorescens WCS374 is a plant growth-promoting rhizobacterium (PGPR), originally isolated from the rhizosphere of potato (22), that produces SA and the green-fluorescent siderophore pseudobactin at low iron availability (33). Strain WCS374 suppresses Fusarium wilt in radish by ISR, and pseudobactin has been identified as one of the WCS374 traits involved in ISR (33). Likewise, these authors reported that other iron-regulated metabolites induce ISR in bioassays at low iron availability, which might be explained by SA production. However, they also suggested that additional siderophores may play a role in WCS374 ISR induction. The aim of this work was to localize and characterize the SA biosynthesis region of strain WCS374 as well as to identify additional siderophores produced by this strain under low iron availability. During the course of this investigation, evidence was obtained that SA biosynthesis in WCS374 is linked to the synthesis of a siderophore which has been chemically identified as pseudomonine.

**MATERIALS AND METHODS**

**Strains, plasmids, and media.** The bacterial strains and plasmids used are listed in Table 1. Pseudomonas strains were grown at 28°C in King's medium B (KB) (32), and Escherichia coli strains were grown at 30°C in Luria-Bertani medium (44). SA production was determined from cultures grown in standard succinate medium (SSM; pH 7.0) (42) for Pseudomonas strains and M9 minimal medium (53) for E. coli strains. All plasmids for sequencing were propagated in E. coli DH5α (Clontech) at 37°C. Antibiotics were added at the following concentrations (micrograms per milliliter) when required: ampicillin, 100; kanamycin (KAN), 50; nalidixic acid (NAL), 25; and tetracycline (TET), 20 (for E. coli) and 40 (for Pseudomonas).

**DNA manipulations.** Standard procedures were used for DNA electrophoresis, DNA transfer from agarose gels to nylon membranes (Boehringer Mannheim Biochemicals), preparation of competent cells, and transformation (53). Small-scale isolation of recombinant plasmids was done by alkaline lysis as described by Birnboim and Doly (9). Purification of plasmid DNA and elution of DNA restriction fragments from agarose gels were performed using Qiagen Inc. systems according to the instructions provided. DNA restrictions and ligations with T4 DNA ligase were conducted with enzymes purchased from Pharmacia according to the manufacturer’s instructions. Hybridization was done with a non-radioactive detection kit from Boehringer Mannheim Biochemicals, and the chemiluminescence method was used to detect hybridization bands.
pms gene expression. Expression of pms genes in cultures under low- and high-iron conditions was studied in reverse transcription-mediated PCR (RT-PCR) experiments. We used mutant 374-08 as an internal gene expression control since it contains the neomycin phosphotransferase gene (neo) (7). This gene, carried by the Tn5 element present in this mutant, is expressed when KAN is present in the incubation medium, and its expression can be compared to that of the pms genes. Total RNA was isolated from strains WCS374 (used as a negative control for SA production), WCS374, and 374-08 with a Qiagen RNeasy kit according to the manufacturer’s instructions. Bacteria were grown in liquid SSM (15 ml) for at least 48 h at 28°C. For high-iron conditions, cells from 48-h-old cultures were spun down and resuspended in 15-ml of fresh SSM, amended with 100 μM FeCl₃. H₂O. The cells were then incubated for 3 h at 28°C. 10⁷ cells from low- and high-iron cultures were used for RNA isolation. RNA A isolated RNA from each culture and used 10 μl of an oligo-dT primer/DEPC-treated water. RNA concentration and quality were determined spectrophotometrically and by gel electrophoresis, respectively. To exclude the possibility of contamination with traces of DNA, RNA samples were treated with 20 U of RNase-free DNase (Pharmacia Biotech) for 30 min at 37°C. RNAs were subsequently purified by the method described in the RNAeasy kit for RNA cleanup. Simultaneously, 50 μl DNA samples of strain WCS374 and mutant 374-08 (2 μg of genomic DNA in 50 μl of DEPC-treated water) were treated with RNase-free DNase, as controls to check the effectiveness of the DNase treatment. cDNA synthesis was carried out using a First-Strand cDNA Synthesis kit.
FIG. 1. (A) Restriction map of the 28-kb insert cloned in plasmid pMB374-07. The two lines above the restriction map represent subclones which showed the phenotypes indicated (SA* and/or Pms*) when harbored by JM218. (B) Physical map of the minimal 5-kb EcoRI fragment responsible for SA biosynthesis. Lines below the restriction map represent different deletion clones and subclones constructed to delimit the minimal region involved in SA biosynthesis in E. coli. Production [(+] or nonproduction [(-)] of SA is indicated. Restriction sites: E, EcoRI; B, BamHI; H, HindIII; S, SalI; Sc, SacI; P, PvuII; RV, EcoRV.

