

Essential PchG-Dependent Reduction in Pyochelin Biosynthesis of *Pseudomonas aeruginosa*

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The biosynthetic genes *pchDCBA* and *pchEF*, which are known to be required for the formation of the siderophore pyochelin and its precursors salicylate and dihydroaeruginoate (Dha), are clustered with the *pchR* regulatory gene on the chromosome of *Pseudomonas aeruginosa*. The 4.6-kb region located downstream of the *pchEF* genes was found to contain three additional, contiguous genes, *pchG*, *pchH*, and *pchI*, probably forming a *pchEFGHI* operon. The deduced amino acid sequences of PchH and PchI are similar to those of ATP binding cassette transport proteins with an export function. PchG is a homolog of the *Yersinia pestis* and *Y. enterocolitica* proteins YbtU and Irp3, which are involved in the biosynthesis of yersiniabactin. A null mutation in *pchG* abolished pyochelin formation, whereas mutations in *pchH* and *pchI* did not affect the amounts of salicylate, Dha, and pyochelin produced. The pyochelin biosynthetic genes were expressed from a vector promoter, uncoupling them from Fur-mediated repression by iron and PchR-dependent induction by pyochelin. In a *P. aeruginosa* mutant lacking the entire pyochelin biosynthetic gene cluster, the expressed *pchDCBA* and *pchEFG* genes were sufficient for salicylate, Dha, and pyochelin production. Pyochelin formation was also obtained in the heterologous host *Escherichia coli* expressing *pchDCBA* and *pchEFG* together with the *E. coli entD* gene, which provides a phosphopantetheinyl transferase necessary for PchE and PchF activation. The PchG protein was purified and used in combination with PchD and phosphopantetheinylated PchE and PchF in vitro to produce pyochelin from salicylate, L-cysteine, ATP, NADPH, and S-adenosylmethionine. Based on this assay, a reductase function was attributed to PchG. In summary, this study completes the identification of the biosynthetic genes required for pyochelin formation from chorismate in *P. aeruginosa*.

The opportunistic human pathogen *Pseudomonas aeruginosa* responds to iron-limiting growth conditions by the production of two major siderophores, pyoverdinin (6, 19) and pyochelin (4, 28). These compounds are released to the extracellular environment, where they form a complex with iron and deliver it to the bacterial cell via their specific membrane receptors, FpvA (23) and FptA (1), respectively. Both siderophores, which contribute to the virulence of *P. aeruginosa* (5, 20, 36), are made by a thiotemplate mechanism (13, 18, 26, 27).

The genes required to make pyochelin and its precursors salicylate and dihydroaeruginoate (Dha) are clustered on the *P. aeruginosa* chromosome, next to the pyochelin receptor gene *fptA* (Fig. 1). Two biosynthetic operons, *pchDCBA* and *pchEF*, whose expression is repressed by the Fur protein in the presence of iron, have been identified (27, 33). Interestingly, pyochelin biosynthesis is autoregulated by a positive-feedback loop which requires the transcriptional regulator PchR. The *pchR* gene is located between the two biosynthetic operons (15, 27) (Fig. 1).

In the initial steps of pyochelin biosynthesis, salicylate is

made from chorismate via isochorismate. These reactions are catalyzed by an isochorismate synthase and an isochorismate-pyruvate lyase encoded by the *pchA* and *pchB* genes, respectively (32; C. Gaille and D. Haas, unpublished results). Salicylate and L-cysteine, adenylated by PchD and PchE, respectively, are loaded onto the peptide synthetase PchE via covalent thioester linkages provided by two posttranslationally added phosphopantetheine prosthetic groups (26, 27). Subsequent condensation and cyclization reactions, catalyzed by PchE, generate an enzyme-bound hydroxyphenyl-thiazoline intermediate (26) which, when released by the action of a thioesterase, produces Dha (alternatively designated hydroxyphenyl-thiazolinyl-carboxylate). A domain of PchF displays such a thioesterase activity (26), and a putative external thioesterase is also encoded by the *pchC* gene (33). Pyochelin formation additionally requires the peptide synthetase PchF, which adenylates a second molecule of L-cysteine and anchors it, again via a phosphopantetheine prosthetic group, to a carrier domain in PchF (26, 27). PchF then catalyzes the condensation of the hydroxyphenyl-thiazoline-S-PchE intermediate with cysteinyl-S-PchF and generates the second thiazoline ring (26). At this stage, the basic skeleton of pyochelin is completed and the second thiazoline ring needs to undergo reduction and methylation before biosynthesis is terminated by pyochelin release from PchF due to cleavage of the remaining thioester bond.

In vitro reconstitution experiments with purified PchE and PchF, primed posttranslationally with phosphopantetheine,

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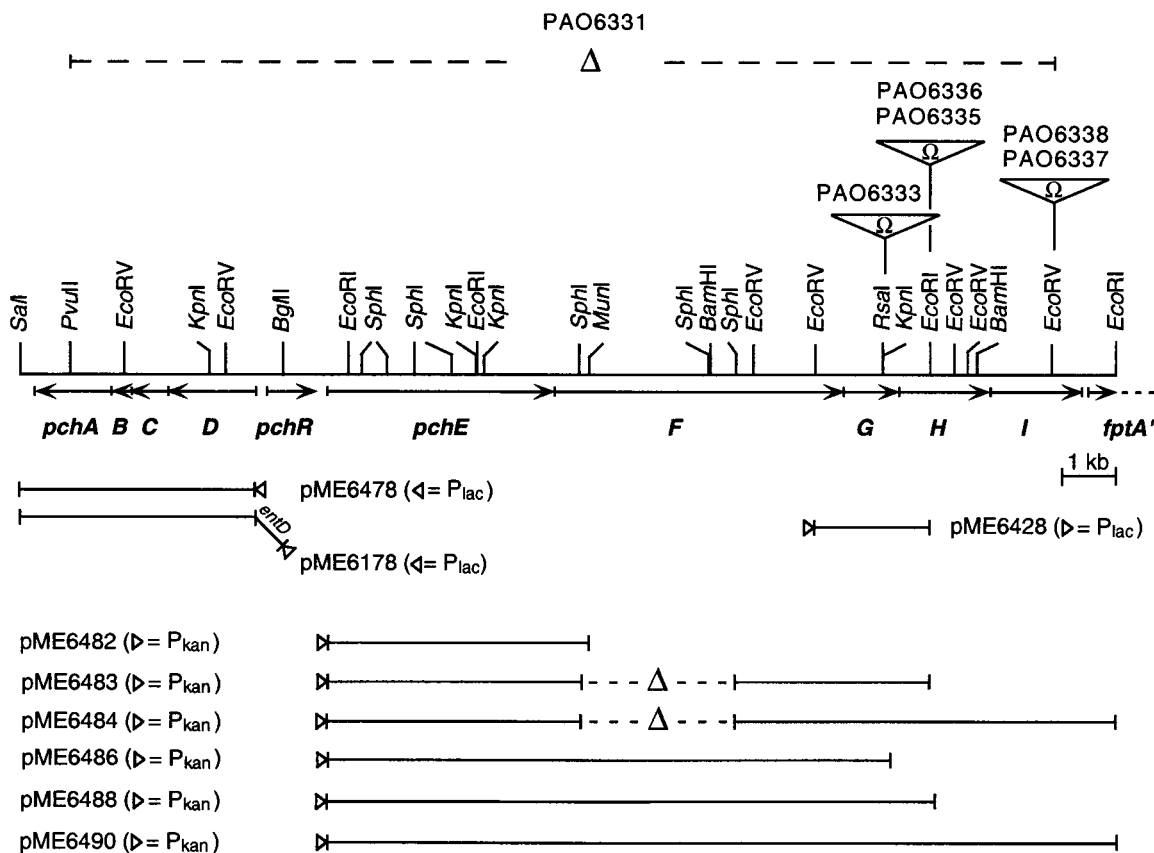


