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PchC Thioesterase Optimizes Nonribosomal Biosynthesis of the Peptide Siderophore Pyochelin in *Pseudomonas aeruginosa*

Cornelia Reimmann,[1]* Hiten M. Patel,[2] Christopher T. Walsh,[2] and Dieter Haas[1]

Département de Microbiologie Fondamentale, Université de Lausanne, Lausanne, Switzerland,[1] and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts[2]

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In *Pseudomonas aeruginosa*, the antibiotic dihydroaeruginoate (Dha) and the siderophore pyochelin are produced from salicylate and cysteine by a thiotemplate mechanism involving the peptide synthetases PchE and PchF. A thioesterase encoded by the *pchC* gene was found to be necessary for maximal production of both Dha and pyochelin, but it was not required for Dha release from PchE and could not replace the thioesterase function specified by the C-terminal domain of PchF. In vitro, 2-aminobutyrate, a cysteine analog, was adenylated by purified PchE and PchF proteins. In vivo, this analog strongly interfered with Dha and pyochelin formation in a *pchC* deletion mutant but affected production of these metabolites only slightly in the wild type. Exogenously supplied cysteine overcame the negative effect of a *pchC* mutation to a large extent, whereas addition of salicylate did not. These data are in agreement with a role for PchC as an editing enzyme that removes wrongly charged molecules from the peptidyl carrier protein domains of PchE and PchF.

Under iron-limiting growth conditions, aerobic bacteria produce siderophores which form complexes with Fe^{3+} in the environment and deliver it, via specific outer membrane receptors, to the bacterial cytoplasm. The two major siderophores made by *Pseudomonas aeruginosa* are pyoverdin (10, 23) and pyochelin (8, 34), both of which contribute to the virulence of this opportunistic human pathogen (9, 24, 43). The biosynthetic genes of the aryl-peptide pyochelin (32, 33, 39, 40) are clustered with the pyochelin receptor gene *fptA* (1) and the regulatory gene *pchR* (21) on the *P. aeruginosa* chromosome. The biosynthetic genes are organized in two divergent operons, *pchDCBA* and *pchEFGHI*. In the presence of iron, *pchDCBA*, *pchEFGHI*, and *fptA* are repressed by the Fur protein (1, 32, 40). During iron limitation, the biosynthesis of pyochelin and its receptor protein is induced by a positive feedback loop involving pyochelin and the transcriptional regulator PchR (17, 21, 32).

Pyochelin is made from salicylate and two molecules of cysteine by a thiotemplate mechanism on a four-protein, 12-domain assembly line (11, 27) (Fig. 1). The salicylate moiety is generated from chorismate by PchA (isochorismate synthase) (15) and PchB (isochorismate pyruvate-lyase) (14, 39) and is subsequently adenylated by PchD (31). Adenylation of L-cysteine is catalyzed by the nonribosomal peptide synthetase PchE, which then covalently links both activated substrates, as thioesters, to two posttranslationally added 4′-phosphopantetheine prosthetic groups on PchE. Condensation, epimerization, and cyclization reactions, also catalyzed by PchE, generate the enzyme-bound intermediate hydroxyphenyl-thiazoline (HPT), which can be released by slow hydrolysis of the thioester bond, to produce dihydroaeruginoate (Dha) (28, 31). This metabolite is found in small amounts in culture supernatants of *P. aeruginosa* (40). The 4′-phosphopantetheinylated (primed) form of the peptide synthetase PchF adenylates a second molecule of L-cysteine and, like PchE, links it covalently via a thioester. PchF subsequently catalyzes the condensation of the PchE-bound intermediate HPT with cysteinyl-PchF