WCS374 was carried out. Upon screening of some 4,000 KmR colonies, 13 nonfluorescent (deficient in production of pseudobactin [Psb-deficient]) mutants on KB agar plates were isolated. However, all of the mutants were still able to grow on KB supplemented with 2,2-bipyridyl. This result could be explained either by the production of SA, which can act as a siderophore, or by the presence of third siderophore other than pseudobactin and SA in strain WCS374.

CAS medium (55) was used to assess the presence of siderophore activity in the Psb-deficient mutants isolated. The mutants tested were still able to produce an orange halo on CAS medium agar plates similar in size to the one produced by wild-type WCS374. Moreover, no significant differences in relative halo size were encountered after 48 h of incubation at two different temperatures: 28°C, optimal for growth of the strains, and 37°C, which is repressive for pseudobactin production (39).

Finally, the presence of a single Tn5 insertion was confirmed by hybridization. All mutants studied contained a unique insertion (data not shown).

All of the Psb-deficient mutants analyzed produced comparable quantities of SA; however, production was significantly lower than for WCS374 (Table 2). Nevertheless, SA produced by WCS374 or the Psb-deficient mutants could not explain the siderophore activity detected in the CAS assay, since SA can induce a transitory color shift on CAS medium agar plates only when applied at high concentrations (data not shown). Interestingly, SSM cultures of Psb-deficient mutants emitted a blue fluorescence under UV irradiation. This attribute facilitated the differentiation between pseudobactin producers (green fluorescence), Psb-deficient mutants (blue fluorescence), and the double mutants (nonfluorescent) described below.

The normal growth abilities displayed under low-iron con-
dations by the Psb-deficient mutants along with the capacity to induce wild-type siderophore activity in CAS medium agar plates, which can be discriminated by different incubation temperatures, indicated the presence of an additional siderophore other than SA and pseudobactin produced by strain WCS374.

Identification of a mutant impaired in production of both SA and a newly detected siderophore and construction of SA^{-} Psb-deficient mutants. During the screening for Psb-deficient mutants, a mutant (374-05) showing enhanced production of pseudobactin compared to that by _P. fluorescens_ WCS374 was also isolated. When analyzed for SA production, the result was negative (Table 2). The results obtained in the CAS medium agar assay revealed that the halo produced at 28°C was not significantly different from the one produced by WCS374 or the Psb-deficient mutants. However, incubation at 37°C showed a strong reduction of the relative halo size, due to repression of pseudobactin synthesis at this temperature (39). Actually, pseudobactin was masking the detection of the additional siderophore in CAS agar plates. In contrast, no reduction in halo size at 37°C was observed for the Psb-deficient mutant 374-08. This observation indicated that mutant 374-05 was impaired in the production of not only SA but also the unidentified siderophore. Yet, siderophore activity was detectable in mutant 374-05 at 37°C.

These results, along with the facts that some bacteria, including _Pseudomonas_ spp., produce more than one siderophore and that SA is a precursor in the biosynthesis of certain siderophores, led us to hypothesize that production of SA and the blue-fluorescent siderophore in strain WCS374 are related. Psb-deficient mutants were derived from mutant 374-05 to investigate whether the SA^{-} phenotype in 374-05 is coupled to siderophore deficiency. The suicide vector system pJQ18 (28) was used to generate a second Tn5 insertion in mutant 374-05. Double mutants were selected on the basis of both Km' and Te', The transposition frequency was lower than that obtained in the first mutagenesis, in accordance with the previously described phenomenon of inhibition of transposition exerted by a preexisting Tn5 in the cell (8). After screening of some 900 clones, three Km' and Te', Psb-deficient (nonfluorescent) colonies with significantly reduced halos in CAS medium were isolated. Moreover, the three mutants (374-05.1, 374-05.8, and 374-05.10) did not show the characteristic blue fluorescence of Psb-deficient mutants in CAS cultures, and as for the parent strain 374-05, SA production was not detectable. This result further supported the link between SA and the unidentified siderophore in WCS374. DNA-DNA hybridization experiments revealed the presence of two bands that hybridized with digoxigenin-labeled Tn5 DNA (data not shown).