FIG. 1. Physical map of the *pchDCBA-pchR-pchEFGHI-fptA* region of *P. aeruginosa* PAO1. The *pchDCBA* operon and the *pchR*, *pchE*, and *fptA* genes have been described previously (1, 15, 27, 32). The Ω Sm/Sp cassette present in mutants PAO6333, PAO6335, PAO6336, PAO6337, and PAO6338 is designated by Ω . Deletions are indicated by Δ . Subclones used for complementation experiments were constructed such that the respective vector promoter (indicated by a triangle) was located upstream of *pchE*, *pchG*, *pchD*, and *entD*. The restriction enzymes used for subcloning experiments are indicated. Note that for *SalI*, *PvuII*, *MunI*, and *RsaI*, only the locations relevant to this study are shown.

and purified PchD have shown that these proteins are able to make the pyochelin backbone from salicylate and L-cysteine but that they are not sufficient to catalyze the modification of the second thiazoline ring (26). A detailed genetic analysis of the pyochelin biosynthetic gene cluster indicates that additional genes located downstream of *pchF* may encode these missing functions (27).

Here we investigate the role of three additional genes, *pchG*, *pchH*, and *pchI*, in pyochelin biosynthesis and translocation and show that PchG, together with PchD and the holoenzyme forms of PchE and PchF, is sufficient to catalyze pyochelin formation from salicylate, L-cysteine, ATP, NADPH, and S-adenosylmethionine (SAM) *in vitro*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Bacteria were usually grown on nutrient agar and in nutrient yeast broth (35) at 37°C. To quantify salicylate, Dha, and pyochelin in culture supernatants of *P. aeruginosa*, strains were grown in GGP medium (3). Minimal medium M9 (29), supplemented with 20 mM sodium succinate and the weak iron chelator 2,2'-dipyridyl (500 μ M), was used as an iron-depleted medium to test pyochelin-mediated iron uptake in pyoverdine-negative mutants of *P. aeruginosa*. Antibiotics, when required, were added to the growth media at the following concentrations: tetracycline, 25 μ g/ml for *Escherichia coli* and 100 μ g/ml for *P. aeruginosa*; ampicillin, 100 μ g for *E. coli*; carbenicillin, 250 μ g/ml for *P. aeruginosa*; spectinomycin, 50 μ g/ml for *E. coli* and 1 mg/ml for *P. aeruginosa*; gentamicin, 10 μ g/ml for *E. coli* and *P. aeruginosa*; and kanamycin, 25 μ g/ml for

E. coli. To counterselect *E. coli* donor cells in matings with *P. aeruginosa*, chloramphenicol was used at 10 μ g/ml.

Construction of plasmids and gene replacement mutants. All plasmids used in this study are listed in Table 1. To uncouple the expression of the *pch* genes from iron and pyochelin control, a two-plasmid system was developed to allow constitutive pyochelin formation in homologous and heterologous backgrounds. First, the *pchDCBA* operon was fused at the ATG start codon of *pchD* to the *lac* promoter (P_{lac}) carried by vector pME6001 by using a linker region containing a ribosome binding site (5'-AAGCTTGATATCGAATTGTGAGCGGATAACAATTTACACAGAATTGATTAAGAGGAGAAATTAAGCATG-3'; the *HindIII* site used for cloning into pME6001 is italicized; the ATG start codon of *pchD* is underlined). This procedure gave plasmid pME6478 (Fig. 1). Then, the *entD* gene, encoding a phosphopantetheinyl transferase from *E. coli*, was excised from pET28b-*entD* by using *XbaI* and *HindIII*, ligated to a *Clal-XbaI* DNA linker (5'-ATCGATAAGCTCTAGA-3'), and cloned into pME6478 between *Clal* and *HindIII* to give pME6178 (Fig. 1). Constitutive expression of the *pchEFGHI* operon or parts thereof was achieved by fusing the ATG start codon of *pchE* to the kanamycin promoter (P_{kan}) present on pME6012 by using a linker region with a ribosome binding site (5'-AGATCTATCGATGCATGCCATGGTACCCAACTTTAAGAAGGAGATATACCCATG-3'; the *BglII* site used for cloning into pME6012 is italicized; the ATG start codon of *pchE* is underlined). In subsequent cloning steps, a series of constructs carrying *pchE* only (pME6482), *pchEG* (pME6483), *pchEGHI* (pME6484), *pchEF* (pME6486), *pchEFG* (pME6488), and *pchEFGHI* (pME6490) were generated (Fig. 1).

To overexpress and purify the PchG protein, its coding region was PCR amplified from genomic DNA of *P. aeruginosa* strain PAO1 by using the forward primer 5'-ATGCCAGAGGAGGCGGAGCATATGAGCGACGTTCTCGTTCCG-3' and the reverse primer 5'-AGCAGGCGCCACAGCACCCTCGAGCGAGGCTTGCTCC-3'; the forward primer introduces a silent mutation in the fourth codon to replace the low-frequency valine codon GTA with a GTT valine codon