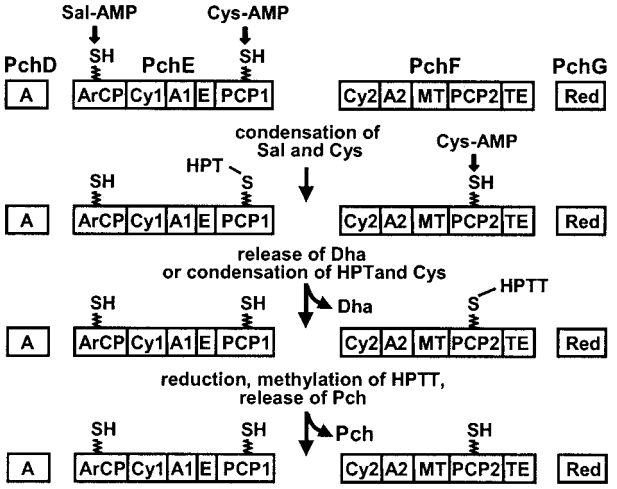

FIG. 1. Model for PchDEFG-dependent biosynthesis of pyochelin from salicylate in *P. aeruginosa* (adapted from references 27, 28, 31, and 33). Adenylated forms of salicylate (Sal) and L-cysteine (Cys) are loaded onto the peptide synthetases PchE and PchF via covalent thioester linkages. PchE-dependent condensation of salicylate with L-cysteine, followed by epimerization and cyclization reactions, generates the intermediate HPT, which can be released from PchE to give Dha or can be coupled to PchF-bound L-cysteine to give hydroxyphenyl-bis-thiazoline (HPTT). After reduction of HPTT by PchG and methylation by PchF, pyochelin (Pch) is released by thioester cleavage. Functional domains are indicated for adenylation (A, A1, and A2), cyclization (Cy1 and Cy2), epimerization (E), thiolation (ArCP for the aryl carrier protein domain and PCP1 and PCP2 for peptidyl carrier protein domains), reduction (Red), methyl transfer (MT) and thioesterase (TE) activity.

* Corresponding author. Mailing address: Département de Microbiologie Fondamentale, Bâtiment de Biologie, Université de Lausanne, CH-1015 Lausanne Dorigny, Switzerland. Phone: 41 21 6925632. Fax: 41 21 6925635. E-mail: Cornelia.Reimmann@imf.unil.ch.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
E. coli S17-1	*thi pro hsdR recA*; chromosomal RP4 (Tra^+ Tc^s Km^s Ap^s)	41
P. aeruginosa strains		
PALS128	*pvdB*	45
PALS128-6	*pvdB pchA*::ω (insertion of 35 bp)	39
PAO1	Wild type	ATCC 15692
PAO6331	Δ*pch* (= Δ*pchDCBA* Δ*pchR* Δ*pchEFGHI*)	33
PAO6339	Δ*pchC*	This study
PAO6342	*pvdB* Δ*pchC*	This study
PAO6357	*pvdB pchA*::ω Δ*pchC*	This study
Plasmids		
pBLS II KS	Cloning vector, Ap^r	Stratagene
pPchE	*pchE* overexpression construct; Km^r	31
pPchF	*pchF* overexpression construct; Km^r	31
pPchFTE	*pchF* overexpression construct with C1606A/S1607A mutations; Km^r	31
pME3087	Suicide vector; ColE1 replicon; Tc^r; EcoRI-KpnI-BamHI-XbaI-PstI-SphI-HindIII polylinker	46
pME3318	pQF10 derivative carrying *pchDCBA*	40
pME6001	pBBR1-based cloning vector; Gm^r	4
pME6012	pVS1-p15A shuttle vector; Tc^r	20
pME6178	pME6001 carrying *entD pchDCBA* under P_{lac} control	33
pME6488	pME6012 carrying *pchEFG* under P_{kan} control	33
pME6815	Suicide plasmid for construction of *pchC* deletion; derivative of pME3087 containing the 3′ part of *pchD*, *pchC* with an in-frame deletion, and the 5′ part of *pchB* on a 1.0-kb BamHI-HindIII fragment	This study
pME6831	pUCPSK carrying *pchDC* on a 2.8-kb EcoRI-ScaI fragment from pME3318	This study
pME6852	pME6001 carrying *entD pchDBA* under P_{lac} control (same as pME6178 but with in-frame deletion in *pchC*)	This study
pME6886	pME6012 carrying $pchEF_{C1606A/S1607A}G$ under P_{kan} control (same as pME6488 but with mutations affecting the PchF thioesterase motif)	This study
pME7538	pUCPSK carrying $pchDC_{S90A}$ (same as pME6831 but with mutation affecting the thioesterase motif)	This study
pUCSK	ColE1-pRO1600 shuttle vector; Ap^r	47
pUK21	Cloning vector; Km^r	44

and thereby generates the second thiazoline ring, which is reduced by the PchG reductase and N-methylated by a tailoring domain of PchF. Hydrolysis of the remaining thioester bond finally releases pyochelin from PchF (27, 33).

The *pch* gene cluster encodes two thioesterases. One of these thioesterases resides in the C-terminal thioesterase domain of PchF (internal thioesterase) (Fig. 1) and releases pyochelin from PchF (31). The other, a type II thioesterase (external thioesterase), is encoded by the *pchC* gene (40), and its function has not been studied previously. Thioesterases carry two conserved sequences: GXSXG, a motif also present in the active sites of serine proteases, lipases, and acyltransferases; and GXHF, which is located approximately 140 amino acids further downstream and whose His residue is essential for catalytic activity (29, 36, 48). Both motifs occur in the thioesterase domain of PchF ($GYCSGX_{176}GGHF$ [31]) and in PchC ($GHSLGX_{126}GGHF$ [40]). Mutations in the first, integrated thioesterase motif of PchF prevent pyochelin release from PchF in vitro (31). The purpose of the present study was to examine the role of the second, external thioesterase, PchC.