Identification of the additional siderophore as the SA-based siderophore pseudomonine. Attempts to identify pyochelin, an SA-based siderophore, in culture supernatants of the strains tested failed. Moreover, neither catechol nor hydroxamate groups were detected in the supernatant of these strains. In contrast, weak positive reactions for both were obtained in pseudobactin producer strains WCS374 and 374-05. This can be explained by the presence of these groups in the structure of this siderophore (26). To identify the siderophore produced by WCS374, culture supernatants of mutant strain 374-08 grown in liquid SSM were used.

(i) Mass spectrometric analysis of pseudomonine. The siderophore preparation was analyzed in the positive-ion mode using FAB mass spectrometry and CID, as well as EI. In both the positive-ion mode FAB mass spectrum recorded from the siderophore isolated in this study and the (unpublished) positive-ion mode FAB mass spectrum recorded by Anthoni and coworkers from pseudomonine (3), a protonated molecule was observed at _m/z_ 331. To obtain structural information, the ion at _m/z_ 331 was submitted to CID tandem mass spectrometry (Fig. 2A). The most abundant fragment ion is observed at _m/z_ 211 and corresponds to the loss of the SA group (Fig. 2B). The SA group fragment is observed at _m/z_ 121. The ion at _m/z_ 110 corresponds to a histidine immonium ion, which is derived from the histamine moiety, while the ions at _m/z_ 82 and 95 also arise by cleavage of the hydrocarbon backbone of the histamine moiety. The ion at _m/z_ 138 corresponds to cleavage in the cyclohexenone moiety with charge retention on the histamine-containing part of molecule. We assign the ion at _m/z_ 204 as deriving from the SA-containing portion of the molecule and being generated by cleavage in the cyclohexenone moiety. This ion is complemented by an ion at _m/z_ 128 that we assign as arising via an analogous cleavage in the cyclohexenone moiety, but now with charge retention on the histamine-containing part of the molecule.

To further verify these assignments, pseudomonine was submitted to H-D exchange and subsequent mass spectrometric analysis. In the FAB mass spectrum, this time recorded in a deuterated glycerol matrix, the pseudomolecular ion shifted by 4 atomic mass units to _m/z_ 335, which is in correspondence with the replacement of three exchangeable protons by deuterium and one deuterium carrying the charge. The CID mass spectrum of the ion at _m/z_ 335 confirmed the assignment of the fragmentation described above, with all fragments indeed incorporating the expected number of D atoms. Figure 2B shows the ions observed in the CID spectrum of the deuterated species and the exchangeable hydrogens.

The EI mass spectrum (data not shown) is directly comparable to the unpublished EI spectrum recorded by Anthoni et
al. (3). The molecular ion is observed at m/z 330, and fragment ions are observed at m/z 82, 95, 110, 121, 127, 138, and 204, corresponding to the fragment ions that were observed in the CID spectrum (see above).

(ii) NMR analysis of pseudomonine. To obtain further structural information from the bioactive compound isolated, the fraction containing the bioactive compound was analyzed by 13C and 1H NMR. The chemical shifts are summarized in Table 3, alongside the NMR data obtained by Anthoni et al. (3). A comparison of the two data sets makes it clear that the chemical shifts observed by both groups are nearly identical.