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
<i>E. coli</i>		
DH5 α	<i>recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1 Δ(lacZYA-argF)U169</i> (ϕ 80dlacZ Δ M15)	29
MT147	<i>thi trpE purE proC leuB lacY mil xyl rpsL azi fhuA tsx supA entC::Km^r</i>	21
S17-1	<i>thi pro hsdR recA</i> ; chromosomal RP4 (Tra ⁺ Tc ^s Km ^s Ap ^s)	34
BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (λ DE3)	Novagen
<i>P. aeruginosa</i>		
PALS128	<i>pvdB</i>	37
PALS128-17	<i>pvdB pchB</i> or <i>pchA</i>	32
PAO1	Wild type	ATCC 15692
PAO6331	Δ <i>pch</i>	This study (Fig. 1)
PAO6333	<i>pchG::ΩSm/Sp</i>	This study (Fig. 1)
PAO6335	<i>pchH::ΩSm/Sp</i>	This study (Fig. 1)
PAO6336	<i>pvdB pchH::ΩSm/Sp</i>	This study (Fig. 1)
PAO6337	<i>pchI::ΩSm/Sp</i>	This study (Fig. 1)
PAO6338	<i>pvdB pchI::ΩSm/Sp</i>	This study (Fig. 1)
Plasmids		
pPchD	<i>pchD</i> overexpression construct; Km ^r	26
pPchE	<i>pchE</i> overexpression construct; Km ^r	26
pPchF	<i>pchF</i> overexpression construct; Km ^r	26
pPchG	<i>pchG</i> overexpression construct derived from pET29b; Km ^r	This study
pET28b- <i>entD</i>	<i>entD</i> overexpression construct; Km ^r	16
pET29b	Expression vector; Km ^r	Novagen
pHP45 Ω	Ω Sm/Sp-containing plasmid; Ap ^r Sm ^r Sp ^r	24
pME3087	Suicide vector; Tc ^r ; ColE1 replicon; <i>EcoRI KpnI BamHI XbaI PstI SphI HindIII</i> polylinker	38
pME3088	Suicide vector; Tc ^r ; ColE1 replicon; <i>EcoRI KpnI DraII XhoI HindIII</i> polylinker	38
pME3300	pLAFR3 carrying the structural genes of PAO1 for the biosynthesis of salicylate, Dha, and pyochelin	27, 32, 33
pME6001	pBBR1-based cloning vector; Gm ^r	2
pME6012	pVS1-p15A shuttle vector; Tc ^r	14
pME6176	pME3088 carrying the 3' part of <i>pchA</i> on a 0.95-kb <i>SalI-PvuII</i> fragment joined to the 3' part of <i>pchI</i> on a 1.2-kb <i>EcoRV-EcoRI</i> fragment	This study
pME6178	pME6001 carrying <i>entD pchDCBA</i> under P _{lac} control	This study (Fig. 1)
pME6180	pME3088 carrying a 4.2-kb <i>EcoRV-EcoRI</i> fragment containing <i>pchG</i> interrupted by an Ω Sm/Sp cassette at <i>RsaI</i>	This study
pME6428	pUCPKS carrying <i>pchG</i> under P _{lac} control on a 2.2-kb <i>EcoRV-EcoRI</i> fragment	This study (Fig. 1)
pME6478	pME6001 carrying <i>pchDCBA</i> under P _{lac} control	This study (Fig. 1)
pME6482	pME6012 carrying <i>pchE</i> under P _{kan} control	This study (Fig. 1)
pME6483	pME6012 carrying <i>pchEG</i> under P _{kan} control	This study (Fig. 1)
pME6484	pME6012 carrying <i>pchEGHI</i> under P _{kan} control	This study (Fig. 1)
pME6486	pME6012 carrying <i>pchEF</i> under P _{kan} control	This study (Fig. 1)
pME6488	pME6012 carrying <i>pchEFG</i> under P _{kan} control	This study (Fig. 1)
pME6490	pME6012 carrying <i>pchEFGHI</i> under P _{kan} control	This study (Fig. 1)
pME6498	pME3087 carrying a 3.8-kb <i>KpnI-BamHI</i> fragment containing part of <i>pchH</i> interrupted by an Ω Sm/Sp cassette at <i>EcoRI</i>	This study
pME6499	pME3087 carrying a 4.6-kb <i>BamHI-EcoRI</i> fragment containing <i>pchI</i> interrupted by an Ω Sm/Sp cassette at <i>EcoRV</i>	This study
pUCPKS and pUCPSK	ColE1-pRO1600 shuttle vectors; Ap ^r	39

(italicized). The amplified product was digested with *NdeI* and *XhoI* and ligated to expression vector pET29b, digested with the same enzymes, to generate plasmid pPchG.

To generate chromosomal insertion and deletion mutations in *pch* genes (Fig. 1), derivatives of the suicide plasmids pME3087 and pME3088 (Table 1) were 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 (40). All mutants were checked by Southern analysis.

DNA manipulation and sequencing. DNA manipulations were carried out as described by Sambrook et al. (29). Small-scale preparation of plasmid DNA was carried out by the cetyltrimethylammonium bromide (CTAB) method (7); large-scale preparation was performed with Qiagen-tip 100 columns (Qiagen Inc.). Chromosomal DNA was prepared as described by Gamper et al. (11). Restriction and DNA-modifying enzymes were used by following the instructions of the manufacturers. DNA fragments were purified from agarose gels with a Gene Clean DNA extraction kit (Bio 101, La Jolla, Calif.). *E. coli* and *P. aeruginosa* were transformed by the standard CaCl₂ procedure (29) or by electroporation (8). Nucleotide sequences were determined on both strands with a dye terminator kit (Perkin-Elmer product no. 402080) and an ABI PRISM 373 sequencer

and, in part, by the Euro Sequence Gene Service. Sequences were compared to the DNA sequence available from the *P. aeruginosa* genome sequencing project (<http://www.pseudomonas.com>) and found to be identical except for a single mismatch at position 1555 (T instead of G) of the *pchI* coding sequence. Nucleotide sequences were analyzed with programs from the University of Wisconsin Genetics Computer Group package (version 9.1).

Identification of salicylate, Dha, and pyochelin in culture supernatants of *P. aeruginosa* and *E. coli*. *P. aeruginosa* strains were grown in GGP medium to stationary phase (optical density at 600 nm, \approx 7 to 11, as measured with a Pharmacia Ultrospec III spectrophotometer), and recombinant *E. coli* strains were grown in nutrient yeast broth for 42 h to reach a final optical density at 600 nm of \approx 2 to 2.5. For high-pressure liquid chromatography (HPLC) analysis, ethyl acetate extracts of acidified culture supernatants were dried by evaporation, dissolved in 60% (vol/vol) methanol–10 mM H₃PO₄, and injected into an HPLC system as described previously (27). Compounds were identified by their retention time and UV spectra. Dha, salicylate, and pyochelins I and II were quantified at 256, 237, 258, and 254 nm, respectively.

Overproduction and purification of Sfp and Pch proteins in *E. coli*. Overproduction and purification of Sfp, PchD, PchE, and PchF proteins have been described previously (25, 26). For production in *E. coli* BL21(DE3) and purifi-

TABLE 2. Effects of *pchG*, *pchH*, and *pchI* mutations on the concentrations of salicylate, Dha, and pyochelin in culture supernatants of *P. aeruginosa*^a

Strain	Mutation	Plasmid	Gene carried	Culture supernatant concn (nmol/ml) of:		
				Salicylate	Dha	Pyochelin
PAO1	None (wild type)			<8.0	69.3 ± 0.6	878.1 ± 59.0
PAO6333	<i>pchG</i> ::ΩSm/Sp			28.4 ± 3.4	5.5 ± 0.2	<2.0
PAO6333	<i>pchG</i> ::ΩSm/Sp	pME6428	<i>pchG</i>	27.7 ± 5.2	99.5 ± 1.4	619.2 ± 14.9
PAO6335	<i>pchH</i> ::ΩSm/Sp			<8.0	75.2 ± 7.3	905.7 ± 71.8
PAO6337	<i>pchI</i> ::ΩSm/Sp			<8.0	61.7 ± 3.0	824.1 ± 10.5

^a GGP medium (30 ml) containing, when required, carbenicillin (250 µg/ml) was inoculated with 0.3-ml samples of cultures grown in the same medium. After incubation at 37°C and 220 rpm for 33 h, supernatants were extracted and analyzed for salicylate, Dha, and pyochelin concentrations by HPLC (see Materials and Methods). The values given represent the means and standard deviations for three independent experiments.

cation of hexahistidine-tagged PchG, all techniques were identical to the methods used for the purification of PchD, PchE, and PchF, except that cultures were grown at 25°C for 24 h without induction.

