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Biosynthesis of Pyochelin and Dihydroaeruginoic Acid Requires the Iron-Regulated *pchDCBA* Operon in *Pseudomonas aeruginosa*

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The high-affinity siderophore salicylate is an intermediate in the biosynthetic pathway of pyochelin, another siderophore and chelator of transition metal ions, in *Pseudomonas aeruginosa*. The 2.5-kb region upstream of the salicylate biosynthetic genes *pchBA* was sequenced and found to contain two additional, contiguous genes, *pchD* and *pchC*, having the same orientation. The deduced amino acid sequence of the 60-kDa PchD protein was similar to those of the EntE protein (2,3-dihydroxybenzoate-AMP ligase) of *Escherichia coli* and other adenylate-forming enzymes, suggesting that salicylate might be adenylated at the carboxyl group by PchD. The 28-kDa PchC protein showed similarities to thioesterases of prokaryotic and eukaryotic origin and might participate in the release of the product(s) formed from activated salicylate. One potential product, dihydroaeruginoate (Dha), was identified in culture supernatants of iron-limited *P. aeruginosa* cells. The antifungal antibiotic Dha is thought to arise from the reaction of salicylate with cysteine, followed by cyclization of cysteine. Inactivation of the chromosomal *pchD* gene by insertion of the transcription and translation stop element ΩSm/Sp abolished the production of Dha and pyochelin, implying that PchD-mediated activation of salicylate may be a common first step in the synthesis of both metabolites. Furthermore, the *pchD*::ΩSm/Sp mutation had a strong polar effect on the expression of the *pchBA* genes, i.e., on salicylate synthesis, indicating that the *pchDCBA* genes constitute a transcriptional unit. A full-length *pchDCBA* transcript of ca. 4.4 kb could be detected in iron-deprived, growing cells of *P. aeruginosa*. Transcription of *pchD* started at tandemly arranged promoters, which overlapped with two Fur boxes (binding sites for the ferric uptake regulator) and the promoter of the divergently transcribed *pchR* gene encoding an activator of pyochelin biosynthesis. This promoter arrangement allows tight iron-mediated repression of the *pchDCBA* operon.

Pseudomonas aeruginosa, a ubiquitous bacterium and opportunistic pathogen, produces three siderophores during growth under iron-limiting conditions: pyoverdin, salicylate, and pyochelin (2, 11, 12, 36, 60). Pyoverdin consists of a dihydroquinoline chromophore attached to a hydroxamate-containing oligopeptide (65). The stability constant of the Fe(III)-pyoverdin complex is about 10^{32} (37). Salicylate is derived from chorismate by rearrangement and subsequent elimination of pyruvate (54). The stability constant of the Fe(III)-trisalicylate complex is ca. 10^{35} in the absence of inorganic phosphate (34, 48). Furthermore, salicylate is a precursor of pyochelin, a low-affinity but active siderophore having a stability constant of ca. 10^5 for the Fe(III)-dipyochelin complex (4, 9, 12, 13). Pyochelin also chelates Zn(II), Cu(II), Co(II), Mo(VI), and Ni(II) ions (61), and hence an important function of pyochelin might be to mobilize these metal ions and deliver them to the cell.

Excess iron represses the synthesis of pyoverdin, salicylate, and pyochelin in *P. aeruginosa* (11, 12, 60). Repression is mediated by the Fur repressor (6, 46), which in the presence of iron binds to the promoters of the *pvdS* and *pchR* genes (33, 42). PvdS, a putative sigma factor, and PchR, a member of the AraC family of transcriptional activators, are positive regulators of pyoverdin and pyochelin synthesis, respectively (15, 26, 27, 40). PchR is required for the synthesis of both pyochelin and the pyochelin receptor FptA (26, 27). Furthermore, PchR negatively regulates its own expression (27). Exogenous pyochelin induces the FptA receptor (24), and iron represses FptA at the transcriptional level in a *fur*$^+$ background (3). Pyoverdin and pyochelin contribute to the virulence of *P. aeruginosa* in compromised mice (10, 38, 41).

Salicylate is incorporated into pyochelin by *P. aeruginosa* (2). The stereochemistry of the two naturally occurring isomers pyochelin I and pyochelin II suggests that salicylate is coupled to one molecule each of D-cysteine and L-cysteine (49). Both cysteine residues cyclize; dehydrogenation and methylation reactions probably complete the synthesis of pyochelin (13). However, nothing is known about the genes and enzymes involved in the reactions converting salicylate to pyochelin. We have previously characterized the *pchBA* genes of *P. aeruginosa*; they code for enzymes catalyzing the formation of salicylate from chorismate, via isochorismate as a likely intermediate (54). Here, we show that the *pchBA* genes are located in the distal part of an operon containing two additional genes, *pchD* and *pchC*, which are involved in the initial steps of pyochelin synthesis from salicylate. This *pchDCBA* operon and the adjacent regulatory gene *pchR* are divergently transcribed from a single, iron-repressible promoter region. We also demonstrate that wild-type *P. aeruginosa* excretes dihydroaeruginoate (Dha) [2′-(2-hydroxyphenyl)-2′-thiazoline-4′-carboxylate], an antibiotic previously detected in *Pseudomonas fluorescens* (7). Dha can be formed enzymatically or nonenzymatically when salicylate activated at the carboxyl group reacts with one molecule of cysteine (4, 7, 29).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
P. aeruginosa		
PAO1	Prototroph	ATCC 15692
PAOA4	*fur-4*	6
PALS128	*pvdB*	62
PALS128-17	*pvdB pchB* or *pchA*	54
PAO6285	*pchD*::ω	This study
PAO6286	*pvdB pchD*::ΩSm/Sp	This study
PAO307	*argC54*	25
PAO951	*proA137*	63
PAO4104	*pyu-9010 dcu-9008 trpE9051*	35
E. coli		
XL1-Blue	*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1*/*F′* (*proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*)	52
DH5α	*recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80d*lacZ*ΔM15)	52
S17-1	*thi pro hsdR recA*; chromosomal RP4 (Tra$^+$ Tc^s Km^s Ap^s)	55
Plasmids		
pQF10	Broad-host-range vector, ColE1-pRO1600 replicon; Ap^r Tc^r	20
pBluescript I KS-	Cloning vector; ColE1 replicon; Ap^r	Stratagene
pNM480	Ap^r; ColE1 replicon, *′lacZY*$^+$	39
pME3087	Suicide vector; Tc^r; ColE1 replicon	64
pKT240	IncQ Mob Ap^r/Cb^r Km^r	5
pME3300	pLAFR3 carrying a 28-kb *Bam*HI-*Hin*dIII fragment of *P. aeruginosa* PAO1 DNA, containing the structural genes for salicylate and pyochelin biosynthesis	54
pME3309	pQF10 carrying a 10-kb *Eco*RI-*Hin*dIII fragment containing the *pchDCBA* genes	54
pME3315	pQF10 carrying a 6.7-kb *Bgl*II-*Kpn*I fragment containing the *pchDCBA* genes	54
pME3318	pQF10 carrying a 4.6-kb *Eco*RI-*Sal*I fragment containing the *pchDCBA* genes	This study
pME3349	pBluescript carrying a 953-bp *Xma*I-*Eco*RI fragment (*Eco*RI site from Tn*1725* insertion 9)	This study
pME6122	pKT240 carrying a 3.7-kb *Eco*RI-*Dra*I fragment containing *pchD′*-*′lacZ* derived from pNM480; Cb^r/Ap^r	This study

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. aeruginosa* and *Escherichia coli* strains used and their plasmids are listed in Table 1. Bacteria were routinely grown at 37°C on nutrient agar and minimal medium E and in nutrient yeast broth (25). Iron-depleted Casamino Acids medium (DCAA medium) (61) was used for production of salicylate, Dha, and pyochelin by *P. aeruginosa*. Chrome azurol S (CAS) agar served as an indicator for pyoverdin and pyochelin (53). When the pyoverdin-, salicylate-, and pyochelin-negative strain PALS128-17 was complemented for pyochelin production, e.g., by plasmid pME3315, an orange halo was formed on CAS agar. Tn*1725* insertions in *pchD* abolished the formation of this halo. Spectinomycin was used at 1,000 μg/ml in *P. aeruginosa*. Other antibiotic concentrations have been specified previously (54).