SA and pseudomonine biosynthesis loci are linked in the strain WCS374 genome: delimitation of the SA biosynthesis region. To localize a recombinant cosmid clone harboring the biosynthetic regions of SA and pseudomonine, heterologous expression was pursued. JM218, a Tn5 Psb-deficient mutant of P. putida WCS358 (39), produces a very small halo on CAS medium agar plates. Both WCS358 (33) and JM218 (Table 2) do not produce SA. Thus, halo restoration could be used as a marker for expression of WCS374 siderophore biosynthetic genes in JM218. Matings were performed using a mixture of the gene library (>8,000 clones; average insert size, 20 kb) in

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FIG. 2. (A) CID mass spectrum of the compound found in the fraction eluting in 100% ACN. (B) Fragmentation scheme for the component with [M + H]+ at m/z 331. Boldface numbers indicate fragment masses of the ions obtained from the deuterated component. Small numbers refer to the atom numbering used to assign the NMR data summarized in Table 3. The H atoms shown in boldface correspond to exchangeable protons.
DH5α as donor strain and JM218 as recipient strain. Helper plasmid pRK2013 (20) was used to aid transfer of the recombinant cosmids. Selection of the transconjugants was made on KB without Mg²⁺ to avoid antagonism with TET, supplemented with TET, KAN, and NAL. After screening 2,150 Tc resistant colonies, 38 were selected. The three clones that fulfilled this criterion (pMB374-07, pMB374-15, and pMB374-31) were also the only ones showing SA production. However, the amount of SA detected was far lower than that obtained for WCS374 (33) (Table 2). SSM culture supernatants of JM218 carrying these recombinant clones showed a blue fluorescence under UV light, which corroborates the observation obtained for the Psb-deficient mutants of WCS374. High-pressure liquid chromatography (HPLC) analysis of the culture supernatant of JM218 carrying pMB374-07 revealed the presence of pseudomonine (data not shown).

According to their EcoRI restriction patterns, the three clones isolated were identical. The insert, with a calculated size of 28 kb, consisted of four fragments of 14, 5.5, 5, and 3.5 kb. A restriction map of plasmid pMB374-07, used in further experiments, is shown in Fig. 1. Subcloning of the different restriction fragments led us to identify the minimal region involved in SA biosynthesis in WCS374 (Fig. 1). A 5-kb EcoRI fragment was sufficient to promote SA synthesis in P. putida JM218 (plasmids p07E3 and p07E3R) and E. coli strains (plasmids pE3 and pE3R) (Table 2). Higher SA levels were obtained for plasmid p07E3R than for plasmid pMB374-07. Plasmids containing only the 5-kb EcoRI fragment were no longer able to induce halo production by JM218 on CAS medium agar plates; only JM218 carrying the entire, originally isolated cosm id pMB374-07 retained this ability. Additionally, the characteristic blue fluorescence detected in the SSM supernatant of JM218(pMB374-07) cultures was weaker for JM218(p07E3R), despite the higher SA levels detected for the latter (Table 2).

These results support the hypothesis that SA and pseudomonine production are linked in strain WCS374. There was a correlation among halo production on CAS medium agar plates and the blue fluorescence displayed by Psb-deficient mutants in SSM. Moreover, all pseudomonine-producing strains showed the ability to synthesize SA, and plasmid pMB374-07 contains closely linked genes for the biosynthesis of both compounds.

**Nucleotide sequence analysis of the SA biosynthesis region.**

Nucleotide sequence analysis of the 5,057-bp EcoRI fragment (56.48% G+C content) revealed the presence of four major open reading frames (ORFs) with high coding probability and rightward polarity (Fig. 1). The first ORF (ORFA) is preceded by a strong potential ribosome binding sequence (RBS) (GGAGGA) that matches the consensus RBS of E. coli (58).

ORFA is 1,176 bp long (positions 207 to 1382), potentially coding for a 391-amino-acid-residue-long protein. A second ORF (ORFB), identified between positions 1409 to 3244, is 1,836 bp long and codes for a putative protein of 611 amino acid residues. At 39 bp downstream from the TGA stop codon of ORFB, a third ORF (ORFC), 1,218 bp long, potentially coding for a 405-amino-acid-residue protein (positions 3284 to 4501). A strong RBS (AAGGAG) is found at 6 bp upstream from the ATG starting codon. Finally, at 14 bp from the TGA stop codon of ORFC, the ATG start codon of ORFD (positions 4516 to 4851) was located. It was only 336 bp long, with a predicted translation product of 111 amino acid residues. An appropriate RBS (AAGGAG) was found at positions 4502 to 4507. The four ORFs identified in the 5-kb region are closely connected, supporting the idea of a polycistronic organization.