Production and detection of salicylate-containing compounds. In vitro reconstitution reactions contained 75 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM ATP, 0.1 mM coenzyme A, 1 mM salicylate, 5 mM L-cysteine, 2.5 µM PchE, 2.5 µM PchF, 4 µM PchD, and 0.5 µM Sfp. When needed, NADPH (2.0 mM), SAM (2.0 mM), and/or PchG (6 µM) was added. Reaction mixtures were incubated at 25°C for 1 h to allow phosphopantetheinylation, prior to initiation of the reaction by the addition of ATP. Incubation was continued for 2 h before the addition of HCl (100 mM final concentration) and extraction with ethyl acetate (1 ml) as reported previously (26). Dried samples were dissolved in 10% acetonitrile and analyzed by HPLC using a Vydac C₁₈ reverse-phase column (4.6 mm by 25 cm) on a Beckman Gold HPLC system with monitoring at 254 nm. The mobile phases used were as follows: mobile phase A, 0.1 ml of formic acid and 0.2 ml of triethylamine in 1 liter of water; and mobile phase B, acetonitrile. Samples were eluted at 1 ml/min using a linear gradient of mobile phase B from 8 to 100% over 30 min. Mass spectrometry was carried out as described previously (26).

Nucleotide sequence accession number. The nucleotide sequence of the *pchG*, *pchH*, and *pchI* genes of *P. aeruginosa* PAO1 has been assigned GenBank accession no. AF074705. The nucleotide sequence of *pchG* from strain PAO1 is almost identical to that from strain PA103 determined by De Voss and coworkers (GenBank accession no. AF116557).

RESULTS

The peptide synthetase genes *pchE* and *pchF* are followed by three additional genes, *pchG*, *pchH*, and *pchI*, in *P. aeruginosa*. Previous work has indicated that the *pchEF* transcriptional unit contains additional genes downstream of *pchF* which may be necessary for pyochelin formation (27). To characterize these genes, the DNA region between *pchF* and the pyochelin receptor gene *fptA* was subcloned from cosmid pME3300 (32) and sequenced. Three open reading frames (ORFs) were identified. The first ORF, designated *pchG*, extends for 1,047 bp. The predicted ATG start codon overlaps with the stop codon of *pchF* and is preceded by a plausible ribosome binding site (AGGA). The second ORF, *pchH*, consists of 1,710 bp. The predicted GTG start codon overlaps with the TGA stop codon of *pchG*. A putative ribosome binding site (GGAG) is located at the 3' end of *pchG*. The stop codon of *pchH* overlaps with the presumed ATG start codon of the third ORF, *pchI*, which consists of 1,722 bp and is also preceded by a plausible Shine-Dalgarno sequence (GGAG). The structural organization of the *pchEFGHI* genes strongly indicates that they belong to the same transcriptional unit, whereas the downstream *fptA* gene has its own iron-regulated promoter, as shown previously (1).

Comparison of the deduced amino acid sequence of *pchG* with the current sequence databases did not convincingly indicate the function for this protein. Some similarity was found only to proteins Irp3 (GenBank accession number Y12527;

28% identity) and YbtU (accession number AL031866; 27% identity), which are required for yersiniabactin formation in *Yersinia enterocolitica* and *Y. pestis*, respectively (12, 22). NrpU, a protein from *Proteus mirabilis* (accession number U46488), which shares 41% identical amino acids with Irp3, is also related to PchG (24% identity). However, no biochemical function has yet been attributed to Irp3, YbtU, and NrpU (12, 22).

The deduced amino acid sequences of PchH and PchI showed similarities to those of various ATP binding cassette (ABC) transport proteins with export function (30 to 35% identical amino acids). All four conserved sequence motifs characteristic of ABC proteins (17) are present in PchH and PchI; the Walker A motif (GPSGSGKST in PchH and GPSG AGKSS in PchI), resembling the consensus core sequence GX XGXGKST, and the Walker B motif (LLLLDEPT in PchH and PchI), resembling the consensus core sequence hhhh DEPT (h being a hydrophobic residue), are necessary for ATP binding and hydrolysis. The ABC signature sequence LSGG, which is likely to be involved in energy transduction, is entirely conserved in PchH and PchI. An additional new motif consisting of a conserved histidine preceded by four hydrophobic residues and followed by a charged residue is present in PchH (VIVHR) and PchI (LVLTHR) about 30 amino acids downstream of the conserved aspartic acid residue of the Walker B motif. PchH and PchI have membrane-spanning domains with six plausible transmembrane helices in their amino-terminal portions, whereas the ATPase domains are located in their carboxy-terminal portions.

Effects of *pchG*, *pchH*, and *pchI* mutations in *P. aeruginosa* on the formation of salicylate, Dha, and pyochelin. To study the role of PchG, PchH, and PchI in pyochelin biosynthesis and translocation, several *P. aeruginosa* mutants were constructed. First, a *pchI* mutation was created by marker exchange using plasmid pME6499, which carries an ΩSm/Sp cassette at the *EcoRV* site in *pchI*. This mutation was made in the chromosome of the wild-type strain PAO1, giving strain PAO6337 (Fig. 1). Similar pyochelin and Dha concentrations were measured in culture supernatants of PAO6337 and PAO1 grown under iron-limiting conditions (Table 2), indicating that PchI is not involved in pyochelin or Dha export. An ΩSm/Sp insertion was also created in *pchH* (at the *EcoRI* site) using the suicide construct pME6498. Again, the mutation was transferred to wild-type strain PAO1, giving strain PAO6335 (Fig. 1). This insertion not only interrupts *pchH* but also has a polar effect on the expression of the downstream *pchI* gene. Salicylate, Dha, and pyochelin concentrations in culture supernatants of PAO6335

TABLE 3. Effects of the *pchEFGHI* genes on salicylate, Dha, and pyochelin formation in *P. aeruginosa*^a

Strain	Chromosomal mutation	Plasmid(s)	Genes carried	Culture supernatant concn (nmol/ml) of the following in the absence (-) or presence ^b (+) of Fe:					
				Salicylate		Dha		Pyochelin	
				-	+	-	+	-	+
PAO1	None (wild type)			<8	<8.0	79 ± 10.0	<1.2	824 ± 26	<2.0
PAO6331	Δpch			<8	<8.0	<1.2	<1.2	<2.0	<2.0
PAO6331	Δpch	pME6178	<i>entDpchDCBA</i>	688 ± 38	428 ± 11	<1.2	<1.2	<2.0	<2.0
PAO6331	Δpch	pME6178 + pME6482	<i>entDpchDCBA</i> + <i>pchE</i>	1,178 ± 93	1,044 ± 118	140 ± 11.7	145 ± 20	<2.0	<2.0
PAO6331	Δpch	pME6178 + pME6483	<i>entDpchDCBA</i> + <i>pchEG</i>	969 ± 118	1,075 ± 107	130 ± 15.6	148 ± 13.9	<2.0	<2.0
PAO6331	Δpch	pME6178 + pME6484	<i>entDpchDCBA</i> + <i>pchEFGHI</i>	720 ± 146	621 ± 72	4 ± 1.7	<1.2	<2.0	<2.0
PAO6331	Δpch	pME6178 + pME6486	<i>entDpchDCBA</i> + <i>pchEF</i>	773 ± 129	1,059 ± 14	104 ± 11.8	101 ± 12.1	<2.0	<2.0
PAO6331	Δpch	pME6178 + pME6488	<i>entDpchDCBA</i> + <i>pchEFG</i>	225 ± 114	477 ± 109	21 ± 3.5	25 ± 2.5	494 ± 60	424 ± 52
PAO6331	Δpch	pME6178 + pME6490	<i>entDpchDCBA</i> + <i>pchEFGHI</i>	233 ± 49	387 ± 13	19 ± 1.0	20 ± 1.2	437 ± 12	349 ± 29
PAO6331	Δpch	pME6478 + pME6488	<i>pchDCBA</i> + <i>pchEFG</i>	57 ± 19	88 ± 26	9 ± 1.7	16 ± 1.4	308 ± 52	296 ± 11

^a GGP medium (30 ml) containing, when required, gentamicin (10 µg/ml) and tetracycline (100 µg/ml) was inoculated with 0.3-ml samples of cultures grown in the same medium. After incubation at 37°C and 220 rpm for 33 h, supernatants were extracted and analyzed for salicylate, Dha, and pyochelin concentrations by HPLC (see Materials and Methods). The values given represent the means and standard deviations for three independent experiments.