In prokaryotic polyketide or nonribosomal peptide syntheses, it is common to find a distinct, external type II thioesterase. Mutational loss of the external thioesterase generally results in a reduced amount of product, and it has been suggested that such enzymes could either assist in product release from the thiotemplate or play a role as editing enzymes that remove wrongly charged substrates or aberrant intermediates from the enzyme complex (19, 36, 37). In vitro, type II thioesterases can regenerate misacylated peptide synthetases resulting from erroneous priming or from loading of an amino acid which cannot be processed (37, 51). Here we provide evidence for such a proofreading role for the type II PchC thioesterase of *P. aeruginosa* in vivo, which results in optimized Dha and pyochelin production.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. Bacteria were usually grown on nutrient agar and in nutrient yeast broth (42) at 37°C. To quantify salicylate, Dha, and pyochelin in culture supernatants of *P. aeruginosa*, strains were grown in GGP medium (6). Antibiotics, when required, were added to the growth media at the following concentrations: tetracycline, 25 μg/ml for *Escherichia coli* and 100 μg/ml for *P. aeruginosa*; ampicillin, 100 μg/ml for *E. coli*; carbenicillin, 250 μg/ml for *P. aeruginosa*; kanamycin, 25 μg/ml for *E. coli*; and gentamicin, 10 μg/ml for *E. coli* and *P. aeruginosa*. To counterselect *E. coli* donor cells in matings with *P. aeruginosa*, chloramphenicol was used at a concentration of 10 μg/ml. 2-Aminobutyrate was purchased from Acros Organics.

Construction of plasmids and gene replacement mutants. All plasmids used in this study are listed in Table 1. The suicide plasmid pME6815 used to create an in-frame deletion in the chromosomal *pchC* gene was constructed as follows. A 0.9-kb XmaI-ScaI fragment carrying most of the *pchC* gene and the 5′ part of *pchB* was cloned into pBluescript (pBLS II KS) between XmaI and EcoRV sites. This plasmid was used as a template in an inverse PCR performed with PchC1 (5′-CTGACCCGGGCCTCGCACATCCCGAC; XmaI site underlined) and PchC2 (5′-GAAGCGGTCCTCGCGACCCGGG; XmaI site underlined) as the primers to amplify a 3.3-kb fragment. The PCR product was cleaved with XmaI and religated. From the resulting plasmid, a 0.32-kb XmaI-HindIII fragment was excised, ligated to a 0.65-kb fragment carrying the 5′ part of *pchC* (upstream of the XmaI site) and part of the flanking *pchD* gene, and cloned into the suicide vector pME3087 between BamHI and HindIII sites. The resulting suicide plasmid, pME6815, carried the flanking regions of *pchC* and a mutated *pchC* gene which lacked codons 53 to 218. For construction of chromosomal *pchC* deletions in *P. aeruginosa* PAO1, PALS128, and PALS128-6, pME6815 was mobilized from *E. coli* S17-1 to the recipient *P. aeruginosa* strains and chromosomally in-

TABLE 2. Effects of a *pchC* mutation on the concentrations of salicylate, Dha, and pyochelin in culture supernatants of *P. aeruginosa*[a]

Strain	Mutation	Plasmid	Genes carried	Salicylate concn (nmol/ml)[b]	Dha concn (nmol/ml)[b]	Pyochelin concn (nmol/ml)[b]
PAO1	Wild type			<8	113 ± 14	1,047 ± 36
PAO6339	Δ*pchC*			1,034 ± 263	45 ± 4	408 ± 13
PAO6339	Δ*pchC*	pME6831	*pchDC*	<8	89 ± 4	1,010 ± 83
PAO6339	Δ*pchC*	pME7538	$pchDC_{S90A}$	961 ± 218	10 ± 3	127 ± 41

[a] GGP medium (30 ml) (6) was inoculated with 0.3-ml portions of cultures grown in the same medium. After incubation at 37°C and 220 rpm for 38 h, supernatants were extracted and analyzed for salicylate, Dha, and pyochelin by HPLC as described previously (33). The values are the means ± standard deviations for three parallel experiments.

[b] Concentration in culture supernatant.

tegrated with selection for tetracycline resistance. Excision of the vector via a second crossing over was accomplished by enrichment for tetracycline-sensitive cells as described previously (50). The resulting *pchC* mutants, PAO6339, PAO6342, and PAO6357, were checked by Southern blot analysis (data not shown). To generate the same mutation in the P_{lac}-*entDpchDBA* expression plasmid pME6852, the 671-bp XmaI-EcoRV fragment of pME6178 was replaced by the corresponding XmaI-EcoRV fragment of pME6815 (173 bp) carrying the *pchC* deletion.