**Protein sequence homology.**

A search in the databases for protein homology yielded relevant data for all predicted translational products of the sequenced region. ORFA (proposed name pmsA, for pseudomonine synthesis) codes for a protein with a predicted molecular weight of 43,102. The deduced amino acid sequence showed similarities with several chorismate-utilizing enzymes: isochorismate synthases (ICSs) of E. coli (EntC; EMBL/GenBank accession number P10377; 45% identity and 73% similarity), Aeromonas hydrophila (AmoA; P23300; 43% identity and 69% similarity), Vibrio cholerae (VibC; 007898; 43% identity and 69% similarity), and P. aeruginosa (PchA; Q51508; 38.5% identity and 52.6% similarity in 192 residues of the carboxy-terminal domain). It also showed moderate homology with malaquilone-specific ICS (MenF) from different bacteria and weak homology with component I of anthranilate synthases (TrpE) and specific ICS (MenF) from different bacteria and weak homology with component I of anthranilate synthases (TrpE) and specific ICS (MenF) from different bacteria. ICS catalyzes the conversion of chorismate to isochorismate. This compound is utilized for the biosynthesis of some quinones and phenolic acid derivatives such as 2,3-dihydroxybenzoic acid (2,3-DHB). ICSs cited above have been reported as key enzymes in the biosynthetic pathway of siderophores such as enterobactin (19), pyochelin and SA (56), amononabactin (6), and vibriobactin (O07898).

The deduced amino acid sequence of ORFB (pmsE) (predicted molecular weight of 67,417) also showed strong similarities with proteins involved in siderophore synthesis: 2,3-dihydroxybenzoate-AMP ligase of E. coli (EntE; P10378; 55% identity and 81% similarity) and B. subtilis (DhbE; P40871; 49.7% identity and 70% similarity in 463) for enterobactin...
synthesis, vibriobactin (VibE; O07899) biosynthesis of *V. cholerae* (45% identity and 74% similarity), pyochelin (PchD; P72175) biosynthesis of *P. aeruginosa* (43% identity and 71% similarity), and yersiniabactin (YbE; Q56950) biosynthesis of *Yersinia pestis* (39% identity and 70% similarity). The function of EntE is the activation of the carboxylate group of 2,3-DHB, via an ATP-dependent PPi-exchange reaction. It is one of the constituents of a membrane-bound multienzyme complex (35). Moderate to weak homologies were found with other enzymes that act via an ATP-dependent covalent binding of AMP to their substrates, such as 4-coumarate coenzyme A ligase, surfactin synthetase subunit 1, and luciferin 4-monoxygenase (luciferase).

The ORFC (*pmsA*) translational product (predicted molecular weight of 45,726) showed high homology with pyridoxal phosphate (pyridoxal-P)-dependent histidine decarboxylases (HDHC) from *Klebsiella planticola* (P28578; 77% identity), *Morganella morgani* (P05034; 76% identity), *Enterobacter aerogenes* (P28577; 75% identity), and *Vibrio anguillarum* (Q56851; 62% identity). Moreover, 39% identity and 72% similarity were also found with HDHC of tomato (*Lycopersicon esculentum*) (P54772). Important features such as the sequence SXHK (positions 231 to 234), found in other pyridoxal-P-dependent carboxylases, the lysine residue that binds the molecule of pyridoxal-P (position 234), and the serine residue (position 324) that binds the adduct formed between pyridoxal-P and the inhibitor α-fluoromethylhistidine (25) are also present in the WCS374-encoded HDHC. However, a common nucleotide sequence found upstream of *hdc* genes from *M. morgani*, *K. planticola* and *E. aerogenes*, and postulated to be a histidine-responsive element (30), was not detected upstream of the putative *hdc* gene of strain WCS374. HDC catalyzes decarboxylation of histidine to histamine. Although histamine production has been reported for different pseudomonads (23), this is to our knowledge the first putative *hdc* gene sequenced for a *Pseudomonas* species. Histamine has been found in the molecular structures of different siderophores: anguibactin from *V. anguillarum* (29), pseudomonine from *P. fluorescens* AH2 (3), and acinetobactin from *Acinetobacter baumannii* (65). It has been shown by mutation analysis that the *hdc* gene is essential for the biosynthesis of anguibactin (62).

