Dihydroaeruginosic acid synthetase and pyochelin synthetase, products of the *pchEF* genes, are induced by extracellular pyochelin in *Pseudomonas aeruginosa*

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The siderophore pyochelin of *Pseudomonas aeruginosa* is derived from one molecule of salicylate and two molecules of cysteine. Two cotranscribed genes, *pchEF*, encoding peptide synthetases have been identified and characterized. *pchE* was required for the conversion of salicylate to dihydroaeruginosate (Dha), the condensation product of salicylate and one cysteine residue and *pchF* was essential for the synthesis of pyochelin from Dha. The deduced PchE (156 kDa) and PchF (197 kDa) proteins had adenylation, thiolation and condensation/cyclization motifs arranged as modules which are typical of those peptide synthetases forming thiazoline rings. The *pchEF* genes were coregulated with the *pchDCBA* operon, which provides enzymes for the synthesis (PchBA) and activation (PchD) of salicylate as well as a putative thioesterase (PchC). Expression of a translational *pchE*'-'*lacZ* fusion was strictly dependent on the PchR regulator and was induced by extracellular pyochelin, the end product of the pathway. Iron replete conditions led to Fur (ferric uptake regulator)-dependent repression of the *pchE*'-'*lacZ* fusion. A translational *pchD*'-'*lacZ* fusion was also positively regulated by PchR and pyochelin and repressed by Fur and iron. Thus, autoinduction by pyochelin (or ferric pyochelin) and repression by iron ensure a sensitive control of the pyochelin pathway in *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, siderophore, pyochelin, non-ribosomal peptide synthesis, autoinduction

INTRODUCTION

Under iron-limiting growth conditions, *Pseudomonas aeruginosa* produces two major siderophores, pyoverdine (Meyer & Abdallah, 1978; Cox & Adams, 1985) and pyochelin (Cox, 1980; Rinehart *et al.*, 1995). The hydroxamate siderophore pyoverdine has a greater affinity for Fe(III) than has pyochelin (Cox & Graham, 1979). Siderophore-mediated iron uptake requires the outer-membrane receptors FpvA (Poole *et al.*, 1993) and

FptA (Ankenbauer & Quan, 1994) which recognize Fe(III)pyoverdine and Fe(III)pyochelin complexes, respectively. Salicylate, the metabolic precursor of pyochelin (Ankenbauer & Cox, 1988), is a strong iron chelator in the absence of inorganic phosphate (Martell & Smith, 1977; Ratledge *et al.*, 1974) and can also promote iron uptake in *P. aeruginosa* (Meyer, 1992). It is not known whether a receptor protein is involved in salicylate-mediated iron uptake. Pyoverdine and pyochelin both contribute to the virulence of the opportunistic pathogen *P. aeruginosa* (Meyer *et al.*, 1996; Cox, 1982). In particular, pyochelin enhances hydroxyl radical formation and epithelial injury when Fe(III) and pyocyanin are present (Britigan *et al.*, 1997).

Excess iron represses the biosynthesis of pyoverdine, salicylate and pyochelin (Cox & Graham, 1979; Cox & Adams, 1985; Visca *et al.*, 1993). Repression occurs via the Fur (ferric uptake regulator) protein, which, in the

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Abbreviations: CTAB, cetyltrimethylammonium bromide; Dha, dihydroaeruginosic acid (dihydroaeruginosate).

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presence of iron, binds to the promoters of *pvdS* and *pchR* (Leoni *et al.*, 1996; Ochsner *et al.*, 1995). The PvdS and PchR proteins positively regulate the biosynthesis of pyoverdinin and pyochelin, respectively. PvdS belongs to a class of alternative sigma factors (Miyazaki *et al.*, 1995; Cunliffe *et al.*, 1995), whereas PchR is an AraC-like regulatory protein (Heinrichs & Poole, 1993). In the presence of pyochelin, PchR activates the expression of *fptA* and *pchR*, whereas in the absence of pyochelin, PchR appears to act as a repressor of the receptor gene and of its own gene (Heinrichs & Poole, 1996).

The biosynthesis of pyochelin requires the Fur-regulated *pchDCBA* operon, which lies next to the divergently transcribed *pchR* gene in *P. aeruginosa* (Serino *et al.*, 1997). The PchA and PchB enzymes convert chorismate to salicylate via isochorismate (Serino *et al.*, 1995; C. Gaille, C. Reimmann & D. Haas, unpublished data). The deduced PchD protein resembles adenylate-forming enzymes and probably activates salicylate at the carboxyl group. The PchC protein shows homology to thioesterases (Serino *et al.*, 1997). Expression of the *pchDCBA* operon of *P. aeruginosa* is also necessary for the formation of the antibiotic and iron-chelator dihydroaeruginosic acid (dihydroaeruginosate; Dha) (Serino *et al.*, 1997). Dha is the condensation product of salicylate and cyclized D-cysteine (Carmi *et al.*, 1994). Formally, pyochelin could be derived from Dha by condensation (amide bond formation), cyclization, reduction and methylation of a second cysteine residue (Rinehart *et al.*, 1995; Serino *et al.*, 1997). Thus, Dha would be the precursor of pyochelin in a linear pathway. However, considering the available evidence, the alternative possibility exists that Dha might be the product of a branch of the salicylate–pyochelin pathway (Serino *et al.*, 1997).

Here we describe two new genes, *pchE* and *pchF*, which encode peptide synthetases involved in the sequential formation of Dha and pyochelin in *P. aeruginosa* and we show that the expression of these genes depends on the presence of extracellular pyochelin. A model is presented in which each step of a linear salicylate–Dha–pyochelin pathway is assigned to specific functions of the *pchDCBA* and *pchEF* gene products.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Bacteria were usually grown on nutrient agar and in nutrient yeast broth (Stanisich & Holloway, 1972) at 37 °C (*Escherichia coli* and *Pseudomonas aeruginosa*) or at 30 °C (*Pseudomonas fluorescens*). Iron-depleted Casamino acids medium (DCAA medium; Visca *et al.*, 1993) was used to study the regulation of the *pchE* gene by iron. Salicylate, Dha and pyochelin were quantified in culture supernatants of *P. aeruginosa* and *P. fluorescens* strains grown in GGP medium containing glycerol and proteose-peptone (Carmi *et al.*, 1994), in which limited iron availability results in the production of large amounts of Dha and pyochelin. Antibiotics, when required, were added to the growth media at the following concentrations: tetracycline, 25 µg ml⁻¹ for *E. coli* and *P. fluorescens* and 100 µg ml⁻¹ for *P. aeruginosa*; ampicillin, 100 µg ml⁻¹ for *E. coli*; carbenicillin,

250 µg ml⁻¹ for *P. aeruginosa*; spectinomycin, 50 µg ml⁻¹ for *E. coli* and 1000 µg ml⁻¹ for *P. aeruginosa*. To counterselect *E. coli* donor cells in matings with *P. aeruginosa* or *P. fluorescens*, chloramphenicol was used at 10 µg ml⁻¹.

Construction of plasmids and gene replacement mutants. All plasmids used in this study are described in Table 1. To construct plasmid pME3384, a *Bam*HI (restriction site underlined) DNA linker (GGATCCGGTGATTGATTGAGCAAGCTTGCTCAATCAATCACCGGATCC) termed ω and carrying translation stops (bold type) in all three reading frames and in both orientations, was inserted at the *Bgl*II site of *pchR*. To construct mutations in the chromosomal *pch* genes of *P. aeruginosa*, derivatives of the suicide plasmids pME3087 and pME3088 (Voisard *et al.*, 1994) 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 (Ye *et al.*, 1995). All gene replacement mutants were checked by Southern blot analysis (data not shown).