The thioesterase motif in the *pchC* gene of plasmid pME7538 was mutated by overlap extension (25) as follows. Two 0.3-kb fragments, fragments A and B, were amplified by PCR from chromosomal DNA of PAO1 by using primer PchCmut1 (5′-CTGCGCGAGCGCCTGCGCCGCGAGC) plus primer PchCmut6 (5′-CAGCGCCGCGCCGAGGGCGTGGCCGAACAG) and primer PchCmut5 (5′-GCGCTGTTCGGCCACGCCCTCGGCGCGGCG) plus primer PchCmut4 (5′-GACTTCCTCGTCGTGCTCGCCGAGG), respectively. PchCmut6 and PchCmut5 are complementary to each other to a large extent and specify a serine-to-alanine mutation in codon 90 (underlined). Equimolar amounts of PCR fragments A and B served as templates for the subsequent PCR amplification with primers PchCmut1 and PchCmut4. From the 0.6-kb PCR product obtained, an internal 0.3-kb XmaI-XhoI fragment was excised and used to replace the corresponding wild-type fragment in pME6831, resulting in the P_{lac}-$pchDC_{S90A}$ expression plasmid pME7538.

Plasmid pME6886, which expresses $pchEF_{C1606A/S1607A}G$ under P_{kan} control and carries a mutation in the PchF thioesterase motif, was constructed by replacing the 311-bp SalI-NotI fragment of pME6488 with the corresponding fragment from pPchFTE. All constructs carrying *pchC* in-frame deletions or point mutations in *pchC* or *pchF* were verified by sequence analysis.

DNA manipulation and cloning procedures. DNA manipulations were carried out as described by Sambrook et al. (35). Small-scale preparation of plasmid DNA was carried out by the cetyltrimethylammonium bromide method (12), and large-scale preparation was performed by using JetStar-Tips (Genomed, Basel, Switzerland). Chromosomal DNA was prepared by the method of Gamper et al. (16). DNA fragments were purified from agarose gels with a Gene Clean DNA extraction kit (Bio 101, La Jolla, Calif.). Transformation of *E. coli* and *P. aeruginosa* was carried out by electroporation (13). Nucleotide sequences were determined with a Big Dye terminator cycle sequencing kit and an ABI-PRISM 373 automatic sequencer (Applied Biosystems). Nucleotide sequences were analyzed with programs of the University of Wisconsin Genetics Computer Group package (version 9.1).

Identification of salicylate, Dha, and pyochelin in culture supernatants of *P. aeruginosa*. *P. aeruginosa* strains were grown in GGP medium for the time indicated. 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 H_3PO_4, and injected into an HPLC system as described previously (32). Compounds were identified by their retention times and UV spectra. Dha and salicylate were quantified at 256 and 237 nm, respectively. Pyochelin, which exists as a mixture of two interconvertible isomers, pyochelin I and pyochelin II (34), was quantified at 258 and 254 nm, respectively.

ATP-$^{32}PP_i$ exchange reactions. PchE and PchF proteins used in the assays were overexpressed and purified in *E. coli* and were converted to their phosphopantetheinyl holoforms as described previously (30, 31). ATP-PP_i exchange reactions were performed by using previously described procedures (31); the reaction mixtures contained 100 nM PchE and 100 nM PchF, respectively, and the amino acid tested at a concentration of 1 mM. Incubation was at 30°C for 20 min.

RESULTS

Inactivation of the PchC thioesterase decreases production of Dha and pyochelin in *P. aeruginosa*. To study the role of PchC, we constructed a chromosomal in-frame deletion in its gene and evaluated the impact of this on the production of Dha and pyochelin. As shown in Table 2, the production of both metabolites was significantly reduced in the *pchC* mutant PAO6339 compared to the production in the wild-type strain, PAO1, and as a consequence, large amounts of the precursor salicylate accumulated. When the PAO6339 mutant was complemented with plasmid pME6831 carrying *pchDC*, production of Dha and pyochelin was restored to the wild-type level. By contrast, plasmid pME7538, which is identical to pME6831 except for a point mutation (Cys90Ala) destroying the essential thioesterase motif of PchC, did not complement the PchC-negative phenotype of PAO6339 (Table 2). Note that, as reported previously, expression of *pchC* requires the presence of the upstream *pchD* gene in *cis* (40); *pchD* was therefore included in these plasmids.

PchC cannot release pyochelin from its thiotemplate, PchF, and is not essential for Dha formation. Since pyochelin is required for full transcriptional expression of the *pchDCBA* and *pchEFGHI* operons by positive autoregulation (32), it is important to express both operons under the control of constitutive promoters when the roles of individual genes in the pyochelin biosynthetic pathway are assessed. We therefore used a two-plasmid system described previously (32) with the deletion mutant PAO6331 lacking *pchDCBA*, *pchR*, and *pchEFGHI*. The *E. coli entD* gene encoding a phosphopantetheinyl transferase with relaxed specificity was coexpressed with the *pchDCBA* operon to ensure constitutive conversion of PchE and PchF to their phosphopantetheinyl holoforms (33). In this system *pchDCBA* and *pchEFG* are sufficient for pyochelin biosynthesis (33), but the amounts of pyochelin and Dha produced under these conditions are two- to threefold lower than the amounts in PAO1, probably because the kanamycin promoter used to drive *pchEFG* expression on pME6488 is weaker than the natural, pyochelin-inducible promoter of the *pchEFGHI* operon. As a consequence, salicylate is not quantitatively incorporated into Dha and pyochelin and accumulates to some extent in PAO6331 carrying pME6178 (*entD pchDCBA*) and pME6488 (*pchEFG*) (33) (Table 3). Pyochelin and Dha formation in PAO6331 carrying pME6852 (*entD pchDBA*) and pME6488 (*pchEFG*) was reduced threefold compared to the formation in PAO6331 carrying pME6178 and pME6488, confirming that *pchC* is important, but not essential, for Dha and pyochelin formation in *P. aeruginosa*. Increased amounts of salicylate accumulated in culture supernatants as a result of reduced incorporation into Dha and pyochelin.