Comparison of the ORFD (*pmsB*) amino acid sequence (predicted molecular size of 13,003 Da) with database entries revealed significant homology to an unknown ORF of *V. vulnificus* (P74964; 60% identity and 83% similarity) and to the salicylate biosynthesis protein PchB of *P. aeruginosa* (56) (57% identity and 69% similarity). It has been hypothesized that PchB has isochorismate-pyruvate lyase activity. PchB together with PchA (a putative isochorismate synthase) are required for SA synthesis in *P. aeruginosa*. Interestingly, PmsB showed similarity to the bifunctional TyrA proteins (chorismate mutase/ prephenate dehydrogenase; T protein) of *Haemophilus influenzae* (P43902), *Erwinia herbicola* (Q02287), and *E. coli* (P07023). The homology was located in the N-terminal domain of TyrA. TyrA proteins are larger than FbsB, and no homology was found with the monofunctional chorismate mutase from *B. subtilis* (24), the smallest natural chorismate mutase known (127 amino acids), and similar in size to FbsB (111 residues). However, lack of sequence homology between chorismate mutases of different species is well known (48).

**Expression of SA genes in *E. coli***. SA was detected in M9 culture supernatants of *E. coli* DH5α cells carrying plasmid pE3 or pE3R (Table 2), suggesting that the putative promoter detected upstream of *pmsC* is functional in *E. coli*. Although high SA levels were detected, irrespective of orientation, enhanced SA production was obtained when the genes were properly oriented with regard to the *lacZ* promoter present in pGEM-3Z (pE3R). SA was also detectable in the spent medium of DH5α cells harboring different deletion clones, as well as subclones (Table 2). Deletions affecting *pmsB* (plasmid pE3-01) (Fig. 1) failed to produce SA in DH5α, indicating that this gene is essential for SA biosynthesis. Deletions affecting genes upstream of *pmsB* (i.e., plasmid pE3R-01 [Fig. 1], a 233-bp deletion derivative affecting the potential promoter region and the first nine codons of *pmsC*) resulted in 10-fold-lower SA production (Table 2). Likewise, DH5α carrying plasmids pJMSal-10 and pJMSal-20 produced detectable amounts of SA (Table 2).

**Expression of pms genes is iron regulated**. DNA sequence analysis identified putative σ^54^ promoter sequences only upstream of *pmsC*, suggesting that *pms* genes might have a poly- cisitronic organization. The presence of two potential Fur boxes suggests that expression of the *pms* genes is iron regulated. Expression of *pms* genes was analyzed by RT-PCR. Total RNA from strains WCS374 and 374-08 grown in SSM was used for cDNA synthesis using primer SAL03. For subsequent PCR experiments, we used several pairs of primers that overlapped different *pms* genes: SAL01-SAL02 to define an internal *pmsB* PCR product, HDC01-SAL02 to yield a PCR product spanning *pmsAB*, and DHBO1-DHB02 to amplify a fragment that partially overlaps the *pmsCE* coding region (Fig. 3A). For cDNA synthesis from neo mRNA, primer KM03 was used. The internal primer pair KM01-KM02 was used for PCR. If a polycistronic mRNA is obtained from the cDNA synthesis using primer SAL03, the predicted PCR products will be detected in all primer combinations designed. The result of this experiment is shown in Fig. 3B. PCR products expected for SAL01-SAL02 (214 bp), HDC01-SAL02 (599 bp), and DHBO1-DHB02 (439 bp) were detected in mutant 374-08 and WCS374 cDNA PCRs. Similarly, the PCR product predicted for KM01-KM02 (405 bp) was detected in mutant 374-08 cDNA but not in WCS374 cDNA. The band sizes were concordant with PCR products obtained in PCR control reactions using WCS374 and 384-08 genomic DNAs as templates. Simultaneously, control PCRs of genomic DNA treated with RNase-free DNase did not yield any product, indicating that PCR bands due to DNA contamination in RNA samples can be excluded.