Chemical synthesis of Dha. D-Cysteine · HCl (0.010 mol) and salicylonitrile (0.005 mol) were incubated in 40 ml of methanol–0.1 M phosphate buffer (pH 6.0) (1:1) at 40°C with stirring for 3 days (29) to give (*S*)-Dha (7). The product was purified by high-pressure liquid chromatography (HPLC) and identified by mass spectroscopy (ionization with NH_3), ^1H and ^{13}C nuclear magnetic resonance, and determination of optical rotation (7, 29).

Identification of salicylate, Dha, and pyochelin in culture supernatants of *P. aeruginosa*. *P. aeruginosa* strains were grown in DCAA medium to stationary phase. Metabolites were extracted from culture supernatants as described previously (54). Salicylate, Dha, and pyochelin were analyzed by thin-layer chromatography (TLC) (54). Dha was visualized by use of UV light and by its brown color after being sprayed with $FeCl_3$ reagent (12). For HPLC analysis, ethyl acetate extracts of acidified culture supernatants (54) were dried by evaporation and dissolved in 400 μl of 70% (vol/vol) methanol + 0.43% H_3PO_4. An aliquot of 100 μl was injected into an HPLC system (54). A binary gradient consisting of solvent A (0.43% H_3PO_4) and solvent B (95% methanol and 0.43% H_3PO_4) was used as follows: 0 to 29 min with 20 to 83% solvent B and 2 min with 83 to 100% solvent B. Elution was carried out at room temperature with a flow rate of 0.8 ml per min. Compounds were identified by their retention times and UV spectra. Dha and salicylic acid were eluted at 19.7 and 20.8 min, respectively. Under the conditions described, (−)-(*R*)-dihydroaeruginoic acid was not separated from (+)-(*S*)-dihydroaeruginoic acid. Dha in culture fluids was identified by HPLC coupled with thermospray-mass spectroscopy and postcolumn addition of ammonium acetate as the ionizing agent. The pyochelin standard was isolated as described previously (13) from a culture filtrate of strain PALS128/pME3300 grown in DCAA medium (54) and purified by HPLC. Pyochelin isomerizes spontaneously to a mixture of pyochelins I and II, with pyochelin I being favored by ca. 3:1 (49). Pyochelins I and II were eluted at 23.9 and 26.9 min, respectively. Total pyochelin equals pyochelin I plus pyochelin II. The identity of purified pyochelin was assessed by UV spectroscopy and by mass spectroscopy (ionization with NH_3). Dha, salicylic acid, and pyochelins I and II were quantified at 258, 237, 261, and 257 nm, respectively. Salicylic acid was also quantified by fluorescence, using a detector set at 233 nm (excitation) and 422 nm (emission) with a 295-nm-cutoff filter and a pulsed xenon lamp (110 Hz, 2.5 W).

DNA manipulation and sequencing. Routine procedures were used for the isolation of DNA and for cloning experiments (16, 52). DNA fragments were excised from low-melting-point agarose gels and purified with a Gene Clean DNA extraction kit (Bio 101, La Jolla, Calif.). Large-scale preparations of plasmid DNA from *E. coli* were made with QIAGEN-tip 100 columns (Qiagen Inc.). *P. aeruginosa* and *E. coli* strains were transformed with plasmid DNA by the standard $CaCl_2$ procedure (52) or by electroporation (21). Subclones for DNA sequencing were generated in pBluescript KS$^-$ (Stratagene) by nested deletions using an exonuclease III Erase-a-base kit (Promega). Sequencing reactions were performed by the dideoxynucleotide method (52) with Sequenase version 2 and [α-^{35}S]dATP or [α-^{32}P]dATP according to the instructions of the manufacturer (U.S. Biochemical Corp.). Both strands were sequenced by using T3, T7, and reverse primers or oligonucleotides complementary to specific internal regions. Compressions were resolved by using 7-deaza-nucleotides as previously described (54).

Plasmid and mutant construction. Transposon Tn*1725* insertions were obtained in pME3315 as described elsewhere (54). A translational *pchD′*-*′lacZ* fusion was constructed by fusing the 0.7-kb *Pst*I-*Eco*RV fragment carrying the proximal part of *pchD* to *′lacZ* by blunt-end ligation of the *Eco*RV site to the *Sma*I site of pNM480. The *pchD′*-*′lacZ* fusion was inserted on a 3.8-kb *Eco*RI-*Dra*I fragment into the broad-host-range vector pKT240 cut with *Eco*RI and *Sma*I, resulting in pME6122. Defined insertion mutations were generated in the chromosomal *pchD* gene of *P. aeruginosa* by marker exchange. Tn*1725* inserted at position 7 in plasmid pME3315 (Fig. 1) was excised with *Eco*RI, creating a 35-bp insertion with an *Eco*RI site (designated ω) (54). The ω mutation was transferred on a 1.6-kb *Bgl*II-*Kpn*I fragment (Fig. 1) to the ColE1-based suicide vector pME3087 (64). The ΩSm/Sp element carrying transcription and translation stops (45) was then integrated, as a 2-kb *Eco*RI fragment, into the *Eco*RI site of ω. The resulting pME3087-*pchD*::ω and pME3087-*pchD*::ΩSm/Sp constructs were each mobilized from *E. coli* S17-1 to strain PAO1 and chromosomally integrated with selection for tetracycline resistance. Excision of the vector via a second crossing-over was obtained by enrichment for tetracycline-sensitive cells (70), producing a weakly polar *pchD*::ω and a strongly polar *pchD*::ΩSm/Sp mutation, respectively, in the PAO1 chromosome. Both mutations were checked by Southern blotting and by testing of their pyochelin-negative phenotype, which was detected by TLC analysis of culture supernatants.

Transduction. The generalized transducing phage E79*tv*-2 (41) was used to map the *pchD*::ΩSm/Sp mutation relative to flanking markers on the chromosome. Prototrophic transductants were selected on minimal medium E and