DNA manipulation and sequencing. Standard DNA manipulations were carried out as described by Sambrook *et al.* (1989). Small-scale preparations of plasmid DNA were obtained by the cetyltrimethylammonium bromide (CTAB) method (Del Sal *et al.*, 1988) and large-scale preparations were performed using Qiagen-tip 100 columns. Chromosomal DNA was prepared according to Gamper *et al.* (1992). Restriction and DNA modifying enzymes were used following the instructions of the manufacturers. DNA fragments were purified from agarose gels with a GeneClean DNA extraction kit (Bio 101). Transformation of *E. coli* and *P. aeruginosa* was done by the standard CaCl₂ procedure (Sambrook *et al.*, 1989) or by electroporation (Farinha & Kropinski, 1990). The complete nucleotide sequence of *pchE* and *pchF* was determined on both strands by a joint effort of Euro Sequence Gene Service (ESGS; Evry, France), Genome Express SA (Grenoble, France) and this laboratory, except for a 2.4 kb *Eco*RI fragment in *pchE* (Fig. 1) which was sequenced on only one strand and compared to the DNA sequence already available from the *P. aeruginosa* genome sequencing project (<http://www.pseudomonas.com>). Nucleotide sequences were analysed with the programs FASTA, GAP, BESTFIT, MOTIFS, ISOELECTRIC, PEPTIDESORT and CODONPREFERENCE using the Genetics Computer group (GCG) package.

Protein expression. *pchE* and *pchF* were cloned under the control of the T7 promoter in pALTER-Ex2 (Fig. 2). In nutrient yeast broth cultures of *E. coli* JM109(DE3) harbouring these constructs, the chromosomal T7 RNA polymerase was induced at a cell density of OD₆₀₀ = 0.5 for 2 h by the addition of 1 mM IPTG. Rifampicin was then added at 200 µg ml⁻¹. Cells were harvested 2 h later and proteins were analysed on a 7.5% (w/v) SDS-polyacrylamide gel as described previously (Serino *et al.*, 1995).

Identification of salicylate, Dha and pyochelin in culture supernatants of *P. aeruginosa* and *P. fluorescens*. Bacterial strains were grown in GGP medium to stationary phase (OD₆₀₀ ≈ 7–11, measured in a Pharmacia Ultrospec III spectrophotometer). For HPLC analysis, ethyl acetate extracts of culture supernatants acidified to pH 1–2 were dried by evaporation and dissolved in 200 µl 60% (v/v) methanol and 10 mM H₃PO₄. An aliquot of 100 µl was injected into an HPLC system (Serino *et al.*, 1995). A binary gradient consisting of solvent A (10 mM H₃PO₄) and solvent B (95% methanol in 10 mM H₃PO₄) was used as follows: 0–29 min with 20–83% solvent B and 2 min with 83–100% solvent B. Elution was carried out at room temperature with a flow rate of 0.8 ml

Table 1. Strains and plasmids

Strain/plasmid	Relevant characteristics/genotype	Source/reference
<i>E. coli</i>		
DH5 α	<i>recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1 Δ(lacZYA-argF) U169 (ϕ80dlacZΔM15)</i>	Sambrook <i>et al.</i> (1989)
JM109(DE3)	Chromosomal T7 polymerase	Promega
S17-1	<i>thi pro hsdR recA</i> ; chromosomal RP4 (Tra ⁺ Tc ^s Km ^s Ap ^s)	Simon <i>et al.</i> (1983)
<i>P. aeruginosa</i>		
PAO1	Wild type	ATCC 15692
PAO1A4	<i>fur-4</i>	Barton <i>et al.</i> (1996)
PAO6274	<i>pchR::ω</i> (insertion of a 42 bp linker in the <i>Bgl</i> III site of <i>pchR</i>)	This work (Fig. 1)
PAO6295	<i>pchF::ΩSm/Sp</i>	This work (Fig. 1)
PAO6296	Chromosomal insertion of Ω Sm/Sp at the <i>Bam</i> HI site downstream of <i>pchF</i>	This work (Fig. 1)
PAO6297	Chromosomal deletion of a 1 kb <i>EcoRV-Pvu</i> II fragment in <i>pchBA</i>	This work
PAO6310	Chromosomal deletion of a 2.4 kb <i>EcoRI</i> fragment in <i>pchE</i> (= <i>pchEΔEcoRI</i>)	This work (Fig. 1)
PAO6312	<i>pchEΔEcoRI::ΩSm/Sp</i>	This work (Fig. 1)
PAO6323	Chromosomal deletion of two <i>Sph</i> I fragments (0.55 and 2.4 kb) in <i>pchF</i>	This work (Fig. 1)
PALS128-17	<i>pudB pchB</i> or <i>pchA</i>	Serino <i>et al.</i> (1995)
<i>P. fluorescens</i>		
P3	Wild type, naturally deficient in salicylate, Dha and pyochelin production	Voisard <i>et al.</i> (1989)
Plasmids		
pALTER-Ex2	T7 expression vector, Tc ^r ; p15a replicon	Promega
pHP45 Ω	Ω Sm/Sp containing plasmid; Ap ^r , Sm/Sp ^r	Prentki & Krisch (1984)
pME3087	Suicide vector; Tc ^r ; ColE1 replicon; <i>EcoRI KpnI BamHI XbaI PstI SphI HindIII</i> polylinker	Voisard <i>et al.</i> (1994)
pME3088	Suicide vector; Tc ^r ; ColE1 replicon; <i>EcoRI KpnI DraII XhoI HindIII</i> polylinker	Voisard <i>et al.</i> (1994)
pME3300	pLAFR3 carrying the structural genes for salicylate, Dha and pyochelin biosynthesis in PAO1	Serino <i>et al.</i> (1995, 1997) (Fig. 1)
pME3302	Derivative of pME3300 with a 3.3 kb <i>Bam</i> HI deletion; carries the structural genes for salicylate and Dha biosynthesis in PAO1	This work (Fig. 1)
pME3384	pME3087 carrying a 1.8 kb <i>EcoRI-SalI</i> fragment containing the <i>pchR</i> gene interrupted by a 42 bp linker (ω) at the <i>Bgl</i> III site	This work
pME6015	pVS1/p15A shuttle vector for construction of translational <i>lacZ</i> fusions; Tc ^r	S. Heeb & U. Schnider, unpublished
pME6122	Translational <i>pchD'-lacZ</i> fusion on pKT240	Serino <i>et al.</i> (1997)
pME6156	pME3088 carrying part of the <i>pchDCBA</i> operon on a 2.3 kb <i>KpnI-SalI</i> fragment, but lacking a 1 kb internal <i>EcoRV-Pvu</i> II fragment in <i>pchBA</i>	This work
pME6406	pME3088 carrying a 5.8 kb <i>EcoRI</i> fragment containing 3.8 kb of the chromosomal region downstream of <i>pchF</i> with a 2 kb Ω Sp/Sm insertion at <i>Bam</i> HI	This work
pME6407	pME3088 carrying a 10.55 kb <i>EcoRI</i> fragment containing <i>pchF</i> , with a 2 kb Ω Sp/Sm insertion at <i>Bam</i> HI	This work
pME6413	pUCPKS carrying an 8.55 kb <i>EcoRI</i> fragment containing <i>pchF</i>	This work (Fig. 1)
pME6421	pME3088 carrying a 5 kb <i>Bgl</i> III- <i>Clal</i> fragment with 5' and 3' ends of <i>pchE</i> but lacking an internal <i>EcoRI</i> fragment of 2.4 kb	This work
pME6422	pME3088 carrying a 7 kb <i>Bgl</i> III- <i>Clal</i> fragment with an internal <i>EcoRI</i> deletion in <i>pchE</i> (see pME6421) and, in addition, an Ω Sp/Sm cassette at the remaining <i>EcoRI</i> site	This work
pME6425	pME6015 carrying a 0.5 kb <i>PstI</i> fragment containing the promoter region of <i>pchE</i> (translational <i>pchE'-lacZ</i> fusion)	This work
pME6427	pME3088 carrying a 5.6 kb <i>EcoRI</i> fragment containing the 5' and 3' ends of <i>pchF</i> but lacking the internal <i>Sph</i> I fragments of 0.55 and 2.4 kb	This work (Fig. 1)
pME6432	pUCPSK carrying a 7.4 kb <i>SmaI-KpnI</i> fragment containing <i>pchF</i>	This work (Fig. 1)
pME6434	pUCPSK carrying a 5.75 kb <i>Bgl</i> III- <i>MunI</i> fragment containing <i>pchE</i>	This work (Fig. 1)
pME6436	pUCPSK carrying a 12.2 kb <i>Bgl</i> III- <i>EcoRI</i> fragment containing <i>pchE</i> and <i>pchF</i>	This work (Fig. 1)
pME6438	pUCPSK carrying an 11.3 kb <i>Bgl</i> III- <i>KpnI</i> fragment containing <i>pchE</i> and <i>pchF</i>	This work (Fig. 1)
pME6441	pME6443 derivative with an in-frame <i>PstI</i> deletion of 2.4 kb in <i>pchE</i>	This work (Fig. 2)
pME6442	pME6441 derivative with an in-frame <i>SphI</i> deletion of 2.95 kb in <i>pchF</i>	This work (Fig. 2)
pME6443	pALTER-Ex2 carrying <i>pchEF</i> on an 11.3 kb <i>NcoI-EcoRI</i> fragment	This work (Fig. 2)
pUCPKS, SK	ColE1-pRO1600 shuttle vectors; Ap ^r	Watson <i>et al.</i> (1996)