^b The medium was supplemented with 100 µM FeCl₃ (iron excess).

were determined and were found to be similar to those in the wild type and in the *pchI* mutant (Table 2). Thus, neither PchH nor PchI appears to be essential for pyochelin or Dha export.

The *pchH* and *pchI* Ω Sm/Sp insertion mutations were also introduced into the pyoverdinin-negative mutant PALS128, resulting in strains PAO6336 and PAO6338, respectively (Fig. 1). These strains grew well in minimal medium supplemented with the weak iron chelator dipyrindyl, suggesting that they could utilize their own, stronger iron chelator pyochelin for iron uptake. In a control experiment, the double pyoverdinin- and pyochelin-negative mutant PALS128-17 (32) grew very poorly in dipyrindyl minimal medium. Therefore, an involvement of PchH and PchI in pyochelin-mediated iron uptake seems unlikely.

An Ω Sm/Sp cassette was also inserted into the *pchG* gene of strain PAO1 using the suicide construct pME6180, resulting in strain PAO6333. As shown in Table 2, no pyochelin was detected in the supernatant of PAO6333 grown under iron-limiting conditions, implying that *pchG* is required for pyochelin biosynthesis. Dha was detected in small amounts, a result which could be explained by the fact that pyochelin is required to induce the transcription of the entire *pchE* operon (27). Although the mutation in PAO6333 is polar, the observed phenotype can be attributed solely to the mutation in *pchG* since, as shown above, insertions of Ω cassettes in *pchH* or *pchI* do not affect Dha and pyochelin production. Moreover, PAO6333 could be fully complemented by introducing plasmid pME6428, which carries *pchG* under the control of the constitutive *lac* promoter (Fig. 1 and Table 2). These results show that PchG is essential for pyochelin production.

Uncoupling salicylate, Dha, and pyochelin biosynthesis from Fur-mediated repression by iron and PchR-dependent induction by pyochelin. We have shown previously that the expression of the two pyochelin biosynthetic operons is positively autoregulated by pyochelin and PchR (27). As a consequence, it can be difficult to study the importance of each gene individually in the biosynthetic pathway. Thus, although *pchG* is clearly needed for pyochelin production, a possible role in Dha formation cannot be excluded. We therefore decided to uncouple the expression of *pchDCBA* and *pchEFGHI* from positive autoregulation by pyochelin as well as from neg-

ative regulation by iron and Fur. To this end, the *pchDCBA* genes, together with the *entD* gene from *E. coli*, were subcloned into vector pME6001 and expressed under the control of the constitutive *lac* promoter in pME6178 (Fig. 1). The *entD* gene, which encodes a phosphopantetheinyl transferase with relaxed specificity, was added to ensure the conversion of the peptide synthetases PchE and PchF to their holoenzyme forms, in case the gene for phosphopantetheinyl transferase of *P. aeruginosa* were under iron and/or pyochelin control as well. This gene, which is not part of the *pchDCBA* and *pchEFGHI* operons, has not yet been identified in *P. aeruginosa*.

To express different combinations of genes from the *pchEFGHI* operon constitutively, a series of plasmids (Fig. 1 and Table 3) were generated from vector pME6012, in which gene expression is driven from the kanamycin promoter. The individual roles of *pchE*, *pchF*, *pchG*, *pchH*, and *pchI* in Dha and pyochelin formation were then studied with PAO6331, a mutant of PAO1 in which the whole pyochelin region encompassing *pchDCBA*, *pchR*, and *pchEFGHI* had been removed by gene exchange using the suicide construct pME6176 (Fig. 1). As expected, salicylate, Dha, and pyochelin were not detectable in supernatants of PAO6331 grown in the presence or in the absence of iron (Table 3). The introduction of pME6178 (Fig. 1) resulted in iron-independent production of salicylate. In agreement with our previous findings (26, 27), constitutive production of Dha required, in addition, the presence of *pchE* on pME6482 (Fig. 1). PchG was not involved in Dha production, as similar amounts were made when pME6482 was replaced by plasmid pME6483, carrying *pchEG*. To test whether PchH and PchI would contribute to Dha export, we measured the content of Dha in PAO6331 carrying pME6178 and pME6484. Surprisingly, the presence of the *pchHI* genes strongly reduced the amounts of Dha and weakly reduced those of salicylate detected in culture supernatants (Table 3). Thus, a function of PchH and PchI in Dha export is unlikely, but an import activity for Dha (and perhaps salicylate) cannot be excluded. Constitutive Dha and pyochelin formation in PAO6331 was achieved with pME6178 and pME6488, carrying *pchEFG*. The requirement for *pchG* was further corroborated by the fact that no pyochelin was made when pME6488 was replaced by pME6486, carrying the *pchEF* genes only. Adding the *pchHI*

genes downstream of *pchEFG* on pME6490 did not result in larger amounts of Dha and pyochelin being produced (Table 3), showing again that this ABC transport system is not necessary for Dha or pyochelin export.

A control experiment showed that the deletion mutant PAO6331 was complemented by the *pchDCBA* and *pchEFG* genes alone, in the absence of the cloned *entD* gene; pyochelin, salicylate, and Dha were produced by strain PAO6331 carrying plasmids pME6478 and pME6488, and the addition of iron did not influence the amounts of these metabolites (Table 3, last entry). Thus, the expression of the chromosomal phosphotransferase gene of *P. aeruginosa* does not appear to restrict pyochelin synthesis when iron is present in the growth medium. We do not know at this stage whether this gene is expressed constitutively or is positively autoregulated by pyochelin, in parallel with the two pyochelin biosynthetic operons (27).

Pyochelin formation in *E. coli* expressing pyochelin biosynthetic genes from *P. aeruginosa*. The results presented in Tables 2 and 3 indicate that the *pchDCBA* and *pchEFG* genes are sufficient to produce pyochelin from chorismate in *P. aeruginosa*. In an attempt to clarify the role of the *pchHI* genes, we also measured pyochelin formation in recombinant derivatives of *E. coli*, a host which is normally unable to make salicylate, Dha, and pyochelin. After 42 h of growth in nutrient yeast broth containing tetracycline and gentamicin for plasmid maintenance, pyochelin concentrations were measured in supernatants from three independent cultures of strain MT147 carrying pME6178 and pME6488. Although the amount of pyochelin made by this strain (21 ± 1.5 nmol/ml) was considerably smaller than that measured in culture supernatants of *P. aeruginosa*, the result confirms that *pchDCBA* and *pchEFG*, together with *entD*, are sufficient for pyochelin biosynthesis in the heterologous host. Replacing *pchEFG* with *pchEFGHI* did not affect pyochelin production (data not shown); hence, the function of PchH and PchI remained elusive. The small amounts of pyochelin produced by *E. coli* recombinants may have been a result of low chorismate availability, since salicylate concentrations measured in culture supernatants of MT147 carrying pME6178 and pME6488 were also much lower (2.3 ± 0.3 nmol/ml) than those found in PAO6331 carrying the same two plasmids (Table 3).