RT-PCR experiments also confirmed that expression of *pmsCEAB* genes is repressed by iron. When WCS374 and 374-08 were incubated during 3 h in SSM amended with 100 μM FeCl₃, a strong reduction of *pmsCEAB* transcript, measured as the corresponding PCR products, was observed (Fig. 3B and C), whereas *neo* gene expression in mutant 374-08 was not affected by iron. A semiquantitative determination of transcript repression was calculated on the basis of dilution series of the cDNA sample used in PCRs. PCR products from cDNA samples of cultures grown under high-iron conditions were approximately 100-fold lower than those from low-iron condi-
tions (Fig. 3C). These results demonstrate that the promoter region detected by DNA analysis is functional and that the pmsCEAB genes may constitute an operon. However, further analysis will be necessary to confirm this organization. Finally, pms gene expression is iron regulated, suggesting that the iron-regulatory elements detected in the promoter region are functional as well.

**DISCUSSION**

Several strains of *Pseudomonas* spp. have been reported to produce more than one type of siderophore under iron-limiting conditions (2, 11, 43, 64). For the PGPR strain *P. fluorescens* WCS374, production of an additional siderophore, besides pseudobactin and SA, was suggested (33). In this work, we show that the additional siderophore in strain WCS374 is pseudomonine, an isoxazolidone previously isolated in culture supernatants of iron-deficient cultures of a *P. fluorescens* strain isolated on spoiled Nile perch from Lake Victoria (Africa) (3).

Two lines of evidence suggest that SA production and pseudomonine synthesis are related. One mutant that we studied (374-05) is impaired in both SA and pseudomonine production, and a recombinant cosmid from the WCS374 gene library carries the genetic information for both traits. Additionally, pseudomonine has been identified as an SA-based siderophore (reference 3 and this work).
sis genes are closely linked and present in the recombinant clone pMB374-07. This plasmid induced both siderophore activity and SA production in *P. putida* WCS358 Psb-deficient strain JM218. pMB374-07 derivative subclones were unable to drive pseudomonine synthesis, suggesting that most of the cloned insert is involved in this trait. However, only 5 kb from pMB374-07 were required to induce SA production in JM218 (plasmids p07E3 and p07E3R) and in *E. coli* cells (plasmids pE3 and pE3R).

Double mutants impaired in the production of both pseudobactin and pseudomonine still showed some siderophore activity in CAS medium. Although additional iron uptake systems could still be functional, the possibility that mutant 374-05 is leaky and able to release small quantities of pseudomonine to the medium cannot be ruled out. As a matter of fact, HPLC analysis of the culture supernatant of mutant 374-05:8 revealed a very small peak at the position of pseudomonine (data not shown), indicating that the mutant indeed is leaky. Preliminary experiments have shown that the Tn5 insertion in mutant 374-05 is located elsewhere in the WCS374 genome and that this mutant is likely affected in pseudomonine transport machinery (J. Mercado-Blanco, unpublished data).

Serino et al. (56) proposed that SA synthesis in *P. aeruginosa* proceeds from chorismate via isochorismate. The two potential SA biosynthesis genes of strain WCS374, *pmsC* and *pmsB*, showed moderate to high similarity to the SA biosynthetic genes *pchB* and *pchA* of *P. aeruginosa* (56); *PchA* would act as an ICS, and *PchB* would act as an isochorismate-pyruvate lyase. Based on sequence similarities, *pmsC* and *pmsB* may function similarly in *P. fluorescens* WCS374. However, the genetic organization is different. In *P. aeruginosa*, *pchB* and *pchA* are the distal genes of the *pchDCBA* operon involved in SA and pyochelin biosynthesis (57). In *P. fluorescens* WCS374, two genes are interspersed between the proposed SA biosynthetic genes (*pmsC* and *pmsB*): a putative *entE* homolog (*pmsE*) and a putative *hdc* gene (*pmsA*). It is unlikely that there is any role in SA synthesis for these latter genes, especially *pmsA* with a totally unrelated activity. We speculated that this genetic arrangement is part of the biosynthetic machinery of pseudomonine, *pmsC* and *pmsB* would contribute to synthesis of the SA moiety present in the pseudomonine structure. The presence of an *hdc* gene (*pmsA*) supports the conclusion that histamine, which is present in the structure of pseudomonine, is a precursor in the synthesis of this siderophore. Anguibactin in *V. anguillarum* (62) and acinetobactin in *A. baumannii* (65) are siderophores that also contain this biogenic amine. Acinetobactin and pseudomonine possess an oxazoline ring instead of a thiazoline ring which is present in anguibactin. Finally, PmsE has striking similarities to proteins involved in the biosynthesis of siderophores such as enterobactin (EntE), pyochelin (PchD), vibriobactin (VibE), and yersiniabactin (YbrE). The EntE protein mediates the activation of a catechol (2,3-DHB) in one of the steps in enterobactin synthesis. As proposed by Serino et al. (57) and previously reported for *entE* of *E. coli* (35), PmsE might act in the same way as EntE, activating the carboxylate group of SA.