min⁻¹. Compounds were identified by their retention times and UV spectra. Salicylate and Dha were eluted at 21.7 and 23.2 min, respectively. Pyochelin isomerizes spontaneously to

a 3:1 mixture of pyochelin I and II (Rinehart *et al.*, 1995). Pyochelin I and II were eluted at 27.6 and 29.7 min, respectively. Dha, salicylate, pyochelin I and II were quantified

Fig. 2. Expression of *pchE* and *pchF* of *P. aeruginosa*. (a) The *pchEF* genes were inserted entirely or in part into the T7 expression vector pALTER-Ex2 using *Nco*I and a pBluescript-derived *Hind*III linker. Note that in pME6441, pME6442 and pME6443 the ATG start codon of *pchE* was placed downstream of a ribosome-binding site provided by the expression vector. This strong ribosome-binding site favours the expression of *pchE* over that of *pchF*. Moreover, T7 RNA polymerase moves much faster than the translating ribosomes (Lopez & Dreyfus, 1996). For this reason, the long, distal *pchF* transcript might be particularly prone to degradation by RNases. As a consequence, the PchE protein band is stronger than the PchF band. The restriction enzymes are indicated as follows: E, *Eco*RI; H, *Hind*III; N, *Nco*I; P, *Pst*I; S, *Sph*I. (b) SDS-polyacrylamide gel (7.5%) of the proteins encoded by *pchEF*. ΔPchE and ΔPchF indicate truncated forms of PchE and PchF, respectively. Cultures of *E. coli* JM109(DE3) carrying the above plasmids were induced with IPTG.

with the TGA stop codon of *pchE*. The putative ribosome-binding site (AGAG) of *pchF* is located at the 3' end of *pchE*. The predicted proteins PchE and PchF consist of 1438 (156420 Da; pI 5.17) and 1809 aa (197201 Da; pI 5.99), respectively. In reasonable agreement with these molecular mass values, polypeptides having apparent molecular masses of about 160 and 180 kDa could be assigned to PchE and PchF, respectively, after overexpression in *E. coli* of *pchEF* from the T7 promoter on plasmid pME6443 (Fig. 2). Plasmid

pME6441 expressed PchF and a truncated form of PchE, due to an in-frame deletion of two *Pst*I fragments in *pchE*. Truncated forms of both PchE and PchF were expressed from pME6442, carrying an additional in-frame deletion of two *Sph*I fragments in *pchF* (Fig. 2). The PchE and PchF proteins could not be visualized when the T7 expression vector pALTER-Ex2 alone was used in a control experiment (Fig. 2) or when strain PAO1 was grown under iron limitation (data not shown).

The deduced PchE and PchF proteins are similar to peptide synthetases

The deduced amino acid sequences of PchE and PchF showed similarities to various peptide synthetases of bacterial origin. The greatest similarity was found to the High-Molecular-Weight Protein 2 (HMWP2), the *irp2* gene product of *Yersinia enterocolitica* (37 and 31% identical amino acids in the full length PchE and PchF, respectively) and to the AngR protein of *Vibrio anguillarum* (31 and 27% identical amino acids in PchE and PchF, respectively). HMWP2 and AngR are involved in non-ribosomal biosynthesis of the thiazoline-containing siderophores yersiniabactin and anguibactin, respectively (Guilvout *et al.*, 1993; Tomalsky *et al.*, 1993; Bearden *et al.*, 1997; Pelludat *et al.*, 1998).

Because of these homologies and the characteristic modular structure of PchE and PchF (see Discussion), we propose that *pchE* and *pchF* encode peptide synthetases which could be involved in the condensation of salicylate with two molecules of cysteine.

Roles of *pchE* and *pchF* in the sequential biosynthesis of Dha and pyochelin

To study the biological functions of *pchE* and *pchF*, we constructed chromosomal in-frame deletions in these genes via marker exchange using the suicide constructs pME6421 and pME6427 (Table 1) and strains were tested in GGP medium for production of salicylate, Dha and pyochelin. In the *pchE* mutant PAO6310 (Fig. 1), the 2.1 kb *Eco*RI deletion completely abolished the formation of both Dha and pyochelin and resulted in salicylate accumulation (Table 2). The mutant PAO6310 could be complemented with plasmid pME6434 carrying the *pchE* gene behind the *lac* promoter (Fig. 1; Table 2). We conclude that the *pchE* gene is necessary for the synthesis of Dha and pyochelin. In fact, the complemented mutant produced four times more Dha than did the wild-type PAO1 (Table 2), suggesting that the primary function of PchE could be to allow Dha synthesis. An in-frame deletion in *pchF* was constructed by deletion of two *Sph*I fragments of 0.55 and 2.4 kb. Transfer of this mutation to the chromosome resulted in the *pchF* mutant PAO6323 (Fig. 1) which still formed salicylate and Dha, but was no longer able to synthesize pyochelin (Table 2), indicating that *pchF* is essential for pyochelin biosynthesis. Introduction of plasmid pME6432 (Fig. 1) expressing the *pchF* gene from a vector promoter fully restored pyochelin formation in

Table 2. Effects of *pchR*, *pchBA*, *pchE* and *pchF* mutations on the synthesis of salicylate, Dha and pyochelin in *P. aeruginosa*

GGP medium (30 ml) containing, when required, carbenicillin (250 µg ml⁻¹) was inoculated with 0.3 ml culture grown in the same medium. After incubation at 37 °C and 220 r.p.m. for 33 h, supernatants were extracted and analysed for salicylate, Dha and pyochelin by HPLC (see Methods). The values given represent the means ± SD of three independent experiments.