To investigate a potential role of PchC in the release of pyochelin from PchF, we tested whether the *pchC* gene was able to

TABLE 3. Impact of thioesterases encoded by *pchC* and *pchF* on salicylate, Dha, and pyochelin formation in *P. aeruginosa*[a]

Strain	Chromosomal mutation	Plasmids	Genes carried	Salicylate concn (nmol/ml)[b]	Dha concn (nmol/ml)[b]	Pyochelin concn (nmol/ml)[b]
PAO1	Wild type			<8.0	60 ± 4.0	748 ± 25
PAO6331	Δ*pch*			<8.0	<1.2	<2
PAO6331	Δ*pch*	pME6178 + pME6488	*entD pchDCBA* + *pchEFG*	256 ± 29	18 ± 2.5	400 ± 74
PAO6331	Δ*pch*	pME6852 + pME6488	*entD pchDBA* + *pchEFG*	636 ± 72	5 ± 0.3	120 ± 15
PAO6331	Δ*pch*	pME6178 + pME6886	*entD pchDCBA* + $pchEF_{C1606A/S1607A}G$	872 ± 194	81 ± 19.7	<2
PAO6331	Δ*pch*	pME6852 + pME6886	*entD pchDBA* + $pchEF_{C1606A/S1607A}G$	757 ± 168	24 ± 1.9	<2

[a] GGP medium (30 ml) containing, when required, gentamicin (10 μg/ml) and tetracycline (100 μg/ml) was inoculated with 0.3-ml portions 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 by HPLC. The values are the means ± standard deviations for three parallel experiments.
[b] Concentration in culture supernatant.

functionally complement a double point mutation (Cys1606Ala/Ser1607Ala) blocking the thioesterase function of *pchF* (31). As illustrated in Table 3, strain PAO6331 carrying pME6852 (*entD pchDBA*) and pME6886 ($pchEF_{C1606A/S1607A}G$) was unable to produce pyochelin because of a lack of both thioesterases. When the *entD pchDCBA* construct pME6178 was used instead of pME6852, pyochelin was not produced either, indicating that PchC could not substitute for the mutated thioesterase function of PchF. Small amounts of Dha were detected in culture supernatants of the wild-type strain, PAO1, as well as in the recombinant strain lacking both thioesterases, probably as a result of nonspecific hydrolysis of the PchE-HPT thioester bond (31).

The recombinant strain lacking the *pchF*-encoded thioesterase (PAO6331 with pME6178 and pME6886) produced more Dha than the pyochelin-producing $pchF^+$ strain (PAO6331 carrying pME6178 and pME6488) produced (Table 3). Similarly, PAO6331 expressing *entD pchDCBA* and *pchE* produces more Dha than its pyochelin-producing counterpart produces (33). It seems that nonspecific hydrolysis of the PchE-HPT thioester bond increases when the pyochelin assembly line is interrupted because of mutations in *pchF*. The fact that more Dha was excreted in the presence of PchC than in the absence of PchC may be explained by a positive effect of PchC on the catalytic efficiency of PchE (see below). In conclusion, PchC does not seem to be essential for releasing Dha from its thiotemplate, PchE.

The PchC-negative phenotype is suppressed by addition of cysteine. We next evaluated a potential role of PchC as an editing enzyme in case of mischarging of PchE and/or PchF during Dha and pyochelin production. Two sources of mischarging were considered: (i) PchD could activate endogenous or exogenously added salicylate analogs and deliver these molecules to the aryl carrier protein domain of PchE; (ii) PchE and PchF could adenylate and subsequently load amino acids other than L-cysteine onto their peptidyl carrier protein (PCP) domains. In both cases, correct assembly of Dha and pyochelin would be hampered, and thus the role of PchC could be to recognize and eliminate wrongly charged molecules by thioester cleavage. The first hypothesis was tested by measuring the influence of PchC on pyochelin formation in a salicylate-requiring mutant to which salicylate was added at different concentrations (10 μM to 1 mM) together with a large excess of various salicylate analogs, such as *p*-aminosalicylate, benzoate, *p*-hydroxybenzoate, *p*-aminobenzoate, or anthranilate. Pyochelin formation was measured semiquantitatively on CAS agar, which allows the production of siderophores to be visualized by the formation of an orange halo around the colonies (38). Since the siderophore pyoverdin would interfere in this assay, we used a *pvdB* (pyoverdin-negative) *pchA* strain, PALS128-6, and its Δ*pchC* derivative PAO6357. None of the salicylate analogs tested reduced the diameter of the halo around the bacterial colonies, regardless of the presence of *pchC*, suggesting that pyochelin formation was not inhibited (data not shown). If the role of PchC were indeed to remove an endogenous mischarged salicylate analog from the aryl carrier protein domain of PchE, then an excess of salicylate should competitively inhibit mischarging and thereby suppress the negative effect of a *pchC* mutation. We therefore tested whether the PchC-negative phenotype could be suppressed by salicylate addition. Whereas pyochelin production was stimulated somewhat in the PchC-positive strain PALS128, this was not the case in the *pchC* mutant PAO6357 (Fig. 2). Pyochelin formation in the corresponding *pchA* mutants depended on salicylate addition, confirming that salicylate was readily taken up (Fig. 2). In the salicylate-requiring *pchC* mutant PAO6357 the concentration of pyochelin formed remained below 200 μM, even when the culture medium was amended with 1 mM salicylate. Taken together, these results do not support the first hypothesis.