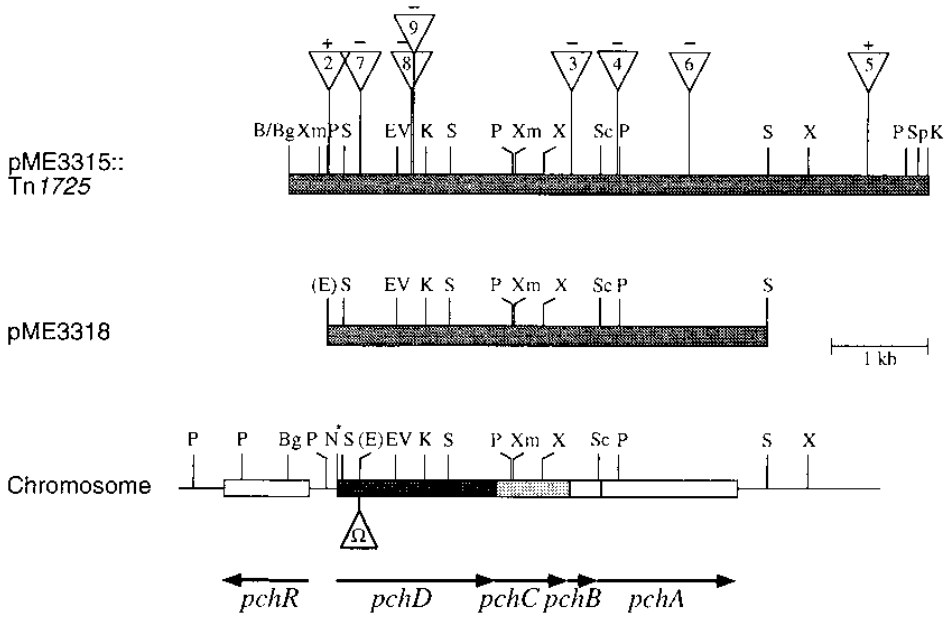

FIG. 1. Physical map of the *pchDCBA-pchR* region of *P. aeruginosa* PAO1. The 2,657-bp DNA region shown in Fig. 2 lies between the 5′ ends of *pchR* and *pchB*. The locations of Tn*1725* insertions in pME3315 (numbered triangles) are indicated. Insertions 2 to 6 have been described previously (54). The ΩSm/Sp element used to construct the *pchD* mutant PAO6286 (triangle below the chromosome) is shown. A 4.6-kb *Eco*RI-*Sal*I fragment, obtained from plasmid pME3315 containing transposon insertion 2, was cloned into vector pQF10, resulting in plasmid pME3318. The restriction enzymes used for subcloning and sequencing in this study are indicated as follows: B/Bg, *Bam*HI-*Bgl*II hybrid site; E, *Eco*RI; (E), *Eco*RI originating from Tn*1725* insertion; EV, *Eco*RV; K, *Kpn*I; N*, *Nla*III (not unique); P, *Pst*I; S, *Sal*I; Sc, *Sca*I; Sp, *Sph*I; X, *Xho*I; Xm, *Xma*I. +, positive for complementation of salicylate and pyochelin synthesis; −, negative for complementation.

scored for cotransduction of the *pchD*::ΩSm/Sp marker on minimal medium E containing spectinomycin.

Computer analysis of the nucleotide sequence. DNA and protein sequences were analyzed by using the following programs of the University of Wisconsin Genetics Computer Group (version 8.0): FASTA for homology searches in the GenEMBL and SwissProt databases, GAP and BESTFIT for comparison of two sequences, PILEUP for analysis of similarities among multiple sequences, PEPPLOT for peptide analysis, MOTIFS for identification of known protein sequence patterns, ISOELECTRIC for prediction of the protein charge, and TESTCODE for prediction of the putative coding region.

RNA isolation and Northern (RNA) blot analysis. Total RNA was isolated from *P. aeruginosa* cells grown in DCAA medium with or without 100 μM $FeCl_3$ by a modification of the hot-phenol extraction method (33). Aliquots of 10 μg of total RNA were denatured in 2 M formaldehyde + 50% (vol/vol) formamide at 65°C for 15 min and electrophoresed at 4 V/cm in a 1% agarose gel containing 2 M formaldehyde in morpholinepropanesulfonic acid (MOPS) buffer (52). The quality of RNA samples was checked by visualization of rRNAs by ethidium bromide staining. Approximate transcript lengths were estimated by comparison with a type II RNA standard (Boehringer). The RNA was transferred onto a nitrocellulose filter (Hybond-C Extra; Amersham) and heat-fixed as described elsewhere (52).

Single-stranded uniformly labeled DNA probes for RNA hybridization and S1 nuclease protection assays of the *pchD* transcripts were generated by a procedure described previously (19, 33). A 327-bp DNA fragment encompassing the 5′ end of *pchD* (from the upstream *Xma*I site to position 378 in *pchD*) (see Fig. 2) was generated by PCR using a biotinylated M13/pUC universal primer (5′-biotin-C GTTGTAAAACGACGGCCAG-3′) and an antisense primer (5′-TTCGGCGA AGCTCTGGTCCTGCCAGT-3′, designated P1a; see Fig. 2) with pME3349 as a template. Amplification reactions were carried out under conditions described previously (33). The PCR product was purified, and the biotinylated single strand was used as a template for the in vitro synthesis of radioactively labeled antisense DNA (33) as follows. A 5-pmol sample of the oligonucleotide P1a was annealed at 58°C with the biotinylated DNA captured on streptavidin beads in a 25-μl reaction mixture containing 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 4 mM dithiothreitol, 20 μM dGTP, 20 μM dTTP, 6.5 U of T7 DNA polymerase (Sequenase version 2.0; U.S. Biochemical Corp.), and 2 μl each of [α-^{32}P]dATP and [α-^{32}P]dCTP (10 mCi/ml). The ^{32}P-labeled DNA strand was eluted from the immobilized template by NaOH-NaCl denaturation (19). Filters containing *pchDCBA* RNA were hybridized to the probe (≥10^7 cpm) under high stringency in a buffered solution containing 6× SSC (0.9 M NaCl–0.09 M sodium citrate, pH 8.0), 0.01 M EDTA, 1% sodium dodecyl sulfate (SDS), 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin, and 50 μg of single-stranded calf thymus DNA per ml at 68°C for 18 h and washed at 68°C by serial passages in 2× SSC (40 min), 2× SSC + 0.5% SDS (30 min), and 0.1× SSC + 0.5% SDS (60 min). The filters were exposed to a Kodak XAR film at −75°C for longer than 1 week with two Lightning Plus (Dupont) intensifying screens.

S1 nuclease mapping and primer extension analysis. The single-stranded probe for the S1 nuclease protection assay of the *pchD* transcripts was generated by using 5 pmol of oligonucleotide P2a (5′-GATAGCGGCGGACGAAGGCG GCGG-3′) complementary to the coding strand of the *pchD* gene (see Fig. 2). The primer was annealed with the streptavidin-bound single-stranded DNA, and cDNA synthesis was carried out under the conditions described above with both [α-^{32}P]dATP and [α-^{32}P]dCTP (10 mCi/ml) used for labeling. The single-stranded DNA probe was mixed with 50 μg of total RNA extracted from *P. aeruginosa* PAO1 exponentially grown in DCAA medium to an optical density at 600 nm (OD_{600}) of 0.12 and coprecipitated by the addition of 0.1 volume of 3 M sodium acetate and 3 volumes of absolute ethanol. S1 nuclease mapping of the *pchD* mRNA was performed as described by Sambrook et al. (52) with a hybridization temperature of 50°C. Controls without RNA, with RNA from iron-proficient cells, or without S1 nuclease were included.

Primer extension reactions were carried out on both *pchD* and *pchR* transcripts essentially as reported elsewhere (33). The oligonucleotides used for priming cDNA synthesis were P3a (5′-GCGGAGATGGACAAAGCGCCCTG C-3′), annealing to the coding strand of *pchR* (see Fig. 2); P4a (5′-GGCGGAG CAATGATGGTGATGGTCAT-3′), annealing to the coding strand of *pchR* (see Fig. 2); and P2a. The oligonucleotides (1 pmol each) were 5′ labeled with 10 U of T4 polynucleotide kinase (New England Biolabs) and 100 μCi of [γ-^{32}P]ATP (Amersham) at 37°C for 1 h. The labeling mixtures were heated at 65°C for 20 min, and the labeled primers were coprecipitated with 100 μg of total RNA by the addition of 0.5 volume of 6 M ammonium acetate and 3 volumes of absolute ethanol. The pellets were washed, redissolved, RNasin treated, and incubated with reverse transcriptase (SuperScript II; Gibco BRL) as described elsewhere (33). Unlabeled primers were used to generate a nucleotide sequence ladder upstream of the *pchD* and *pchR* genes with a T7 sequencing kit (Pharmacia) or a cycle sequencing kit (AmpliCycle; Perkin-Elmer) with [α-^{32}P]dATP. S1 nuclease-protected fragments and primer extension products were run in parallel to the sequencing reactions to map the transcription initiation sites.