Strain	Mutation	Plasmid	Gene(s) carried	Salicylate (nmol ml ⁻¹)*	Dha (nmol ml ⁻¹)*	Pyochelin (nmol ml ⁻¹)*
PAO1	Wild type	—	—	1.4 ± 0.2	93.6 ± 25.8	838.8 ± 90.6
PAO6310	<i>pchE</i> (non-polar)	—	—	42.1 ± 3.4	< 1.2	< 2.0
PAO6310	<i>pchE</i> (non-polar)	pME6434	<i>pchE</i>	2.3 ± 0.5	381.1 ± 38.1	615.5 ± 125.1
PAO6312	<i>pchE</i> (polar)	—	—	38.8 ± 1.1	< 1.2	< 2.0
PAO6312	<i>pchE</i> (polar)	pME6434	<i>pchE</i>	114.8 ± 30.1	139.2 ± 32.6	< 2.0
PAO6312	<i>pchE</i> (polar)	pME6436	<i>pchEF</i>	1.8 ± 0.6	12.7 ± 2.8	574.0 ± 91.9
PAO6312	<i>pchE</i> (polar)	pME6438	<i>pchEF</i>	1.3 ± 0.3	37.1 ± 6.5	< 2.0
PAO6323	<i>pchF</i> (non-polar)	—	—	43.2 ± 1.4	3.7 ± 0.6	< 2.0
PAO6323	<i>pchF</i> (non-polar)	pME6432	<i>pchF</i>	45.3 ± 11.0	72.1 ± 16.6	704.3 ± 24.1
PAO6295	<i>pchF</i> (polar)	—	—	43.2 ± 3.9	3.3 ± 0.8	< 2.0
PAO6295	<i>pchF</i> (polar)	pME6413	<i>pchF</i>	1118.0 ± 184.5	128.0 ± 18	554.7 ± 71.4
PAO6295	<i>pchF</i> (polar)	pME6432	<i>pchF</i>	308.8 ± 37.9	17.6 ± 3.4	< 2.0
PAO6274	<i>pchR</i>	—	—	17.1 ± 2.3	< 1.2	< 2.0
PAO6297	<i>pchBA</i>	—	—	< 0.04	< 1.2	< 2.0
PAO6296	Ω insertion†	—	—	0.9 ± 0.5	98.1 ± 26.7	861.7 ± 81.4

* Content (ml culture supernatant)⁻¹.

† This Ω insertion is located downstream of *pchEF* (Fig. 1).

PAO6323 (Table 2). The regulation of the pathway (discussed below) accounts for the relatively low amounts of Dha excreted by the *pchF* mutant PAO6323. In conclusion, these data are consistent with a linear biosynthetic pathway in which salicylate is first converted to Dha by PchE (hence designated Dha synthetase), followed by the transformation of Dha to pyochelin mediated essentially by PchF (designated pyochelin synthetase).

pchE and *pchF* form a transcriptional unit

The sequence organization of *pchE* and *pchF* points to an operon structure. To test this hypothesis, we constructed polar mutations in the chromosomal *pchE* and *pchF* genes by marker exchange using the suicide constructs pME6422 and pME6407 (Table 1). Insertion of an ΩSm/Sp cassette at the site of the *EcoRI* deletion in *pchE* produced the mutant PAO6312 and an ΩSm/Sp insertion at the unique *BamHI* site in *pchF* gave the mutant PAO6295 (Fig. 1). The polar *pchE* mutant PAO6312 was unable to form Dha and pyochelin, as expected (Table 2). Introduction of the *pchE*-carrying plasmid pME6434 complemented this polar mutant for the production of Dha but not for pyochelin, indicating that *pchE* and *pchF* are indeed organized as an operon. The polar *pchE* mutant PAO6312 could be complemented for pyochelin production by plasmid pME6436 which carries *pchEF* plus an additional 1.6 kb fragment downstream of *pchF* (Fig. 1). The Dha levels

remained rather low in strain PAO6312(pME6436), perhaps because of a channeling effect. The polar *pchF* mutant PAO6295 did not produce pyochelin and formed small amounts of Dha. Plasmid pME6413 carrying *pchF* plus the 1.6 kb fragment downstream of *pchF* restored pyochelin formation and also increased the levels of salicylate and Dha produced (Table 2).

The polar *pchE* mutant PAO6312 was not complemented for pyochelin production by a plasmid, pME6438, carrying only *pchEF* (Fig. 1, Table 2). Similarly, the polar *pchF* mutant PAO6295 was not restored for pyochelin synthesis by a plasmid, pME6432, expressing only *pchF* (Fig. 1, Table 2). These observations indicate that the *pchEF* transcriptional unit contains one or more additional genes downstream of *pchF* which participate in the production of pyochelin. The nucleotide sequence downstream of *pchF* indicates the presence of an ORF of >742 nt (*pchG*) whose biochemical function is unknown at present. An ΩSm/Sp insertion constructed 2.0 kb downstream of the 3' end of *pchF*, in strain PAO6296 (Fig. 1), had no effect on the production of salicylate, Dha and pyochelin (Table 2) and may thus lie outside of the extended *pchEF* transcriptional unit.

Finally, the role of salicylate as a precursor of Dha and pyochelin (Ankenbauer & Cox, 1988; Serino *et al.*, 1997) was confirmed by the construction of the *pchBA* deletion mutant PAO6297, using pME6156 for delivery of the 1.0 kb deletion to the chromosomal *pchBA* genes

Table 3. Regulation of *pchEF* by iron, Fur and PchR

Plasmid pME6425 carrying a *pchE'*-*lacZ* fusion was introduced into each strain. DCAA medium (50 ml) was inoculated with 10 µl of a culture grown in nutrient yeast broth containing tetracycline and grown at 37 °C and 220 r.p.m. for 15 h to OD₆₀₀ ≈ 0.8. –Fe, no iron; +Fe, medium supplemented with 100 µM FeCl₃ (iron excess). β-Galactosidase activities are expressed in thousands of Miller units (kU) and are mean values from three experiments ± SD.

Strain	Genotype	β-Galactosidase activity (kU)	
		–Fe	+Fe
PAO1	Wild type	4.79 ± 0.09	< 0.01
PAO1A4	<i>fur-4</i>	9.97 ± 1.04	1.86 ± 0.46
PAO6274	<i>pchR</i>	< 0.01	< 0.01

(Table 1). Strain PAO6297 was devoid of salicylate, Dha and pyochelin (Table 2). Addition of salicylate restored pyochelin production, whereas addition of Dha did not (data not shown). Thus, salicylate can cross-feed a salicylate-negative mutant, in agreement with the results of Ankenbauer & Cox (1988), whereas external Dha cannot enter the pyochelin pathway, perhaps because of a permeability barrier.

Expression of *pchEF* is repressed by iron with Fur and positively controlled by PchR

The *pchE* promoter is presumed to be located directly downstream of the *pchR* gene (Heinrichs & Poole, 1993; Fig. 1). A sequence strongly resembling the consensus Fur (ferric uptake regulator) binding site (Calderwood & Mekalanos, 1987; Fig. 3) was found 45 bp upstream of the ATG start codon of the *pchE* gene, suggesting that *pchEF* may be iron-regulated. This hypothesis was tested using strain PAO1(pME6425) (*pchE'*-*lacZ*) in DCAA medium. This medium is iron-deficient, limiting growth of *P. aeruginosa* to low cell densities corresponding to OD₆₀₀ ≈ 0.5–1.0 and consequently low yields of Dha and pyochelin are obtained (data not shown). By contrast, the GGP medium used for the excretion experiments above (Table 2) allows high cell yields and iron availability appears to be less restricted than in DCAA medium.

The expression of the *pchE'*-*lacZ* fusion was strongly repressed when DCAA medium was supplemented with 100 µM FeCl₃, whereas high expression was observed during iron limitation (Table 3). In the Fur mutant PAO1A4 grown under iron-limitation, the *pchE'*-*lacZ* expression level was twofold higher than that obtained in PAO1. After iron addition, the expression of *pchE'*-*lacZ* was partially derepressed, as expected for this leaky Fur mutant (Barton *et al.*, 1996). Thus, Fur is responsible for iron repression of *pchE* expression. By analogy with the *pchD*, *pchR* and *fptA* promoters (Serino *et al.*, 1997; Heinrichs & Poole, 1993, 1996), we

postulate that Fur repression of *pchE* is mediated via the Fur box upstream of *pchE* (Fig. 3).

To test whether expression of the *pchEF* genes depends on the transcriptional regulator PchR, we constructed the mutant PAO6274 (Fig. 1) by marker exchange using the suicide construct pME3384 which carries within the *pchR* gene a linker having translation stops in all three reading frames. This *pchR* mutant produced some salicylate but failed to excrete measurable amounts of Dha and pyochelin (Table 2). No expression of the translational *pchE'*-*lacZ* fusion on pME6425 was observed in the *pchR* mutant (Table 3), implying that PchR is essential for expression of the *pchEF* genes.