To evaluate the second hypothesis, we tested whether the PchC-negative phenotype could be suppressed by addition of

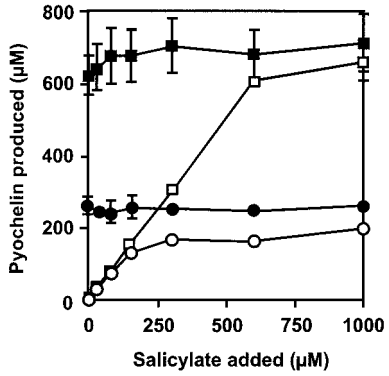

FIG. 2. Pyochelin formation by the pyoverdin-negative *P. aeruginosa* strains PALS128 (■), PAO6342 (*pchC*) (●), PALS128-6 (*pchA*) (□), and PAO6357 (*pchA pchC*) (○) in GGP medium amended with different amounts of salicylate. The growth conditions, extraction, and analysis of pyochelin were as described in Table 2, footnote *a*. Means ± standard deviations for three parallel experiments are shown.

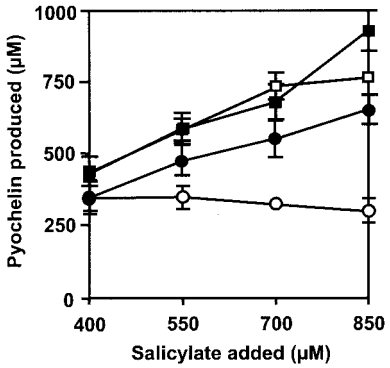

FIG. 3. Effect of cysteine on pyochelin formation by *P. aeruginosa* strains. In GGP medium amended with different amounts of salicylate, pyochelin formation was measured in strain PALS128-6 (*pvdB pchA*) grown without cysteine (□) and with 2 mM L-cysteine (■), as well as in strain PAO6357 (*pvdB pchA pchC*) grown without cysteine (○) and with 2 mM L-cysteine (●). The growth conditions, extraction, and analysis of pyochelin were as described in Table 2, footnote *a*. Means ± standard deviations are shown.

L-cysteine to GGP medium, which is rich in amino acids except for L-cysteine (15). As illustrated in Fig. 3, addition of 2 mM L-cysteine to a medium containing salicylate at concentrations between 400 and 850 μM enabled strain PAO6357 (*pvdB pchA pchC*) to produce almost the same amounts of pyochelin as its *pchC*$^+$ parent PALS128-6 produced. By contrast, we confirmed that in the absence of L-cysteine, the *pchC*-negative mutant produced significantly less siderophore than the *pchC*-positive strain produced. Similar results were obtained when the growth medium was amended with 5 mM L-cysteine (data not shown). These data are in agreement with a role of PchC in the removal of wrongly charged substrates from the PCP domains of PchE and/or PchF.

Which amino acid might be misloaded onto these enzymes in vivo? ATP-PP_i exchange assays were performed to test whether purified PchE and PchF could adenylate amino acids other than L-cysteine. Both enzymes were highly specific for L-cysteine; of all the natural amino acids tested, only L-serine was activated by PchE and PchF at levels above background levels (Fig. 4). PchE was found to load radiolabeled L-serine instead of L-cysteine onto the PCP1 domain in vitro, but no subsequent condensation products could be detected (data not shown).