Nucleotide sequence accession number. The nucleotide sequence of the 2,657-bp segment containing *pchD* and *pchC* has been deposited at GenBank under accession no. PAX82644.

RESULTS

The salicylate biosynthetic genes *pchBA* are preceded by two genes, *pchD* and *pchC*. The salicylate- and pyochelin-negative mutant PALS128-17 of *P. aeruginosa* is complemented by plas-

mid pME3315 for the synthesis of both siderophores (Fig. 1). This plasmid contains a 6.7-kb genomic fragment including the salicylate biosynthetic genes *pchB* and *pchA* (54). Insertions of transposon Tn*1725* were generated in pME3315. Three insertions (designated 7, 8, and 9; Fig. 1 and 2) lying in a 2.5-kb region upstream of *pchB* abolished complementation of strain PALS128-17, as did others (insertions 3, 4, and 6) in *pchB* and *pchA* which were previously described (54). Since Tn*1725* insertions are strongly polar, this finding implies that the *pchBA* genes may be part of a transcriptional unit starting approximately 2.5 kb upstream of *pchB*. The nucleotide sequence of this region (2,657 bp) was determined from *P. aeruginosa* PAO1 via pME3318 (Fig. 1) and found to contain two open reading frames (ORFs) designated *pchD* and *pchC* (Fig. 2). The 244-bp *Xma*I-*Sal*I fragment encompassing the probable 5′ end of *pchD* (Fig. 2) was identical to the *pchR* promoter region, which is present in the opposite orientation. The *pchR* gene and its promoter have been characterized previously (26, 42). These results indicate that the *pchR* and *pchDCBA* genes are divergently oriented (Fig. 1).

The G+C content of *pchD* and *pchC* was 69.8%, with a strong (86.2%) bias for G or C at the third codon position, which is characteristic of protein structural genes in *P. aeruginosa* (66). The ORF designated *pchD* extends for 1,638 bp, and the postulated ATG initiator codon is preceded by a plausible ribosome binding site (GGAGA). The second ORF, *pchC*, consists of 750 bp, and the predicted ATG start codon overlaps with the TGA stop codon of *pchD*. The putative ribosome binding site (GGAG) of *pchC* is located at the 3′ end of *pchD*. The *pchB* gene immediately follows *pchC* (Fig. 2). The predicted products of the *pchD* and *pchC* genes consist of 546 amino acids (M_r, 59,631; pI 6.21) and 250 amino acids (M_r, 27,546; pI 5.92), respectively. In reasonable agreement with these M_r values, polypeptides having apparent molecular masses of about 63 and 29 kDa could be assigned to PchD and PchC, respectively, after overexpression of the *pchDCBA* genes from the T7 promoter on a vector plasmid and analysis by SDS-polyacrylamide gel electrophoresis. In this experiment, which was carried out with *P. aeruginosa*, the PchB and PchA proteins previously described (54) were also detected (data not shown).

The PchD and PchC proteins are similar to adenylate-forming enzymes and thioesterases, respectively. The deduced amino acid sequence of PchD showed the greatest similarity with 2,3-dihydroxybenzoate-AMP ligase from *E. coli*, the EntE protein (46% identity, 67% similarity in an overlap of 526 amino acids) (56), and with the same enzyme from *Bacillus subtilis*, the DhbE protein (53% identity, 70% similarity in the same overlap) (1) (Fig. 3). In *E. coli*, the EntE enzyme activates the carboxyl group of 2,3-dihydroxybenzoate by forming an enzyme-bound adenylate product, which is a precursor of the siderophore enterobactin (51). Three motifs that occur in the superfamily of adenylate-forming enzymes are conserved in PchD (Fig. 3): motif 1 (SGGTTGTPKL), resembling the consensus core sequence SGTTGXPKGV, and motif 2 (RLLQVGG), resembling the consensus core sequence GELCIGG, are typical of ATP-binding proteins, whereas motif 3 (YRTGD), resembling the consensus core sequence YXTGD, occurs in cation ATPases (57, 59, 71). PchD also showed some similarity to adenylate-forming peptide synthetases, e.g., SyrB of syringomycin synthesis in *Pseudomonas syringae* (27% identity) (71), SrfA1 of surfactin synthesis in *B. subtilis* (26% identity) (8), and GrsB of gramicidin S synthesis in *Bacillus brevis* (25% identity) (32). These enzymes all have conserved motifs 1 to 3 (57, 71). However, PchD lacked additional motifs which occur in these peptide synthetases and may be involved in amino acid activation by adenylation (57).

PchC appears to be a member of the family of thioesterases. The similarity was highest (42% identity in an overlap of 244 amino acids) with the PabT protein, which is supposed to be involved in the activation of *p*-aminobenzoate during candicidin synthesis in *Streptomyces griseus* (14). PchC also had 30% identity with GrsT, which takes part in the thiotemplate assembly of gramicidin in *B. brevis* (32) (Fig. 4). However, the precise biochemical functions of PabT and GrsT are unknown. Furthermore, a significant level of identity (30% in 219 amino acids) exists between PchC and thioesterase II (fatty acyl-thioester hydrolase) of rat mammary gland (47) and other thioesterases of eukaryotic origin. These enzymes release the fatty acid product from the thiotemplate that is used for product assembly. Thioesterases and lipases contain the consensus sequence GXSXG with an active-site serine residue (30, 67). This motif (GHSFG) is conserved in PchC, as is another sequence (GGHFY) found in several thioesterases (Fig. 4) (59). The histidine residue in the second motif is important for thioester hydrolysis (67).

In conclusion, the sequence comparison of PchD and PchC with proteins in the database suggests that salicylate may be activated by PchD-mediated adenylation of the carboxyl group and that the product(s) formed subsequently may be released from a thioester linkage by PchC.

Dha is an extracellular metabolite of *P. aeruginosa*. The wild-type strain PAO1 grown under iron limitation in DCAA medium produced extracellular salicylate and pyochelin (Table 2), as noted previously (12, 60). In addition to these compounds, culture supernatants contained the salicylate derivative Dha, an antibiotic previously found in *P. fluorescens* (7) but not yet reported for *P. aeruginosa*. Dha was identified as an extracellular product of strain PAO1 by comparison with chemically synthesized Dha: the R_f of 0.56 in TLC (in chloroform-acetic acid-ethanol [90:5:2.5]), the retention time (19.7 min) in HPLC, the UV and visible spectra, and the mass spectra all were the same. The amounts of Dha excreted by strain PAO1 were smaller than those of salicylate and pyochelin (Table 2) and did not suffice to determine the optical rotation of the product in the culture fluids examined.