Pyochelin induces the expression of the *pchEF* and *pchDCBA* operons

Extracellular pyochelin (or ferric pyochelin) is known to be required for expression of the *pchR* regulator and the *fptA* receptor genes (Heinrichs & Poole, 1993, 1996; Ankenbauer & Quan, 1994). Therefore, we tested whether pyochelin also plays a role in the expression of the *pchEF* and *pchDCBA* operons. *pchE'*-*lacZ* activity was about 100-fold lower in the *pchE* mutant PAO6310 and in the *pchF* mutant PAO6323 than in the wild-type PAO1 growing in GGP medium (Table 4). Low level *pchE'*-*lacZ* expression was also found in the *pchBA* deletion mutant PAO6297. No β-galactosidase activity was detected in a *pchR*-negative background (strain PAO6274; Table 4). Since none of the four mutants was able to produce pyochelin (Table 2), these data suggest that either pyochelin or its precursors (salicylate and Dha) could be responsible for high-level *pchE'*-*lacZ* expression in strain PAO1. Therefore, salicylate, Dha, or pyochelin was added to the culture medium. Salicylate induced *pchE'*-*lacZ* expression about 15-fold in the *pchBA*-negative mutant PAO6297, whereas no increase was observed in the other mutants PAO6310, PAO6323 and PAO6274 (Table 4). Since addition of salicylate to the growth medium restores pyochelin formation to the *pchBA* mutant, the inducing compound may not be salicylate itself but its products Dha or pyochelin. Dha added to the culture medium had no effect on *pchE'*-*lacZ* expression in any of the strains tested (Table 4). By contrast, when the growth medium was amended with pyochelin, *pchE'*-*lacZ* expression was induced about 100-fold in strains PAO6310, PAO6323 and PAO6297, but not in the *pchR* mutant PAO6274. We conclude that pyochelin (or ferric pyochelin), together with the PchR protein, is required for high-level expression of the *pchEF* operon. This fact helps explain the rather small amounts of Dha detected in supernatants of the *pchF* mutants PAO6323 and PAO6295 (Table 2): due to the absence of the inducer pyochelin, expression of *pchE* was low in these *pchF* mutants and therefore little Dha was made.

Pyochelin also induced the *pchDCBA* operon, as shown by experiments using a translational *pchD'*-*lacZ* fusion on plasmid pME6122 (Table 4). In the *pchE*, *pchF*, *pchBA* and *pchR* mutants, the β-galactosidase levels

Fig. 3. Fur recognition sequences in the promoter regions of *pchR*, *fptA*, *pchDCBA* and *pchEF*. The locations of the Fur boxes are indicated relative to the ATG initiation codons. Transcriptional start sites mapped in this region (Serino *et al.*, 1997; Ankenbauer & Quan, 1994) are marked by asterisks. Bold letters in the Fur boxes represent nucleotides matching those in the Fur binding consensus sequence of *E. coli* (Calderwood & Mekalanos, 1987). Conserved nucleotides (bold letters) upstream of the Fur box in the four promoters might be involved in PchR binding.

Table 4. Effects of salicylate, Dha and pyochelin on the expression of *pchE'*-*lacZ* and *pchD'*-*lacZ* translational fusions

GYP culture medium (15 ml) containing tetracycline (for maintenance of pME6425) or carbenicillin (for pME6122) was inoculated with 0.15 ml of a culture grown in the same medium and incubated at 37 °C and 220 r.p.m. for 24 h. Salicylate, Dha or pyochelin were added to the culture medium at the following final concentrations: salicylate, 3.6 µM; (S)-Dha, 44.8 µM; pyochelin, 102 µM. Values shown are the means of duplicate experiments. Variation between parallel measurements was < 20 % in general. BD, Below the limit of detection (< 5 Miller units). β-Galactosidase activity is expressed in thousands of Miller units (kU).

Strain/plasmid	Chromosomal marker	β-Galactosidase activity (kU)			
		–	+ Salicylate	+(S)-Dha	+ Pyochelin
PAO1(pME6425)	–	20.4	22.0	18.5	25.8
PAO6310(pME6425)	<i>pchE</i>	0.2	0.2	0.3	24.6
PAO6323(pME6425)	<i>pchF</i>	0.2	0.2	0.2	24.2
PAO6297(pME6425)	<i>pchBA</i>	0.2	3.3	0.2	23.5
PAO6274(pME6425)	<i>pchR</i>	BD	BD	BD	BD
PAO1(pME6122)	–	23.0	33.2	23.6	27.9
PAO6310(pME6122)	<i>pchE</i>	0.7	0.9	0.9	34.8
PAO6323(pME6122)	<i>pchF</i>	0.9	0.7	0.9	32.2
PAO6297(pME6122)	<i>pchBA</i>	0.9	5.5	1.1	38.8
PAO6274(pME6122)	<i>pchR</i>	0.8	0.7	0.8	0.6

were reduced 30-fold compared to the expression observed in the wild-type PAO1 (Table 4). Addition of pyochelin to the *pchE*, *pchF* and *pchBA* mutants restored the expression to wild-type levels. Addition of salicylate to the growth medium of the *pchBA*-negative mutant PAO6297 carrying pME6122 induced β-galactosidase activity about sevenfold but had no effect on the other mutants tested. Again, Dha had no effect on *pchD'*-*lacZ* expression (Table 4). Whereas PchR was strictly required for the expression of *pchEF*, this regulator appears to be less important for the expression of the *pchDCBA* operon in that a basal expression of the *pchD'*-*lacZ* fusion was observed in the absence of PchR or pyochelin (Table 4). In fact, the *pchR* mutant PAO6274 produced some salicylate, but no Dha and pyochelin (Table 2).

Previously, we have demonstrated Fur-dependent repression of the *pchDCBA* operon by iron (Serino *et al.*,

1997). Taken together, our results show that the *pchDCBA* and *pchEF* genes are regulated in parallel: negatively by iron and positively by pyochelin, the end product of the pathway.

DISCUSSION

Evidence for a linear chorismate–salicylate–Dha–pyochelin pathway in *P. aeruginosa*

The *pchEF* transcriptional unit characterized in this study extends the pyochelin biosynthetic gene region described previously which consists of the *pchR* regulatory gene (Heinrichs & Poole, 1993) and the *pchDCBA* operon (Serino *et al.*, 1995, 1997). Pyochelin is structurally related to the siderophores yersiniabactin of *Y. enterocolitica* and anguibactin of *V. anguillarum*. These three siderophores have a common Dha core (reviewed

Fig. 4. Modular structures of PchE and PchF. Conserved sequence motifs in the predicted cyclization (Cy), adenylation (A), thiolation (T), epimerization (Ra) and methylation (M) domains (Marahiel, 1997; Konz *et al.*, 1997; Stachelhaus & Marahiel, 1995; Haese *et al.*, 1993) are indicated. Note that the proposed adenylation, condensation/cyclization and thiolation domains of PchE and PchF appear to be sufficiently conserved to justify their assignments. The epimerization, methylation and reduction domains proposed are more speculative.

by Konz *et al.*, 1997). The salicylate moiety of the core can be hydroxylated (in the case of anguibactin) or additional amino acid residues can be added to Dha (in

the case of pyochelin and yersiniabactin). The sequence similarities of the deduced PchE and PchF proteins with the peptide synthetases HMWP2 of yersiniabactin bio-

synthesis and AngR of anguibactin biosynthesis (reviewed by Zocher & Keller, 1997; Marahiel, 1997) clearly indicate that Dha and pyochelin are formed by a non-ribosomal, thiotemplate mechanism. Genetic and *in vivo* biochemical data obtained here allow us to assign unequivocal biosynthetic functions to PchE and PchF of *P. aeruginosa*. Mutants blocked in the *pchF* gene (PAO6323, PAO6295) are unable to produce pyochelin but excrete Dha. Mutants impaired in *pchE* function are deficient in both Dha and pyochelin. A polar *pchE* mutant (PAO6312) complemented with *pchE*⁺, which is able to express PchE but not PchF, produces Dha but lacks pyochelin. A non-polar *pchE* mutant (PAO6310) is restored for production of Dha and pyochelin by complementation with *pchE*⁺ (Table 2). Thus, PchE is needed to synthesize Dha from salicylate and PchF is required to synthesize pyochelin from Dha. In this linear pathway, the functions of the *pchEF* genes are confirmed by heterologous expression in *P. fluorescens* P3: strain P3 carrying pME3300 (*pchR*⁺ *pchDCBA*⁺ *pchEF*⁺) produces salicylate, Dha and pyochelin, whereas strain P3 carrying pME3302 (*pchR*⁺ *pchDCBA*⁺ *pchE*⁺*F*⁻) produces salicylate and Dha.