2-Aminobutyrate strongly inhibits Dha and pyochelin formation in a *pchC* mutant. In the ATP-PP_i exchange assays for PchE and PchF, the cysteine analog 2-aminobutyrate was activated with good efficiency (Fig. 4). Previously, 2-aminobutyrate had been shown to be activated and loaded instead of L-cysteine onto the PCP domain of HMWP1 (high-molecular-weight protein 1), a PchE-like nonribosomal peptide synthetase involved in the biosynthesis of the siderophore yersiniabactin in *Yersinia pestis* (22). We therefore tested whether this analog interfered with Dha and pyochelin formation by *P. aeruginosa* in vivo. When 2-aminobutyrate was added to the growth medium at a final concentration of 5 mM, pyochelin formation was very strongly decreased in *pchC* mutant PAO6339, and no Dha was detected (Table 4). In the absence of pyochelin, the expression of the *pchDCBA* and *pchEFG* genes required for salicylate, Dha, and pyochelin formation is low because the positive feedback regulation operating in this biosynthetic pathway is disrupted (32). It is therefore not surprising that addition of 2-aminobutyrate to the *pchC* mutant

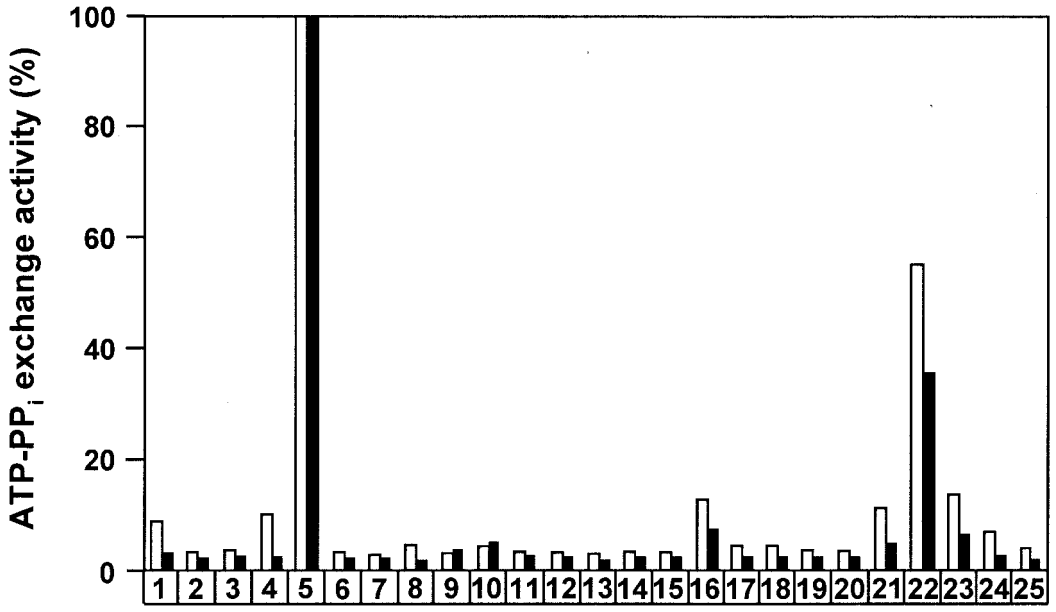

FIG. 4. Relative activation of amino acids by PchE (open bars) and PchF (solid bars). ATP-PP_i exchange activities were measured with proteinogenic amino acids (bars 1 to 20) and nonproteinogenic amino acids (bars 21 to 24) at a concentration of 1 mM. The highest exchange activity measured with L-cysteine was defined as 100%. The standard deviations of values in this assay were ≤20%. Bars 1, L-alanine; bars 2, L-arginine; bars 3, L-asparagine; bars 4, L-aspartate; bars 5, L-cysteine; bars 6, L-glutamate; bars 7, L-glutamine; bars 8, L-glycine; bars 9, L-histidine; bars 10, L-isoleucine; bars 11, L-leucine; bars 12, L-lysine; bars 13, L-methionine; bars 14, L-phenylalanine; bars 15, L-proline; bars 16, L-serine; bars 17, L-threonine; bars 18, L-tryptophan; bars 19, L-tyrosine; bars 20, L-valine; bars 21, L-homoserine; bars 22, L-2-aminobutyrate; bars 23, *S*-methyl-L-cysteine; bars 24, DL-allylglycine; bars 25, no amino acid added.

TABLE 4. Impact of 2-aminobutyrate on salicylate, Dha, and pyochelin formation

Strain	Genotype	Growth medium[a]	Salicylate concn (nmol/ml)[b]	Dha concn (nmol/ml)[b]	Pyochelin concn (nmol/ml)[b]
PAO1	Wild type	GGP	<8.0	87 ± 16	869 ± 111
PAO1	Wild type	GGP + 2-ABA	<8.0	62 ± 10	552 ± 28
PAO6339	Δ*pchC*	GGP	708 ± 100	70 ± 4	422 ± 57
PAO6339	Δ*pchC*	GGP + 2-ABA	87 ± 40	<1.2	40 ± 22

[a] GGP medium (30 ml) containing, where indicated, DL-2-aminobutyrate (2-ABA) at a final concentration of 5 mM, was inoculated with 0.1-ml portions of cultures grown in GGP medium. After incubation at 37°C and 220 rpm for 38 h, supernatants were extracted and analyzed for salicylate, Dha, and pyochelin by HPLC. The values are the means ± standard deviations for three parallel experiments.