PchG is required for pyochelin formation in vitro. As reported previously (26), the in vitro reconstitution reaction containing PchD, PchE, PchF, salicylate, ATP, and L-cysteine results in the formation of hydroxyphenyl-thiazolanyl-cysteine (HPT-cys) and hydroxyphenyl-thiazolyl-thiazolanyl-carboxylic acid (HPTT-COOH) (Fig. 2, trace f). The addition of PchG alone did not alter the HPLC chromatogram (Fig. 2, trace e); however, the addition of PchG and NADPH produced a new product (indicated by an asterisk in trace d) that eluted between the standards for HPT-cys and HPTT-COOH. Mass spectrometric analysis showed this peak to have a mass of 311 Da. This mass corresponds to that of the predicted $[M + H]^+$ ion for desmethyl-pyochelin (hydroxyphenyl-thiazolanyl-thiazolidinyl-carboxylic acid), in which the second thiazoline ring of the precursor hydroxyphenyl-bis-thiazolanyl-carboxylic acid has been reduced. The autoxidation of the middle ring, which is observed in the formation of HPTT-COOH, is not observed in this product.

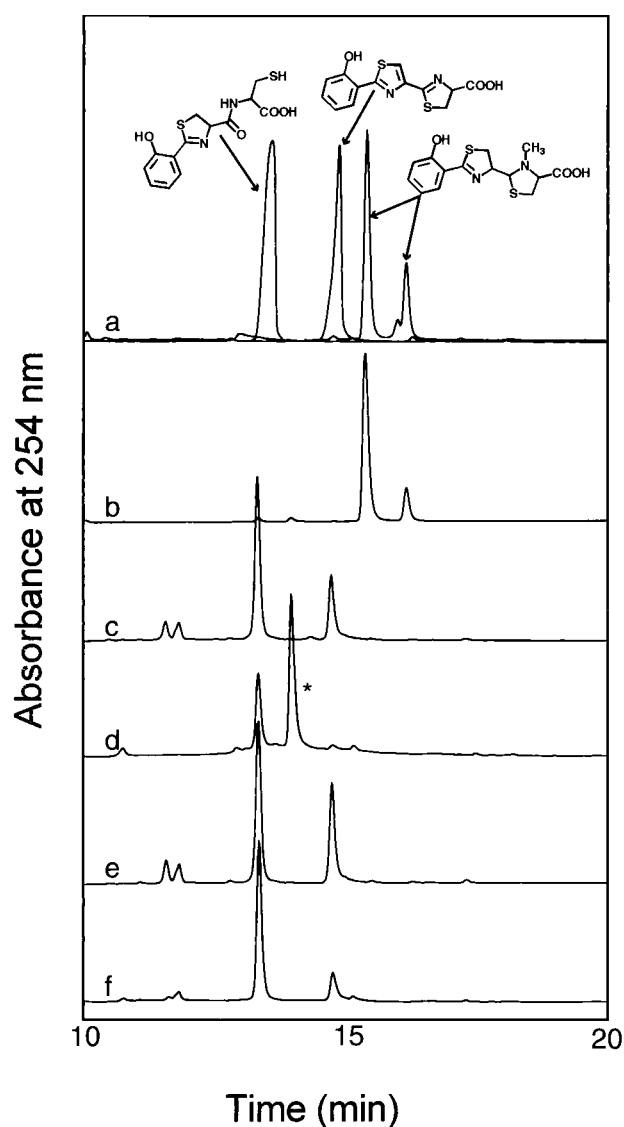


FIG. 2. HPLC elution profile showing intermediates and end products of pyochelin formation in vitro. Trace a, elution profile of the synthetic standards (from left to right) HPT-cys and HPTT-COOH (26) as well as a pyochelin standard purified from *P. aeruginosa* (as a 3:1 mixture of stereoisomers). Traces b to f, enzymatic products of in vitro reactions with mixtures containing 75 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM ATP, 0.1 mM coenzyme A, 1 mM salicylate, 5 mM L-cysteine, 2.5 μM PchE, 2.5 μM PchF, 4 μM PchD, and 0.5 μM Sfp. In addition, traces b to e also contained 6 μM PchG, traces b and c also contained 2.0 mM SAM, and traces b and d also contained 2.0 mM NADPH. The identity of the peak indicated by an asterisk was determined by mass spectrometry and corresponds to desmethyl-pyochelin (see Fig. 3). Note that peak sizes were normalized to show the identity of peaks and that they do not reflect relative amounts of products.

When SAM was added and NADPH was omitted, only HPT-cys and HPTT-COOH were produced (Fig. 2, trace c). Full reconstitution of pyochelin synthetase activity was observed upon inclusion of PchG, NADPH, and SAM (Fig. 2, trace b). The two peaks which had the same retention time as authentic pyochelin (Fig. 2, trace a) and which were observed in the full reconstitution were isolated and subjected to mass spectrometric analysis. The mass of each peak (325 Da) was in