The *pchD* gene is necessary for the biosynthesis of Dha and pyochelin. Insertion 7 of Tn*1725* in pME3315 (Fig. 1) served to generate two defined mutations in the *pchD* gene of *P. aeruginosa*. In vitro excision of Tn*1725* gave rise to a 35-bp insertion, designated ω, containing an *Eco*RI site (58). The transcription and translation stop element ΩSm/Sp (45) was inserted into this *Eco*RI site. Both insertion mutations were transferred to the chromosome of the wild-type strain PAO1 (Fig. 1) by marker exchange via the suicide plasmid pME3087 (see Materials and Methods). The ΩSm/Sp insertion in strain PAO6286 (Fig. 1) completely abolished the production of Dha and pyochelin and, moreover, exerted a strongly polar effect on the expression of the *pchBA* genes, i.e., salicylate was no longer produced by the mutant (Table 2). Plasmid pME3318, which carries the entire *pchDCBA* region (Fig. 1), complemented strain PAO6286 for the production of all three metabolites (Table 2). The ω insertion mutant PAO6285 did not produce Dha and pyochelin but excreted salicylate, albeit in small amounts (Table 2). This indicates that the 35-bp insertion had a weakly polar effect on *pchBA*, presumably as a consequence of premature termination of *pchD* translation. Plasmid pME3318 in strain PAO6285 restored the production of salicylate, Dha, and pyochelin (Table 2). The ω and Ω insertions were also introduced into a pyoverdin-negative mutant of *P.*

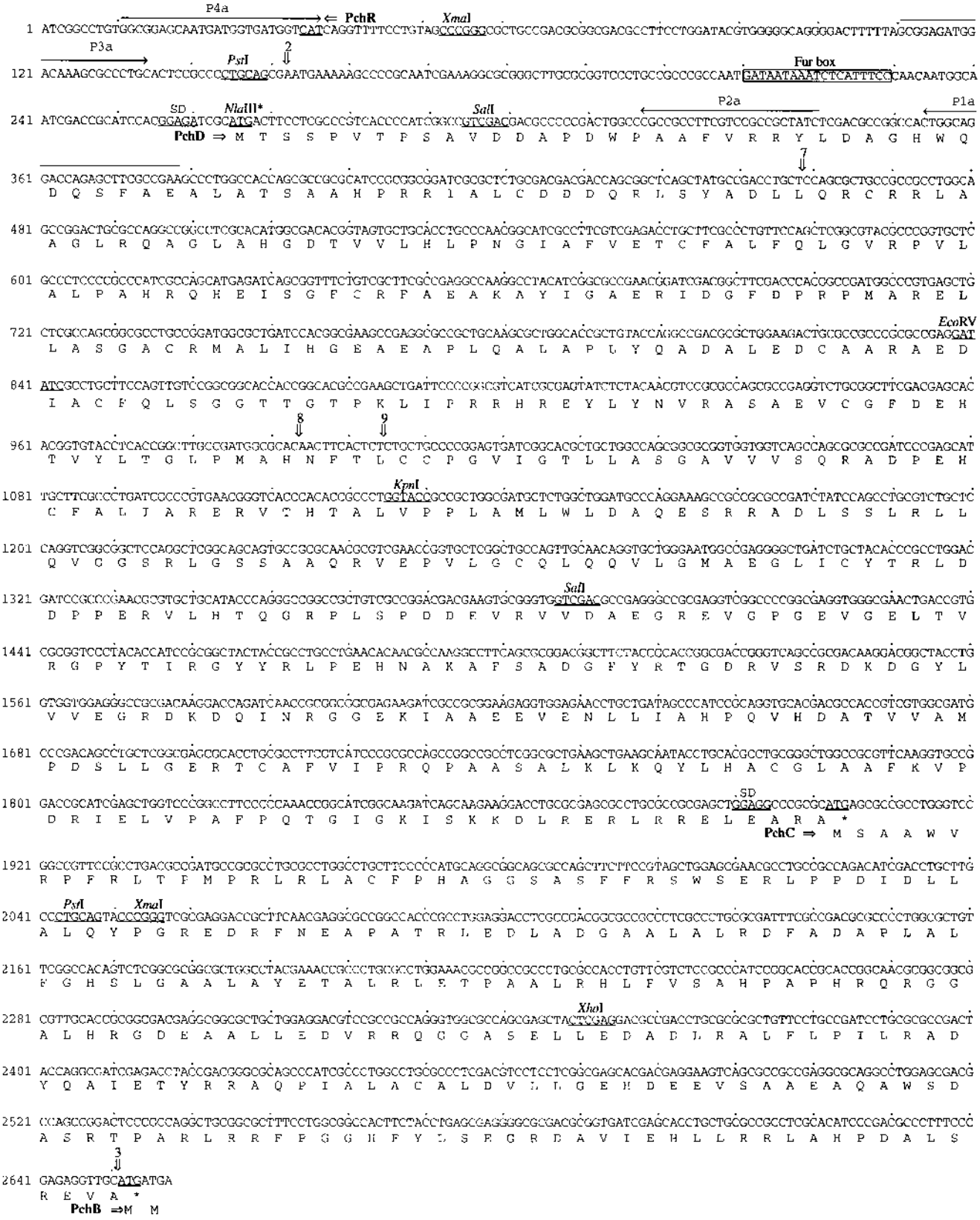

FIG. 2. Nucleotide and deduced amino acid sequences of the 2,657-bp DNA region from *P. aeruginosa* PAO1 containing the genes *pchD* and *pchC*. The putative start codons, potential ribosome binding sites (SD), and restriction sites (underlining) and the positions of transposon Tn*1725* insertions (vertical arrows) are indicated. Insertion 7 was used to construct the mutants PAO6285 and PAO6286. A sequence (Fur box) homologous to the *E. coli* consensus Fur binding site is shown for the *pchD* promoter (see Fig. 5C for further details). The positions of primers used for S1 nuclease mapping, primer extension analysis, and Northern blotting (horizontal arrows above the nucleotide sequence) are also shown.

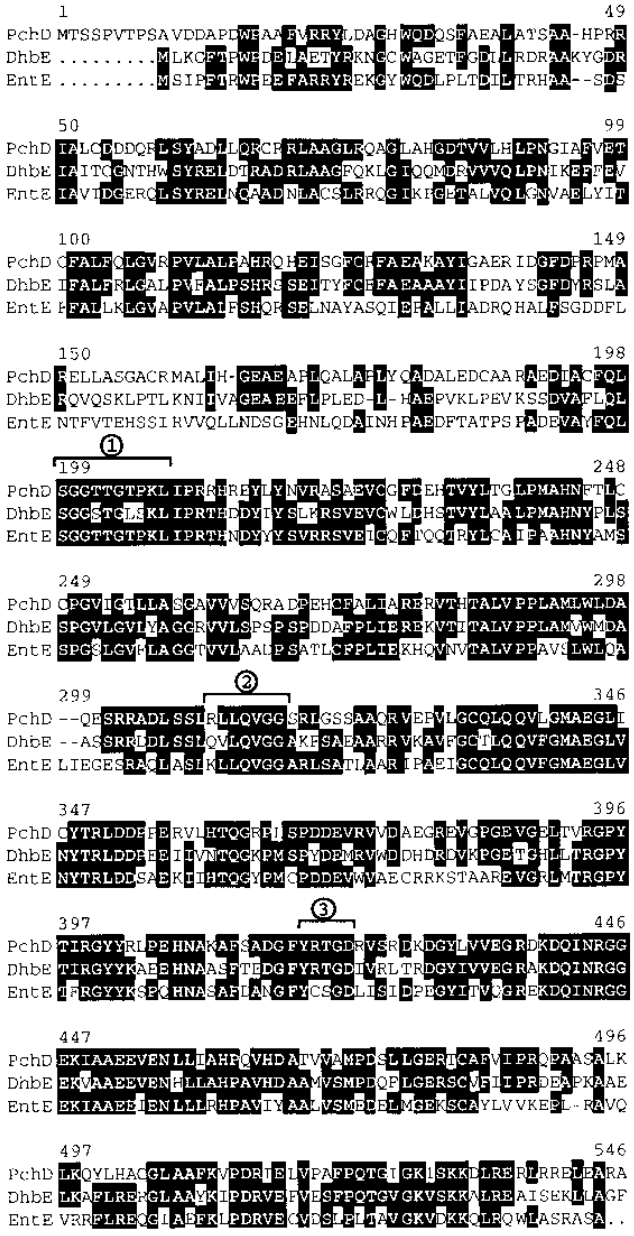

FIG. 3. Alignment of the deduced amino acid sequence of PchD with the *E. coli* EntE and the *B. subtilis* DhbE proteins. The sequences were aligned by using the computer program PILEUP (Genetics Computer Group version 8.0). Identical amino acids (black boxes) are indicated. Conserved motifs (1 to 3) are explained in the text.