[b] Concentration in culture supernatant.

also reduced the production of salicylate (Table 4) as the amount of pyochelin produced under these conditions was not sufficient to allow high-level expression of the *pch* biosynthetic genes. By contrast, addition of 5 mM 2-aminobutyrate to a culture of the wild-type strain, PAO1, had only a modest effect on the production of pyochelin and Dha (Table 4), supporting the hypothesis that the PchC type II thioesterase is able to remove 2-aminobutyrate from PchE and PchF.

DISCUSSION

In this study, we demonstrated that PchC, an external type II thioesterase encoded by the pyochelin gene cluster, is important for optimal production of the siderophore pyochelin and of its precursor, the antibiotic Dha. Type II thioesterase genes have been identified in many gene clusters specifying the biosynthesis of nonribosomal peptides (3, 7, 26, 40) and polyketides (5, 18, 49). Mutational loss of these genes generally results in a decreased amount of product formed, and in vitro, type II thioesterases can regenerate misacylated thiol groups of the 4′-phosphopantetheine cofactors attached to the PCP domains of nonribosomal peptide synthetases (37). In vivo, misacylated enzymes may be formed either when a wrong amino acid is activated and loaded onto the PCP domain or when the 4′-phosphopantetheinyl transferase uses acyl coenzyme A instead of free coenzyme A as the 4′-phosphopantetheine donor (37). Similarly, during polyketide biosynthesis, type II thioesterases are thought to be responsible for hydrolyzing aberrant cofactor-bound acyl groups (19).

Our data support such a proofreading role for PchC in *P. aeruginosa*. The production of Dha and pyochelin was strongly decreased in the *pchC*-negative mutant but not in the wild type, especially when the growth medium was amended with 2-aminobutyrate (Table 4). This cysteine analog was found to be activated by both PchE and PchF and thus could compete with cysteine for loading onto the PCP domains of these nonribosomal peptide synthetases. If 2-aminobutyrate instead of L-cysteine were attached to PchE and PchF, the formation of thiazoline rings would not occur and Dha and pyochelin assembly would be prevented. In the wild type, the most likely function of the PchC enzyme is to remove the wrongly charged substrate, thereby regenerating peptide synthetase activity. To our knowledge, this is the first evidence for a proofreading role of a type II thioesterase in vivo.

There are two indications that a wrong substrate loaded onto PchE causes the enzyme to stall rather than to form aberrant products. (i) Under the conditions used in this study, strain PAO1 converted salicylate quantitatively to Dha and pyochelin. In the *pchC* mutant, however, which produced less Dha and pyochelin, large amounts of salicylate accumulated (Tables 2 to 4), indicating that little, if any, salicylate is coupled to substrates other than L-cysteine by PchE. (ii) Although PchE was able to load L-serine instead of L-cysteine onto the PCP1 domain, no subsequent condensation products were observed in vitro. In addition, HPLC analysis of culture supernatants of the *pchC* mutant (Tables 2 to 4) did not reveal aberrant condensation products of salicylate.

The role of PchC in removing wrongly charged molecules from the PCP domain of PchE and possibly the PCP domain of PchF is corroborated by the fact that addition of L-cysteine to the growth medium largely suppressed the adverse effect of a *pchC* mutation, probably by ensuring that L-cysteine rather than its potential natural competitors (e.g., L-serine) is charged on PchE (and PchF). A stimulating effect of L-cysteine on pyochelin biosynthesis has been noticed previously (2, 15) and may also be important in the wild type when the amount of salicylate available is greater than the amount of L-cysteine. In the experiments shown in Fig. 2 and 3, the amount of salicylate was controlled by exogenous salicylate added to a salicylate-requiring mutant.

Given the fact that in vitro some type II thioesterases hydrolyze PCP-bound peptides (37), we considered the possibility that PchC might detach the biosynthetic intermediate Dha and/or participate in the release of the final product, pyochelin, from the thiotemplate. Whereas it is clear from the results shown in Table 3 that PchC does not cleave the pyochelin-PchF thioester bond, we cannot entirely rule out involvement of PchC in Dha release from PchE since the production of this antibiotic compound was greater in a *pchC*-positive background than in the absence of *pchC*. However, it seems more likely that the editing function of PchC accounts for this effect, as some Dha was formed even in the absence of thioesterase activities encoded by both *pchC* and *pchF* (Table 3).

In conclusion, we showed that the PchC thioesterase is not essential for the release of Dha from PchE and does not participate in the release of pyochelin from PchF. Our data are consistent with PchC having a quality control function in pyochelin biosynthesis by removing wrongly charged molecules from the PCP domains of PchE and PchF. Further support for this conclusion comes from a recent study (51) which demonstrated that a type II thioesterase removes misloaded amino acids from tyrocidine synthetase prior to peptide bond formation and thereby restores the activity of enzyme modules stalled with unprocessed aminoacyl intermediates.

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