PchE and PchF are members of the large group of multifunctional peptide synthetases

Sequence analysis of PchE and PchF revealed modular protein structures that are typical of peptide synthetases (Marahiel, 1997; Zocher & Keller, 1997). Peptide synthetases are large proteins consisting of different modules, each of which is responsible for the activation and incorporation of a single amino acid into the final peptide (Marahiel, 1997; Konz *et al.*, 1997). Typical modules consist of an acylation (A) domain (about 550 aa), which recognizes and activates the substrate amino acid and a thiolation (T) domain (about 100 aa) carrying the cofactor 4'-phosphopantetheine bound to a conserved serine residue. The thiol group of the cofactor undergoes thioester bond formation with the activated amino acid. A third domain of about 350 aa, termed condensation (C) domain, is involved in peptide bond formation. In addition, peptide synthetases may have tailoring domains such as an epimerization domain (about 400 aa) or a N-methylation domain (about 420 aa) when incorporated amino acids are modified correspondingly (Marahiel, 1997).

The deduced amino acid sequences of PchE and PchF revealed acylation domains in which most of the typical motifs (A1–A10) described by Marahiel (1997) are conserved (Fig. 4). Thiolation domains containing invariant serine residues were found in both PchE (Ser-46 and Ser-1385) and PchF (Ser-1442). A putative cyclization domain with well-conserved sequence motifs Cy1–Cy7 was identified in the N-terminal parts of both PchE and PchF (Fig. 4). This specialized form of condensation domain is assumed to catalyse both peptide bond formation using activated cysteine and cyclization of cysteine, resulting in thiazoline ring formation (Konz *et al.*, 1997). Between sequence motifs

A8 and A9 of the acylation domains in PchE and PchF, putative tailoring domains are inserted. Sequence comparison of the respective domain in PchE (aa 960–1300) revealed 30% identity with amino acid residues of ALR2, an alanine racemase of *E. coli* (Lobocka *et al.*, 1994), suggesting that this domain might be responsible for epimerization of L-cysteine to the D-form. Sequence motifs characteristic of epimerization domains of other peptide synthetases (Stachelhaus & Marahiel, 1995) were located between aa 1059 and 1195 of PchE (Fig. 4); however, their conservation was rather weak. The tailoring domain of PchF contains the sequence motif LLELGAGTA (M; Fig. 4) which resembles the signature sequence VLE/DXGXGXG described for S-adenosyl-methionine-dependent methyltransferases (Haese *et al.*, 1993). However, the last glycine residue, which is usually conserved in these enzymes, is lacking from the sequence motif in PchF. The C-terminal part of PchF had 33% identical amino acids in a 117 aa overlap with an alcohol dehydrogenase from *Alcaligenes eutrophus* (Jendrossek *et al.*, 1988). This additional domain of PchF, which has no counterpart in PchE, might carry out the reduction of the second thiazoline ring of pyochelin.

These structural considerations and the biochemical properties of the *pchDCBA* and *pchEF* mutants (Serino *et al.*, 1995, 1997; this study) lead us to propose a model for the pyochelin pathway in *P. aeruginosa* (Fig. 5). This model is strongly inspired by the elegant biochemical work on the enzymes of enterobactin biosynthesis in *E. coli* (Gehring *et al.*, 1997, 1998).

The initial steps of the pathway, the conversion of chorismate to salicylate (Serino *et al.*, 1995), are catalysed by isochorismate synthase (PchA) and isochorismate-pyruvate lyase (PchB). These enzymic reactions do not require organic cofactors (C. Gaille, C. Reimann & D. Haas, unpublished data). Salicylate is thought to be adenylated by PchD (Serino *et al.*, 1997), which is similar to 2,3-dihydroxybenzoate-AMP ligase (EntE) of *E. coli* (Rusnak *et al.*, 1989). Activated salicylate is then expected to be anchored, as a thioester, on a thiol template. This reaction may occur by a mechanism which has been studied in detail in the enterobactin pathway where the EntE enzyme transfers activated 2,3-dihydroxybenzoate (or its analogue salicylate) to phosphopantetheinylated EntB (Gehring *et al.*, 1997). In pyochelin synthesis, PchE has an obligatory role in the conversion of salicylate to Dha (Table 2) and, therefore, is a logical candidate for accepting activated salicylate. PchE has two putative acceptor sites at serine residues 46 and 1385 (Fig. 4), both of which could be modified by a 4'-phosphopantetheinyl group. Either acceptor site could form the postulated salicylate thioester. The model (Fig. 5) further predicts that the PchE protein will activate and fix one molecule of cysteine (probably the L-isomer) at the second acceptor site. PchE could then couple the activated carboxy group of salicylate to the amino group of fixed cysteine, in an amide-bond-forming reaction. The domain structure of PchE also suggests that this protein may carry out cyclization (thiazoline formation) and isomerization of

Fig. 5. Model for the biosynthesis of salicylate, Dha and pyochelin from chorismate in *P. aeruginosa*. The model is discussed in the text. Ppant, 4'-phosphopantetheine; SAM, S-adenosyl-methionine.

cysteine. Release of the reaction product from the PchE template by a thioesterase will yield (S)-Dha. This isomer has been identified as a natural product in *P. fluorescens* PFM2 (Carmi *et al.*, 1994) and as a constituent of pyochelin in *P. aeruginosa* (Rinehart *et al.*, 1995). In *P. aeruginosa* the stereochemistry of free Dha has not yet been worked out. The thioesterase function is probably provided by PchC (Serino *et al.*, 1997).

In the wild-type PAO1 only small amounts of Dha are released, the bulk being converted to pyochelin (Table 2). The model in Fig. 5 predicts that PchE-bound Dha reacts with L-cysteine carried by the PchF protein at the phosphopantetheinylated serine residue 1442. Further tasks of PchF may be the cyclization of cysteine, the reduction of the thiazoline ring and N-methylation of the thiazolidine ring formed (Fig. 5); admittedly, the PchF sequence provides only limited evidence for reduction and methylation functions. Release of pyochelin from PchF may be mediated either by the putative PchC thioesterase or by a potential thioesterase domain in PchF (not shown). A similar situation exists in surfactin synthesis of *Bacillus subtilis* where a thioesterase domain of surfactin synthetase 3 and a separate thioesterase protein cooperate in surfactin synthesis (Schneider & Marahiel, 1998).

Two important aspects of pyochelin biosynthesis are not addressed by the present model (Fig. 5). First, no pyochelin pathway-specific phosphopantetheinyl transferase gene has yet been identified. In enterobactin synthesis, a specialized phosphopantetheinyl transferase (EntD) attaches the cofactor to EntB and to EntF (Gehring *et al.*, 1997), whereas transferases involved in other non-ribosomal peptide syntheses may display cross-pathway activities (Marahiel, 1997; Ku *et al.*, 1997). Second, it is unclear whether active transport is required to export pyochelin and Dha. In pyoverdinin biosynthesis of *P. aeruginosa*, the *pvdE* gene is assumed to have an ATP-dependent pyoverdinin export function (McMorran *et al.*, 1996). The genomic region located

between the *pchF* and *fptA* genes (Fig. 1) might harbour some of the missing *pch* genes.