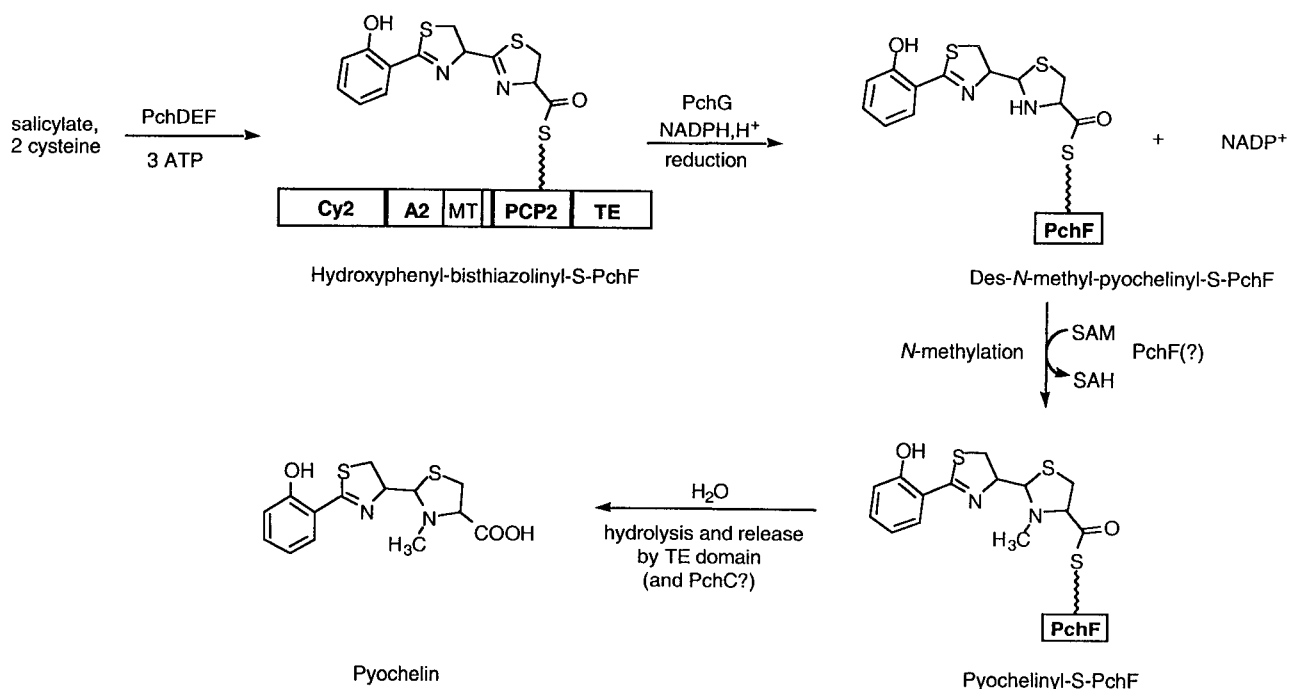


FIG. 3. Action of PchG as a thiazolanyl reductase on an acyl-S-PchF intermediate in pyochelin biosynthesis. PchG reduces hydroxyphenyl-bisthiazolanyl-S-PchF to the des-*N*-methyl-pyochelanyl-S-acyl enzyme intermediate. The latter is then methylated by the putative methyltransferase (MT) domain of PchF and finally hydrolytically released from PchF. Domain structure of PchF: Cy2, cyclization; A2, adenylation; PCP2, peptidyl carrier protein. SAH, *S*-adenosyl-homocysteine; TE, thioesterase (26).

good agreement with that of the predicted $[M + H]^+$ ion and was identical to the mass observed for the pyochelin standard purified from *P. aeruginosa*. Thus, both mass analysis and HPLC confirmed that the two peaks observed in Fig. 2, trace b, corresponded to the isomeric mixture (pyochelins I and II) of natural pyochelin (trace a), as previously reported (28).

DISCUSSION

We have shown in this study that the *pchG* gene, which is located downstream of *pchEF*, is essential for pyochelin formation in *P. aeruginosa*. Thus, we have identified all genes required to make pyochelin from chorismate, with the exception of a phosphopantetheinyl transferase gene, whose activity is necessary for priming the peptide synthetases PchE and PchF. This result was confirmed by the fact that transfer of the *pchDCBA* and *pchEFG* genes to *E. coli* resulted in pyochelin formation in this heterologous host.

The *pchHI* genes located immediately downstream of *pchG* encode proteins that resemble the subfamily of ABC transporters with export function. PchH and PchI each carry an ATP binding domain and a membrane-spanning domain on the same polypeptide, a configuration which is typical for bacterial export systems (9, 31). The absence of an adjacent gene coding for a periplasmic binding protein further supports a role for PchH and PchI in an export function rather than an import function. Based on these structural characteristics and on the likely coregulation of *pchHI* with the *pchEFG* genes, it was conceivable that PchH and PchI could be involved in pyochelin export. However, mutations in either *pchH* or *pchI* did not alter the amount of pyochelin (or Dha) present in culture supernatants. Thus, PchH and PchI may have another, as-yet-

unknown transport function, or Dha and pyochelin may be exported by other transporters.

Two ABC transport genes, *ybtP* and *ybtQ*, have been identified in the yersiniabactin cluster in *Y. pestis*. Their corresponding proteins have the structural characteristics of ABC exporters. However, they are not necessary for yersiniabactin secretion but rather appear to play a role in iron uptake (10). We could not obtain evidence for an involvement of PchH and PchI in pyochelin-mediated iron uptake in *P. aeruginosa*. However, in the absence of pyochelin synthesis (due to the absence of *pchF*), the overexpressed *pchHI* genes significantly reduced the amount of Dha excreted (Table 3). One possible interpretation of this finding may be that PchH and PchI import Dha, thereby allowing the recycling of this compound. Unfortunately, there is no simple assay for such a function, in that free Dha does not act as a biosynthetic precursor of pyochelin (27).

When the essential nature of PchG for pyochelin formation had been established by the genetic knockout results, the specific function of PchG as an enzyme was assayed *in vitro* in the presence of purified PchD, PchE, and PchF proteins. Only in the presence of both pure PchG protein and the cosubstrates NADPH and SAM was pyochelin produced in these incubations, starting from salicylate, cysteine, and ATP. Since we have previously shown that a tricyclic acyl enzyme intermediate (26) accumulates on PchF and is not released in the absence of PchG, we conclude that PchG is a catalytic protein whose action is required before the mature pyochelin acyl chain can be released from its covalent tether on PchF. PchG is weakly similar to biliverdin reductase (30), also an NADPH-dependent imine reductase, supporting the assignment in Fig. 3 of PchG as a thiazolanyl-S-enzyme-reductase converting bisthiazolanyl-S-PchF to the des-*N*-methyl-pyochelanyl-S-PchF acyl

enzyme intermediate. SAM is additionally required for catalytic turnover; while it has not yet been proven whether the *N*-methyltransferase activity resides in PchG or PchF, we have argued (26) that a 35-kDa insert in the A domain of PchF is likely to be the methyltransferase. This question needs to be resolved by future mutagenesis experiments. Thus, in Fig. 3, *N* methylation produces mature pyochelinylnyl-*S*-PchF, which is presumed then to be a substrate for rapid hydrolysis by the C-terminal thioesterase domain of PchF or by PchC.

From these results, we conclude that PchG is an imine reductase, reducing thiazoline to a thiazolidine ring. There is mechanistic analogy between PchG and other NADPH-dependent imine reductases, for example, dihydrofolate reductase. Only after PchG action will the nitrogen in the thiazolidine ring be sufficiently basic and electron rich to attack the electron-deficient methyl group in SAM to yield the *N*-methylthiazolidine moiety characteristic of mature pyochelin. This initial elucidation of a thiazoline reductase activity of PchG is likely to presage equivalent reductase functions of Irp3 and YbtU, which are involved in the biosynthesis of the related but structurally more complex siderophore yersiniabactin (12, 22).