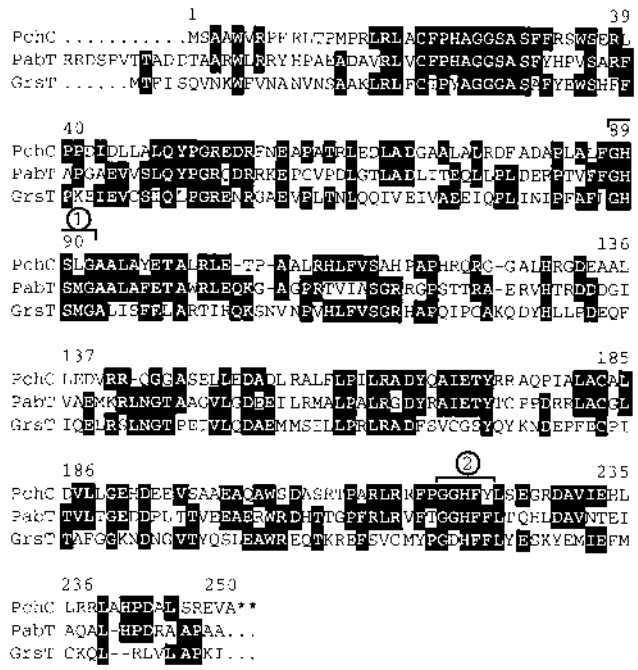

FIG. 4. Alignment of the deduced amino acid sequences encoded by the *pchC* gene from *P. aeruginosa*, *pabT* from *S. griseus*, and *grsT* from *B. brevis*. Identical amino acid residues (black boxes) are indicated. Motifs 1 and 2 are explained in the text.

aeruginosa and caused similar effects on salicylate, Dha, and pyochelin synthesis (data not shown).

In the complementation tests carried out with the multicopy plasmid pME3318, the extracellular concentrations of Dha and pyochelin reached wild-type levels, whereas the concentration of salicylate was higher than in the wild type (Table 2). We tentatively conclude from this result that the *pchD* gene, probably together with *pchC*, is necessary, but not sufficient, for the synthesis of Dha and pyochelin.

Transductional mapping of the *pchD* gene. Physical mapping data have placed *pchR* (43) and *fptA* (3) on *Spe*I fragment A and *Dpn*I fragment F, located in the 23-min region of the chromosome (28). Since *pchD* is adjacent to *pchR* (Fig. 1) and close to *fptA* (26), *pchD* should be present on the same *Spe*I and *Dpn*I fragments. This was confirmed by U. Römling (49a). Moreover, *pchD* could be cotransducible with other loci such as *trpE* and *argC* (at ca. 22 min) or *proA* (at ca. 24 min). A transducing lysate of phage E79*tv*-2 was prepared from strain PAO6286 (*pchD*::ΩSm/Sp) and used to transduce strains PAO4104 (*trpE*), PAO307 (*argC*), and PAO951 (*proA*) to prototrophy. The spectinomycin resistance marker encoded by ΩSm/Sp was cotransducible with *trpE* (12%), *argC* (38%), and *proA* (9%). This is consistent with a marker order of *trpE-argC-pchDCBA-proA*.

Determination of *pchD* and *pchR* transcriptional start sites. The initiation sites of the *pchDCBA* transcripts were determined by primer extension and S1 nuclease mapping. Primer extension analysis was carried out with oligonucleotide P2a (Fig. 2). The primer extension products were compared with the nucleotide sequence obtained by using the same primer (Fig. 5A, lane 1); two transcripts were detected with 5′ ends

TABLE 2. Effects of weakly and strongly polar *pchD* mutations on the synthesis of salicylate, Dha, and pyochelin in *P. aeruginosa*

Strain	Relevant genotype	Metabolite excreted (nmol/OD_{600} unit)[a]		
		Sal	Dha	Pch
PAO1	Wild type	11.2	3.2	51.9
PAO6285	*pchD*::ω	1.7	<0.1	<0.1
PAO6285/pME3318	*pchD*::ω/*pchDCBA*$^+$	23.0	3.6	52.3
PAO6286	*pchD*::ΩSm/Sp	<0.01	<0.1	<0.1
PAO6286/pME3318	*pchD*::ΩSm/Sp/*pchDCBA*$^+$	16.6	3.5	55.0

[a] Strains were grown in DCAA medium to stationary phase (24 h), and culture supernatants were analyzed by HPLC (see Materials and Methods). Values are expressed as nanomoles of product per cell mass in cultures. Cell mass is expressed as OD_{600} units. Sal, salicylate; Pch, pyochelin.