Positive autoregulation of pyochelin biosynthesis

The expression of both the *pchDCBA* and *pchEF* genes was positively controlled by extracellular pyochelin or ferric pyochelin. In different pyochelin-negative mutants, expression occurred at a low basal level unless pyochelin was added. The *pchD'*-*'lacZ* and *pchE'*-*'lacZ* fusions were induced 30- and 100-fold, respectively and induction depended on a functional PchR protein (Table 4). Previously, pyochelin-dependent expression has been reported for the pyochelin receptor FptA and the PchR regulator itself (Heinrichs & Poole, 1993, 1996). The nucleotide sequences upstream of the Fur box in the *pchE*, *pchD*, *pchR* and *fptA* promoters showed a number of conserved nucleotides (Fig. 3), some of which might be involved in PchR recognition. Induction of the outer-membrane receptors by specific siderophores present in the medium is common in pseudomonads (Crosa, 1997). It could be argued that *Pseudomonas* strains which take up extracellular (ferric) pyochelin by inducing a specific receptor would have no need to synthesize pyochelin *de novo*. In the case of *P. aeruginosa*, such a view is an oversimplification since, as we have shown here, pyochelin will induce its own biosynthetic genes. This positive feedback mechanism allows a rapid adaptation of the bacterial cells to iron-depleted environments. Interestingly, yersiniabactin induces its own biosynthetic genes in *Y. enterocolitica* and *Y. pestis* (Pelludat *et al.*, 1998; Bearden *et al.*, 1997) and pyoverdinin up-regulates its own expression in *P. aeruginosa* (Stintzi *et al.*, 1997). Thus, positive autoregulation may be a general phenomenon in siderophore biosynthetic pathways. In the case of pyochelin biosynthesis, positive autoregulation comes to a halt when the system has provided the cells with sufficient intracellular iron, which switches off the synthesis of pyochelin and of the FptA receptor.

Does pyochelin exert its inducing effect in the cytoplasm or from without, e.g. by interacting with the FptA receptor? In the second case, the inducing signal could be generated at the cell surface or in the periplasm and then transmitted to PchR via an unidentified signal transduction pathway, akin to that of the ferric citrate regulatory system in *E. coli* (Braun, 1997). There is some circumstantial evidence from the data of Heinrichs & Poole (1996) that activation of *fptA* expression by pyochelin may involve the functional FptA receptor itself. However, in that study no attempt was made to dissociate the uptake functions from the inferred regulatory functions of FptA. At any rate, the present data do not allow us to decide whether pyochelin activates the PchR protein directly, by physical interaction, or indirectly, via a receptor-dependent signal transduction pathway.

Conclusion

As a rule, the end product of a biosynthetic pathway represses or feedback-inhibits the key enzyme(s) of the pathway in bacteria. Certain extracellular metabolites disobey this textbook rule as they stimulate their own synthesis. Autoinduction is a well-known phenomenon in cell-cell communication mediated by *N*-acyl homoserine lactones, which for this reason are sometimes called autoinducers (Fuqua & Greenberg, 1998). However, autoinduction also characterizes the synthesis of the siderophores pyochelin (this study), pyoverdine (Stintzi *et al.*, 1997) and yersiniabactin (Pelludat *et al.*, 1998). It would not be surprising if autoinduction of extracellular metabolites turned out to be a widespread phenomenon in bacterial adaptation.

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REFERENCES

- Ankenbauer, R. G. & Cox, C. D. (1988). Isolation and characterization of *Pseudomonas aeruginosa* mutants requiring salicylic acid for pyochelin biosynthesis. *J Bacteriol* **170**, 5364–5367.
- Ankenbauer, R. G. & Quan, H. N. (1994). FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. *J Bacteriol* **176**, 307–319.
- Barton, H. A., Johnson, Z., Cox, C. D., Vasil, A. I. & Vasil, M. L. (1996). Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and micro-aerobic environments. *Mol Microbiol* **21**, 1001–1017.
- Bearden, S. W., Fetherston, J. D. & Perry, R. D. (1997). Genetic organization of the yersiniabactin biosynthetic region and con-

- struction of avirulent mutants in *Yersinia pestis*. *Infect Immun* **65**, 1659–1668.
- Braun, V. (1997). Surface signaling: novel transcription initiation mechanism starting from the cell surface. *Arch Microbiol* **167**, 325–331.
- Britigan, B. E., Rasmussen, G. T. & Cox, C. D. (1997). Augmentation of oxidant injury to human pulmonary epithelial cells by the *Pseudomonas aeruginosa* siderophore pyochelin. *Infect Immun* **65**, 1071–1076.
- Calderwood, S. B. & Mekalanos, J. J. (1987). Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. *J Bacteriol* **169**, 4759–4764.
- Carmi, R., Carmeli, S., Levy, E. & Gough, F. J. (1994). (+)-(S)-Dihydro-aeruginic 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. & Adams, P. (1985). Siderophore activity of pyoverdine in *Pseudomonas aeruginosa*. *Infect Immun* **48**, 130–138.
- Cox, C. D. & Graham, R. (1979). Isolation of an iron-binding compound from *Pseudomonas aeruginosa*. *J Bacteriol* **137**, 357–364.
- Coyne, M. J., Jr & Goldberg, J. B. (1995). Cloning and characterization of the gene (*rfc*) encoding O-antigen polymerase of *Pseudomonas aeruginosa* PAO1. *Gene* **167**, 81–86.
- Crosa, J. H. (1997). Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. *Microbiol Mol Biol Rev* **61**, 319–336.
- Cunliffe, H. E., Merriman, T. R. & Lamont, I. L. (1995). Cloning and characterization of *pvdS*, a gene required for pyoverdine synthesis in *Pseudomonas aeruginosa*: PvdS is probably an alternative sigma factor. *J Bacteriol* **177**, 2744–2750.
- Del Sal, G., Manfioletti, G. & Schneider, C. (1988). A one-tube plasmid DNA mini-preparation suitable for sequencing. *Nucleic Acids Res* **16**, 9878.
- Farinha, M. A. & Kropinski, A. M. (1990). High efficiency electroporation of *Pseudomonas aeruginosa* using frozen cell suspensions. *FEMS Microbiol Lett* **58**, 221–225.
- Fuqua, C. & Greenberg, E. P. (1998). Self perception in bacteria: quorum sensing with acylated homoserine lactones. *Curr Opin Microbiol* **1**, 183–189.
- Gamper, M., Ganter, B., Polito, M. R. & Haas, D. (1992). RNA processing modulates the expression of the *arcDABC* operon in *Pseudomonas aeruginosa*. *J Mol Biol* **226**, 943–957.
- Gehring, A. M., Bradley, K. A. & Walsh, C. T. (1997). Enterobactin biosynthesis in *Escherichia coli*: isochorismate lyase (EntB) is a bifunctional enzyme that is phosphopantetheinylated by EntD and then acylated by EntE using ATP and 2,3-dihydroxybenzoate. *Biochemistry* **36**, 8495–8503.
- Gehring, A. M., Mori, I. & Walsh, C. T. (1998). Reconstitution and characterization of the *Escherichia coli* enterobactin synthetase from EntB, EntE and EntF. *Biochemistry* **37**, 2648–2659.
- Guilvout, I., Mercereau-Puijalon, O., Bonnefoy, S., Pugsley, A. P. & Carniel, E. (1993). High-Molecular-Weight Protein 2 of *Yersinia enterocolitica* is homologous to AngR of *Vibrio anguillarum* and belongs to a family of proteins involved in nonribosomal peptide synthesis. *J Bacteriol* **175**, 5488–5504.
- Haese, A., Schubert, M., Herrmann, M. & Zocher, R. (1993).