In vivo SA biosynthesis was achieved in heterologous backgrounds such as in *P. putida* and *E. coli* cells (Table 2). Similarly, *pms* genes were expressed under the control of their own promoter in *E. coli* (Table 2). Only when ORFD (*pmsB*) was not present was SA production impaired. When *pmsB* was provided alone (plasmid pJMSa1-10), SA was still detectable in M9 medium supernatant (Table 2). Thus, *pmsB* has been shown to be essential for SA synthesis in *E. coli*. This observation indicates that PmsB can use endogenous substrates provided by *E. coli* cells. However, upstream genes appear to provide additional substrates to produce high SA levels (plasmids pE3 and pE3R [Table 2]). Serino et al. (56) concluded that *PchB* in *P. aeruginosa* seems to be involved in the conversion of isochorismate to SA, but it is unable to synthesize SA directly from chorismate. The fact that SA was detected in DH5α harboring deletion plasmids affecting *pmsC* indicates that this gene is not essential for SA biosynthesis in *E. coli* but is important to reach the SA levels produced by pE3R. An explanation may be that indigenous ICS activity complements the absence of PmsC in deletion clones. Alternatively, substrates provided by *E. coli* can be used, with less specificity, by PmsB. In contrast, SA was not detected in JM218 culture supernatants when *pmsB* was provided alone (pLASa1-10). PmsB has a certain degree of homology with the N-terminal domain of different chorismate mutases. An interesting possibility, which require further experimental support, is that the putative PmsB can use other substrates, including chorismate. In this sense, moderate homology (29% identity and 59% similarity) was found with the 129-amino-acid-residue protein PapB from *Streptomyces pristinaespiralis*, a protein identified as a mutase involved in the biosynthesis of 4-methylaminol-1-phenylalanine (accession number P72541). It is also interesting that PmsB showed homology with an unknown ORF in *V. vulnificus*. This bacterium synthesizes vulnibactin, a siderophore which contains two residues of SA (46). These two residues are involved in the formation of oxazoline rings with L-threonine, the same arrangement as encountered in pseudomonine. Thus, PmsB, PchB, and the latter ORF may constitute a family of proteins involved in synthesis of the SA moiety in this type of siderophore.

Results of homology analysis presented in this work, along with the results of gene expression by RT-PCR as well as Fe-mediated SA synthesis repression studies performed earlier by our group (33), show that (i) the promoter region detected upstream of *pmsC* is functional, (ii) expression of the *pms* genes is iron regulated, and (iii) the *pmsCEAB* transcript is polycistronic. However, the existence of additional promoters in the *pms* region cannot be completely ruled out. Differences in efficiency in primer binding can be considered, although no obvious differences were observed when DNA templates were used in PCR control experiments (Fig. 3B).

The specific contributions of pseudobactin, pseudomonine, and SA in iron uptake in strain WCS374 are not known. On the other hand, it has been demonstrated that pseudobactin and SA induce resistance in radishes against *Fusarium* wilt (33). These authors reported that a pseudobactin mutant was still able to induce resistance at low iron availability, hypothesizing that SA or a different siderophore, which now has been confirmed to be pseudomonine, was responsible for disease suppression. The possible role of pseudomonine in plant disease suppression and ISR will be further investigated.
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