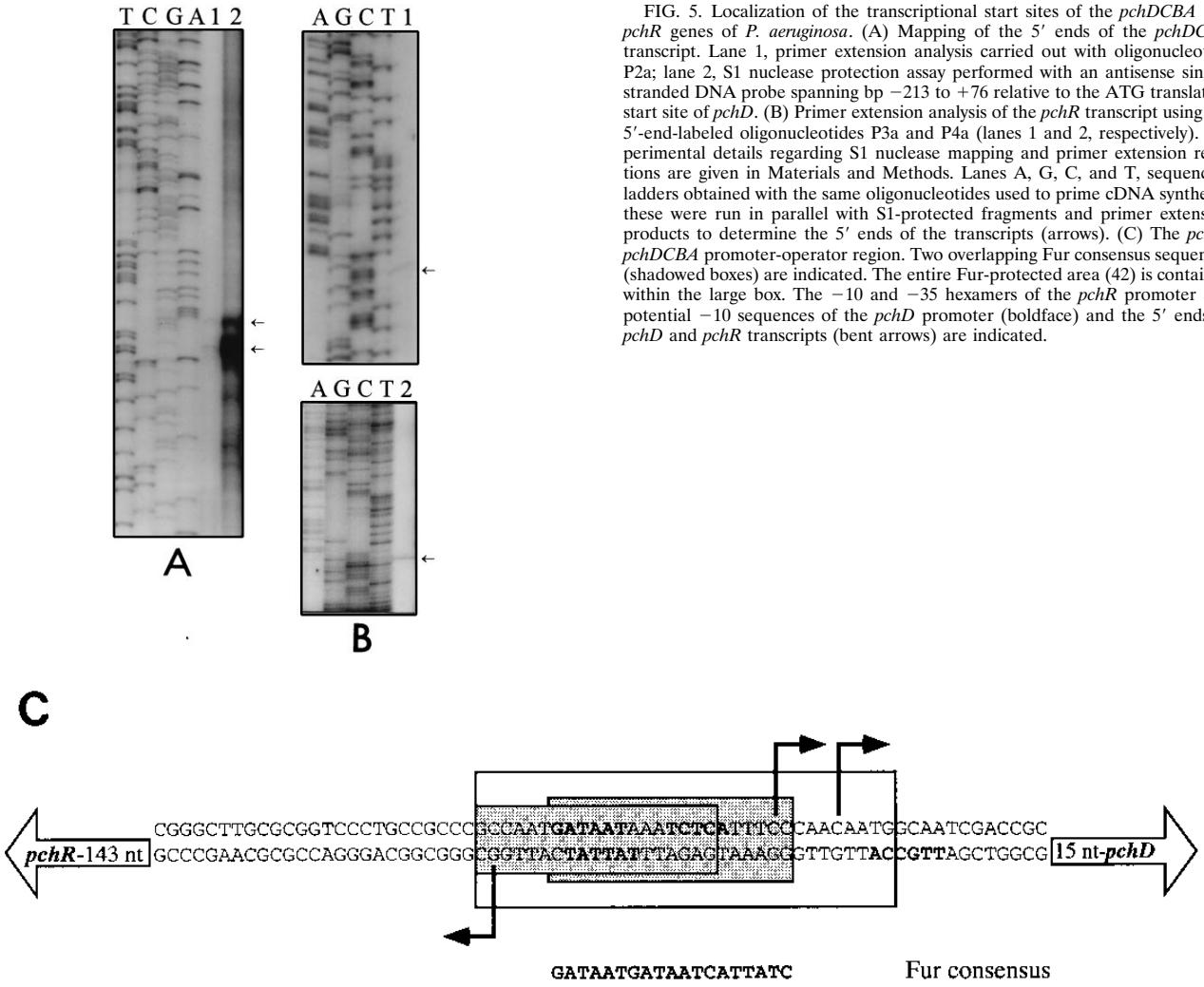

FIG. 5. Localization of the transcriptional start sites of the *pchDCBA* and *pchR* genes of *P. aeruginosa*. (A) Mapping of the 5′ ends of the *pchDCBA* transcript. Lane 1, primer extension analysis carried out with oligonucleotide P2a; lane 2, S1 nuclease protection assay performed with an antisense single-stranded DNA probe spanning bp −213 to +76 relative to the ATG translation start site of *pchD*. (B) Primer extension analysis of the *pchR* transcript using the 5′-end-labeled oligonucleotides P3a and P4a (lanes 1 and 2, respectively). Experimental details regarding S1 nuclease mapping and primer extension reactions are given in Materials and Methods. Lanes A, G, C, and T, sequencing ladders obtained with the same oligonucleotides used to prime cDNA synthesis; these were run in parallel with S1-protected fragments and primer extension products to determine the 5′ ends of the transcripts (arrows). (C) The *pchR-pchDCBA* promoter-operator region. Two overlapping Fur consensus sequences (shadowed boxes) are indicated. The entire Fur-protected area (42) is contained within the large box. The −10 and −35 hexamers of the *pchR* promoter and potential −10 sequences of the *pchD* promoter (boldface) and the 5′ ends of *pchD* and *pchR* transcripts (bent arrows) are indicated.

corresponding to the C residues located at bp 228 and 233 (Fig. 2 and 5C). For S1 nuclease mapping, a single-stranded antisense DNA probe spanning the region from −213 to +76 bp relative to the ATG translation start site of *pchD* was hybridized with total RNA extracted from early-exponential-phase, iron-limited *P. aeruginosa* cells. Two major protected fragments comigrating with the C residues at bp 228 and 233 of the reference sequence (Fig. 5A, lane 2) confirmed the primer extension experiment. In addition, several smaller S1 nuclease-protected fragments were observed (Fig. 5A, lane 2), which could derive from endonucleolytic cleavage of the major transcripts. No signals were detected in RNA samples from iron-rich cells (data not shown).

The transcriptional start site of the *pchR* gene was determined on the opposite strand by two independent primer extension experiments using oligonucleotides P3a and P4a (Fig. 2). A single initiation site was found corresponding to the G residue at bp 206 (Fig. 2 and 5B). The promoters for the divergently transcribed *pchR* and *pchDCBA* genes overlap (Fig. 5C). As shown by Ochsner et al. (42), this promoter region contains two overlapping Fur boxes and is protected by the Fur repressor (Fig. 5C). Both Fur boxes have 68% identity with the consensus Fur binding site (43). The *pchR* promoter shows extensive similarity to the −10 and −35 consensus sequences for σ^{70}-dependent RNA polymerase (50); the −10 hexamer lies entirely within one Fur box (Fig. 5C, left). The *pchD* promoter is likely to be positively controlled by the PchR protein and does not contain obvious −35 sequences; the putative −10 regions (see Discussion) are both located within the second Fur box (Fig. 5C, right).

In summary, our results suggest that the *pchR-pchDCBA* control region acts bidirectionally and can be coordinately regulated by the Fur repressor protein.

Northern blot analysis and transcriptional regulation of the *pchDCBA* cluster. To estimate the approximate size of the RNA transcript encoded by the *pchDCBA* cluster and to investigate whether iron regulates the expression of these genes, we performed Northern blot analyses with total cellular RNA from *P. aeruginosa* PAO1. Bacteria were grown in low-iron medium to early (OD_{600} = 0.10) and late (OD_{600} = 0.49) exponential phase and in high-iron medium to early exponential phase (OD_{600} = 0.16). An antisense single-stranded DNA probe encompassing the 5′ region of *pchD* was labeled to high activity and used to detect the full-length *pchDCBA* mRNA by hybridization. Three signals were observed with the RNA extracted from iron-deficient cells during early exponential phase (Fig. 6, lane 1), whereas only the lower two bands were observed with RNA preparations from cells in late exponential

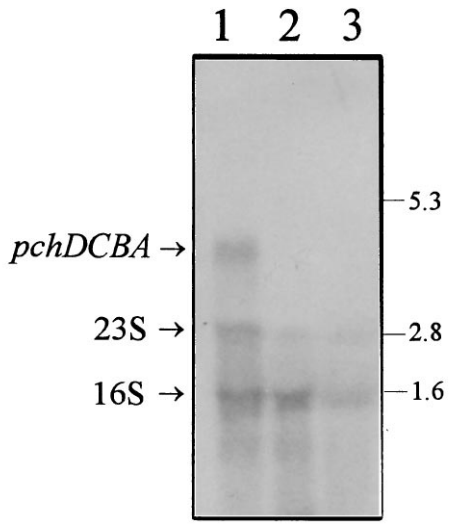

FIG. 6. Iron-dependent transcriptional regulation of the *pchDCBA* cluster of *P. aeruginosa*. Total RNA was isolated from cells grown under conditions of iron deficiency (DCAA medium) and iron sufficiency (DCAA medium supplemented with 100 μM $FeCl_3$). The OD_{600}s of the bacterial cultures were 0.10 (3-h growth, lane 1) and 0.49 (6-h growth, lane 2) for the iron-poor conditions and 0.16 (3-h growth, lane 3) for the iron-rich conditions. Total RNA samples (10 μg) were hybridized with a single-stranded antisense DNA probe, as outlined in Materials and Methods. The positions of RNA standards (with sizes indicated in base pairs) are shown on the right; bands corresponding to 16S and 23S rRNAs and to the *pchDCBA* transcript are indicated on the left.

phase (Fig. 6, lane 2) and from iron-rich cells (Fig. 6, lane 3). These two bands, which are located at the positions of 23S and 16S rRNA species, can result from unspecific binding of the probe and have previously been noticed in Northern blots with various RNA preparations extracted from *P. aeruginosa* (22). The upper RNA hybridization signal, which was detected specifically in iron-limited cells, had a size of ca. 4.4 kb (Fig. 6, lane 1) and, considering the length of the *pchDCBA* cluster, most likely corresponds to the full-length *pchDCBA* transcript. Thus, we suggest that the *pchDCBA* transcript is polycistronic and that transcription of this gene cluster starts upstream of the translation initiation codon of the *pchD* gene (Fig. 5C) and ends at a putative transcription terminator downstream of *pchA* (54).

Iron repression of the *pchDCBA* genes was confirmed with a translational *pchD'-'lacZ* fusion constructed in pME6122. Strain PAO1 carrying this plasmid showed a β-galactosidase activity of 22,000 ± 3,700 Miller units (mean ± standard deviation) after aerobic growth in iron-deficient DCAA medium. When the medium was supplemented with 50 μM $FeCl_3$, the activity of the *pchD'-'lacZ* fusion was strongly repressed (<50 Miller units). In the *fur* mutant PAO1A4, which is partially derepressed for pyoverdin synthesis (6), the *pchD'-'lacZ* fusion resulted in 31,600 ± 7,500 (iron limitation) and 3,500 ± 600 (with added iron) Miller units (means ± standard deviations). These results can be explained by Fur binding to both Fur boxes (42) present in the *pchR-pchD* promoter region.