- Molecular characterization of the enniatin synthetase gene encoding a multifunctional enzyme catalysing *N*-methyldepsi-peptide formation in *Fusarium scirpi*. *Mol Microbiol* 7, 905–914.
- Heinrichs, D. E. & Poole, K. (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.
- Heinrichs, D. E. & Poole, K. (1996). PchR, a regulator of ferripyochelin receptor gene (*fptA*) expression in *Pseudomonas aeruginosa*, functions both as an activator and as a repressor. *J Bacteriol* 178, 2586–2592.
- Jendrossek, D., Steinbuechel, A. & Schlegel, H. G. (1988). Alcohol dehydrogenase gene from *Alcaligenes eutrophus*: subcloning, heterologous expression in *Escherichia coli*, sequencing and location of Tn5 insertions. *J Bacteriol* 170, 5248–5256.
- Konz, D., Klens, A., Schörgendorfer, K. & Marahiel, M. A. (1997). The bacitracin biosynthesis operon of *Bacillus licheniformis* ATCC 10716: molecular characterization of three multi-modular peptide synthetases. *Chem Biol* 4, 927–937.
- Ku, J., Mirmira, R. G., Liu, L. & Santi, D. V. (1997). Expression of a functional non-ribosomal peptide synthetase module in *Escherichia coli* by coexpression with a phosphopantetheinyl transferase. *Chem Biol* 4, 203–207.
- Leoni, L., Ciervo, A., Orsi, N. & Visca, P. (1996). Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeruginosa*: effect of Fur and PvdS on promoter activity. *J Bacteriol* 178, 2299–2313.
- Lobocka, M., Hennig, J., Wild, J. & Klopotoski, T. (1994). Organization and expression of the *Escherichia coli* K-12 *dad* operon encoding the smaller subunit of D-amino acid dehydrogenase and the catabolic alanine racemase. *J Bacteriol* 176, 1500–1510.
- Lopez, P. J. & Dreyfus, M. (1996). The *lacZ* mRNA can be stabilized by the T7 late mRNA leader in *E. coli*. *Biochimie* 78, 408–415.
- McMorran, B. J., Merriman, M. E., Rombel, I. T. & Lamont, I. L. (1996). Characterization of the *pvdE* gene which is required for pyoverdine synthesis in *Pseudomonas aeruginosa*. *Gene* 176, 55–59.
- Marahiel, M. A. (1997). Protein templates for the biosynthesis of peptide antibiotics. *Chem Biol* 4, 561–567.
- Martell, A. E. & Smith, R. M. (1977). Other organic ligands. In *Critical Stability Constants*, vol. 3, pp. 181–201. Edited by A. E. Martell & R. M. Smith. New York: Plenum.
- Meyer, J. M. (1992). Exogenous siderophore-mediated iron uptake in *Pseudomonas aeruginosa*: possible involvement of porin OprF in iron translocation. *J Gen Microbiol* 138, 951–958.
- Meyer, J. M. & Abdallah, M. A. (1978). The fluorescent pigment of *Pseudomonas aeruginosa*: biosynthesis, purification and physico-chemical properties. *J Gen Microbiol* 107, 130–138.
- Meyer, J. M., Neely, A., Stintzi, A., Georges, C. & Holder, I. A. (1996). Pyoverdine is essential for virulence of *Pseudomonas aeruginosa*. *Infect Immun* 64, 518–523.
- Miyazaki, H., Kato, H., Nakazawa, T. & Tsuda, M. (1995). A positive regulatory gene, *pvdS*, for expression of pyoverdine biosynthetic genes in *Pseudomonas aeruginosa* PAO. *Mol Gen Genet* 248, 17–24.
- Ochsner, U. A., Vasil, A. I. & Vasil, M. L. (1995). Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. *J Bacteriol* 177, 7194–7201.
- Pelludat, C., Rakin, A., Jacobi, C. A., Schubert, S. & Heesemann, J. (1998). The yersiniabactin biosynthetic gene cluster of *Yersinia enterocolitica*: organization and siderophore-dependent regulation. *J Bacteriol* 180, 538–546.
- Poole, K., Neshat, S., Krebes, K. & Heinrichs, D. E. (1993). Cloning and nucleotide sequence analysis of the ferripyoverdine receptor gene *fptA* of *Pseudomonas aeruginosa*. *J Bacteriol* 175, 4597–4604.
- Prentki, P. & Krisch, H. M. (1984). *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* 29, 303–313.
- Ratledge, C., Macham, L. P., Brown, K. A. & Marshall, B. J. (1974). Iron transport in *Mycobacterium smegmatis*: a restricted role for salicylic acid in the extracellular environment. *Biochim Biophys Acta* 372, 39–51.
- Rinehart, K. L., Staley, A. L., Wilson, S. R., Ankenbauer, R. G. & Cox, C. D. (1995). Stereochemical assignment of the pyochelins. *J Org Chem* 60, 2786–2791.
- Rusnak, F., Faraci, W. S. & Walsh, C. T. (1989). Subcloning, expression and purification of the enterobactin biosynthetic enzyme 2,3-dihydroxybenzoate-AMP ligase: demonstration of enzyme-bound (2,3-dihydroxybenzoyl)adenylate product. *Biochemistry* 28, 6827–6835.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schneider, A. & Marahiel, M. A. (1998). Genetic evidence for a role of thioesterase domains, integrated in or associated with peptide synthetases, in non-ribosomal peptide biosynthesis in *Bacillus subtilis*. *Arch Microbiol* 169, 404–410.
- Serino, L., Reimann, C., Baur, H., Beyeler, M., Visca, P. & Haas, D. (1995). Structural genes for salicylate biosynthesis from chorismate in *Pseudomonas aeruginosa*. *Mol Gen Genet* 249, 217–228.
- Serino, L., Reimann, C., Visca, P., Beyeler, M., Della Chiesa, V. & Haas, D. (1997). Biosynthesis of pyochelin and dihydroaeruginosic acid requires the iron-regulated *pchDCBA* operon in *Pseudomonas aeruginosa*. *J Bacteriol* 179, 248–257.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vitro* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio-technology* 1, 784–790.
- Stachelhaus, T. & Marahiel, M. A. (1995). Modular structure of peptide synthetases revealed by dissection of the multifunctional enzyme GrsA. *J Biol Chem* 270, 6163–6169.
- Stanisich, V. A. & Holloway, B. W. (1972). A mutant sex factor of *Pseudomonas aeruginosa*. *Genet Res* 19, 91–108.
- Stintzi, A., Meyer, J.-M. & Poole, K. (1997). The *pvc* gene cluster of *Pseudomonas aeruginosa*: role in the synthesis of the pyoverdine chromophore and regulation by the exotoxin A regulator PtxR. VI International Congress on *Pseudomonas*: Molecular Biology and Biotechnology. Madrid, Spain 1997.
- Tomalsky, M. E., Actis, L. A. & Crosa, J. H. (1993). A single amino acid change in AngR, a protein encoded by pJM1-like virulence plasmids, results in hyperproduction of anguibactin. *Infect Immun* 61, 3228–3233.
- Visca, P., Ciervo, A., Sanfilippo, V. & Orsi, N. (1993). Iron-regulated salicylate synthesis by *Pseudomonas* spp. *J Gen Microbiol* 139, 1995–2001.
- Voisard, C., Keel, C., Haas, D. & Défago, G. (1989). Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. *EMBO J* 8, 351–358.
- Voisard, C., Bull, C. T., Keel, C., Laville, J., Maurhofer, M., Schnider, U., Défago, G. & Haas, D. (1994). Biocontrol of root

diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches. In *Molecular Ecology of Rhizosphere Microorganisms*, pp. 67–89. Edited by F. O. Gara, D. N. Dowling & B. Boesten. Weinheim: VCH.

Watson, A. A., Alm, R. A. & Mattick, J. S. (1996). Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. *Gene* **172**, 163–164.

Ye, R. W., Haas, D., Ka, J. O., Krishnapillai, V., Zimmermann, A.,

Baird, C. & Tiedje, J. M. (1995). Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *J Bacteriol* **177**, 3606–3609.

Zocher, R. & Keller, U. (1997). Thiol template peptide synthesis systems in bacteria and fungi. *Adv Microbiol Physiol* **38**, 85–131.

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