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REFERENCES

- Ankenbauer, R. G., and H. N. Quan. 1994. FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. *J. Bacteriol.* **176**:307-319.
- Blumer, C., S. Heeb, G. Pessi, and D. Haas. 1999. Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc. Natl. Acad. Sci. USA* **96**:14073-14078.
- Carmi, R., S. Carmeli, E. Levy, and F. J. Gough. 1994. (+)-(-)-Dihydroaeruginic acid, an inhibitor of *Septoria tritici* and other phytopathogenic fungi and bacteria, produced by *Pseudomonas fluorescens*. *J. Nat. Prod.* **57**:1200-1205.
- Cox, C. D. 1980. Iron uptake with ferripyochelin and ferric citrate by *Pseudomonas aeruginosa*. *J. Bacteriol.* **142**:581-587.
- Cox, C. D. 1982. Effect of pyochelin on the virulence of *Pseudomonas aeruginosa*. *Infect. Immun.* **36**:17-23.
- Cox, C. D., and P. Adams. 1985. Siderophore activity of pyoverdinin in *Pseudomonas aeruginosa*. *Infect. Immun.* **48**:130-138.
- Del Sal, G., G. Manfioletti, and C. Schneider. 1988. A one-tube plasmid DNA mini-preparation suitable for sequencing. *Nucleic Acids Res.* **16**:9878.
- Farinha, M. A., and A. M. Kropinski. 1990. High efficiency electroporation of *Pseudomonas aeruginosa* using frozen cell suspensions. *FEMS Microbiol. Lett.* **58**:221-225.
- Fath, M. J., and R. Kolter. 1993. ABC transporters: bacterial exporters. *Microbiol. Rev.* **57**:995-1017.
- Fetherston, J. D., V. J. Bertolino, and R. D. Perry. 1999. YbtP and YbtQ: two ABC transporters required for iron uptake in *Yersinia pestis*. *Mol. Microbiol.* **32**:289-299.
- Gamper, M., B. Ganter, M. Polito, and D. Haas. 1992. RNA processing modulates the expression of the *arcDABC* operon in *Pseudomonas aeruginosa*. *J. Mol. Biol.* **226**:943-957.
- Gehring, A. M., E. DeMoll, J. D. Fetherston, I. Mori, G. F. Mayhew, F. R. Blattner, C. T. Walsh, and R. D. Perry. 1998. Iron acquisition in plague: modular logic in enzymatic biogenesis of yersiniabactin by *Yersinia pestis*. *Chem. Biol.* **5**:573-586.
- Handfield, M., D. E. Lehoux, F. Sanschagrín, M. J. Mahan, D. E. Woods, and R. C. Levesque. 2000. *In vivo*-induced genes in *Pseudomonas aeruginosa*. *Infect. Immun.* **68**:2359-2362.
- Heeb, S., Y. Itoh, T. Nishijyo, U. Schneider, C. Keel, J. Wade, U. Walsh, F. O'Gara, and D. Haas. 2000. Small, stable shuttle vectors based on the minimal pVSI replicon for use in Gram-negative, plant-associated bacteria. *Mol. Plant Microbe Interact.* **13**:232-237.
- Heinrichs, D. E., and K. Poole. 1993. Cloning and sequence analysis of a gene (*pchR*) encoding an AraC family activator of pyochelin and ferripyochelin receptor synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* **175**:5882-5889.
- Lambalot, R. H., A. M. Gehring, R. S. Flugel, P. Zuber, M. LaCelle, M. A. Marahiel, R. Reid, C. Koshia, and C. T. Walsh. 1996. A new enzyme superfamily—the phosphopantetheinyl transferases. *Chem. Biol.* **3**:923-936.
- Linton, K. J., and C. F. Higgins. 1998. The *Escherichia coli* ATP-binding cassette (ABC) proteins. *Mol. Microbiol.* **28**:5-13.
- Merriman, T. R., M. E. Merriman, and I. L. Lamont. 1995. Nucleotide sequence of *pvdD*, a pyoverdinin biosynthetic gene from *Pseudomonas aeruginosa*: PvdD has similarity to peptide synthetases. *J. Bacteriol.* **177**:252-258.
- Meyer, J. M., and M. A. Abdallah. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *J. Gen. Microbiol.* **107**:319-328.
- Meyer, J. M., A. Neely, A. Stintzi, C. Georges, and I. A. Holder. 1996. Pyoverdinin is essential for virulence of *Pseudomonas aeruginosa*. *Infect. Immun.* **64**:518-523.
- Ozenberger, B. A., T. J. Brickman, and M. A. McIntosh. 1989. Nucleotide sequence of *Escherichia coli* isochorismate synthetase gene *entC* and evolutionary relationship of isochorismate synthetase and other chorismate-utilizing enzymes. *J. Bacteriol.* **171**:775-783.
- Pelludat, C., A. Rakin, C. A. Jacobi, S. Schubert, and J. Heesemann. 1998. The yersiniabactin biosynthetic gene cluster of *Yersinia enterocolitica*: organization and siderophore-dependent regulation. *J. Bacteriol.* **180**:538-546.
- Poole, K., S. Neshat, K. Krebs, and D. E. Heinrichs. 1993. Cloning and nucleotide sequence analysis of the ferripyoverdinin receptor gene *fpvA* of *Pseudomonas aeruginosa*. *J. Bacteriol.* **175**:4597-4604.
- Prentki, P., and H. M. Krusch. 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**:303-313.
- Quadri, L. E. N., P. H. Weinreb, M. Lei, M. M. Nakano, P. Zuber, and C. T. Walsh. 1998. Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases. *Biochemistry* **37**:1585-1595.
- Quadri, L. E. N., T. A. Keating, H. M. Patel, and C. T. Walsh. 1999. Assembly of the *Pseudomonas aeruginosa* nonribosomal peptide siderophore pyochelin: *in vitro* reconstitution of aryl-4,2-bisthiazoline synthetase activity from PchD, PchE, and PchF. *Biochemistry* **38**:14941-14954.
- Reimann, C., L. Serino, M. Beyeler, and D. Haas. 1998. Dihydroaeruginic acid synthetase and pyochelin synthetase, products of the *pchEF* genes, are induced by extracellular pyochelin in *Pseudomonas aeruginosa*. *Microbiology* **144**:3135-3148.
- Rinehart, K. L., A. L. Staley, S. R. Wilson, R. G. Ankenbauer, and C. D. Cox. 1995. Stereochemical assignment of the pyochelins. *J. Org. Chem.* **60**:2786-2791.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schluchter, W. M., and A. N. Glazer. 1997. Characterization of cyanobacterial biliverdin reductase. *J. Biol. Chem.* **272**:13562-13569.
- Schneider, E., and S. Hunke. 1998. ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiol. Rev.* **22**:1-12.
- Serino, L., C. Reimann, B. Baur, M. Beyeler, P. Visca, and D. Haas. 1995. Structural genes for salicylate biosynthesis from chorismate in *Pseudomonas aeruginosa*. *Mol. Gen. Genet.* **249**:217-228.
- Serino, L., C. Reimann, P. Visca, M. Beyeler, V. Della Chiesa, and D. Haas. 1997. Biosynthesis of pyochelin and dihydroaeruginic acid requires the iron-regulated *pchDCBA* operon in *Pseudomonas aeruginosa*. *J. Bacteriol.* **179**:248-257.
- Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vitro* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio/Technology* **1**:784-790.
- Stanisich, V. A., and B. W. Holloway. 1972. A mutant sex factor of *Pseudomonas aeruginosa*. *Genet. Res.* **19**:91-108.
- Takase, H., H. Nitani, K. Hoshino, and T. Otani. 2000. Impact of siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. *Infect. Immun.* **68**:1834-1839.
- Visca, P., L. Serino, and N. Orsi. 1992. Isolation and characterization of *Pseudomonas aeruginosa* mutants blocked in the synthesis of pyoverdinin. *J. Bacteriol.* **174**:5727-5731.
- Voisard, C., C. T. Bull, C. Keel, J. Laville, M. Maurhofer, U. Schneider, G. Défago, and D. Haas. 1994. Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches, p. 67-89. *In* F. O'Gara, D. Dowling, and B. Boesten (ed.), *Molecular ecology of rhizosphere microorganisms*. VCH Publishers, Weinheim, Germany.
- Watson, A., R. A. Alm, and J. S. Mattick. 1996. Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. *Gene* **172**:163-164.
- Ye, R. W., D. Haas, J. O. Ka, V. Krishnapillai, A. Zimmermann, C. Baird, and J. M. Tiedje. 1995. Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *J. Bacteriol.* **177**:3606-3609.