DISCUSSION

Our discovery of Dha in culture fluids of *P. aeruginosa* has several implications. This antibiotic is known to inhibit a range of fungi as well as some bacteria (7). Many clinical isolates of *P. aeruginosa* have antifungal activity and may suppress the development of *Candida albicans* during lung infection (31). The antifungal properties have not been defined chemically; they might be related to Dha production. Furthermore, Dha is an Fe(III) chelator and inhibitor of DNA replication at the level of ribonucleotide reductase, resulting in a cytostatic effect on murine neoplasms (18). It may be worthwhile to test whether Dha, like salicylate and pyochelin (9, 36), can act as a siderophore in *Pseudomonas* spp. Finally, Dha is a likely precursor of two extracellular metabolites found in various *P. aeruginosa* strains, aeruginoic acid [2′-(2-hydroxyphenyl)-thiazole-4′-carboxylic acid] (13, 68) and aeruginol [2′-(2-hydroxyphenyl)-4′-hydroxymethylthiazole] (69). Formally, aeruginoic acid arises from Dha by ring oxidation. Reduction of the carboxyl group of aeruginoic acid then produces aeruginol. However, it is not known whether strain PAO1 makes these Dha derivatives, and the biological functions of these compounds have not been described.

Dha and pyochelin synthesis requires the activity of the *pchD* gene. The extensive sequence similarity of the PchD protein with 2,3-dihydroxybenzoate-AMP ligase of *E. coli* and *B. subtilis* suggests that salicylate may be adenylated by PchD in the presence of ATP. In fact, 2,3-dihydroxybenzoate-AMP ligase of *E. coli* can use salicylate as an alternative substrate in vitro (51). By analogy with this enzyme, the PchD protein might form an enzyme-bound salicylate-AMP complex (compound X in Fig. 7). This complex might react with cysteine to produce (*S*)-Dha. The *S* configuration has been noted at the C-4′ atom of Dha synthesized by *P. fluorescens* (7) and in pyochelin produced by *P. aeruginosa* (49), implying that D-cysteine is the likely presursor of Dha. Thus, if L-cysteine were the primary substrate, racemization to D-cysteine would have to occur either prior to the reaction with salicylate-AMP or during assembly of Dha on the enzyme-substrate complex. However, we did not verify the stereochemistry of Dha produced by strain PAO1 in vivo, and our preliminary attempts to detect the formation of Dha from salicylate, cysteine, and ATP in vitro were unsuccessful.

Dha may be an intermediate in pyochelin synthesis (Fig. 7; upper route). The best evidence comes from the salicylate-positive, Dha- and pyochelin-negative phenotype of the *pchD* mutant PAO6285 (Table 2). Nevertheless, until Dha incorporation into pyochelin has been demonstrated, a branched pathway cannot be excluded. In this scenario, Dha and pyochelin would originate from compound X in separate branches (Fig. 7). At least some genes involved in the transformation of compound X into pyochelin are located in the vicinity of the *pchR-pchDCBA* region on the cosmid pME3300 (54; our unpublished results). Salicylate is used as a carbon source by many fluorescent pseudomonads, including some strains of *P. aeruginosa*. Since strain PAO does not degrade salicylate (44), a salicylate-catabolic branch in this strain is not documented.

The *pchD* and *pchC* genes are located immediately upstream of the salicylate biosynthetic genes *pchBA* (54). There are three arguments for the *pchDCBA* genes constituting an operon. (i) Tn*1725* insertions 7, 8, and 9 in the *pchD* gene abolished salicylate formation, i.e., expression of the *pchBA* genes (Fig. 1). (ii) The polar ΩSm/Sp insertion in *pchD* had the same effect (Table 2). (iii) The *pchD* transcript obtained from iron-deprived, growing cells had a size (ca. 4.4 kb) corresponding to a full-length *pchDCBA* transcript (Fig. 6). Thus, transcription of the *pchDCBA* genes appears to depend solely on the two promoters mapped upstream of *pchD* (Fig. 5).

The *pchD* promoters contain two potential −10 sequences (Fig. 5C). The hexamer GATAAT, which is also found in the coliphage λ promoter P_R, is recognized by σ^{70} RNA polymerase of *P. aeruginosa* (23). The second −10 sequence postulated, TCTCA, is similar to the −10 consensus sequence TCTgA for the σ^E-like transcription factor AlgU in *P. aerugi-*

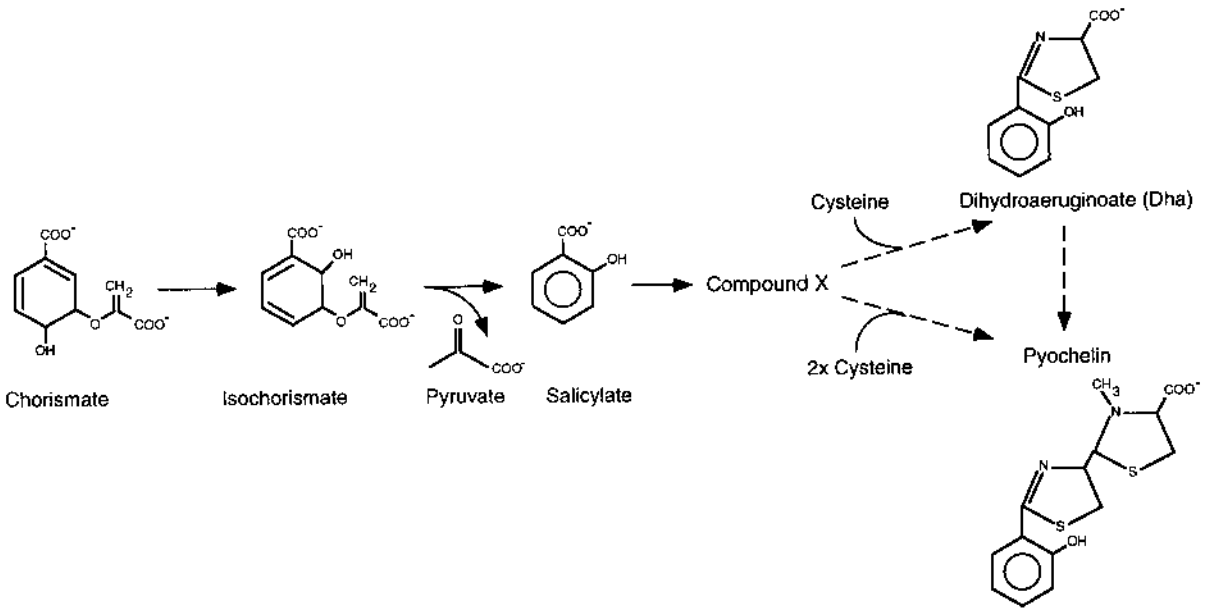

FIG. 7. Proposed pathway of Dha and pyochelin biosynthesis in *P. aeruginosa*. Compound X might be salicylate · AMP bound to an enzyme complex (see text).

nosa (17). Several putative alternative σ factors, including PvdS, appear to be present in *P. aeruginosa* and regulated by iron and Fur (15, 40, 43). These σ factors, like AlgU and σ^E, are in the extracytoplasmic σ-factor family (17, 43). It is possible that some of these σ factors might recognize the downstream promoter of *pchD*. The arrangement of two overlapping Fur boxes in the bidirectional *pchR-pchD* promoters suggests that Fur-mediated iron repression shuts off pyochelin synthesis doubly. First, Fur represses transcription of *pchR* (42). The resulting depletion of the activator PchR will reduce the expression of the pyochelin biosynthetic genes (26), including *pchDCBA*. Second, a repressing action of Fur on the *pchD* promoter itself can be predicted. This dual control could account for the observed tight repression of the *pchD'-'lacZ* fusion in the wild-type strain PAO1 by excess iron in the culture medium.

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