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Iron acquisition with the natural siderophore enantiomers pyochelin and enantio-pyochelin in *Pseudomonas* species

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Abstract The bacterial siderophore pyochelin is composed of salicylate and two cysteine-derived heterocycles, the second of which is modified by reduction and N-methylation during biosynthesis. In *Pseudomonas aeruginosa,* the first cysteine residue is converted to its D-isoform during thiazoline ring formation, whereas the second cysteine remains in its L-configuration. Stereochemistry is opposite in the *Pseudomonas fluorescens* siderophore enantio-pyochelin, in which the first ring originates from L-cysteine and the second ring from D-cysteine. Both siderophores promote growth of the producer organism during iron limitation and induce the expression of their biosynthesis genes by activating the transcriptional AraC-type regulator PchR. However, neither siderophore is functional as an iron carrier or as a transcriptional inducer in the other species, demonstrating that both processes are highly stereospecific. Stereospecificity of pyochelin/enantio-pyochelin-mediated iron uptake is ensured at two levels: (i) by the outer membrane siderophore receptors and (ii) by the cytosolic PchR regulators.

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Introduction

Iron is a cofactor of many redox-dependent enzymes and therefore essential for most living organisms including bacteria. Despite its abundance, iron is not freely available to microorganisms under aerobic conditions, as it forms poorly soluble ferric hydroxides in the environment and is bound to transport and storage proteins in host organisms. To acquire iron, bacteria have developed sophisticated strategies involving the production of iron chelators termed siderophores. Released to the environment, siderophores bind iron with high affinity and transport it to the bacterial cytoplasm via specific outer membrane receptors (Guerinot 1994; Andrews et al. 2003; Wandersman and Delepelaire 2004). The Gram-negative bacterium *Pseudomonas aeruginosa* produces two siderophores, pyoverdine and pyochelin (Pch), and, in addition, utilizes a large number of xenosiderophores for iron uptake (Bodilis and Cornelis 2009; Cornelis 2010), a mechanism known as siderophore piracy. Structurally related pyoverdines are produced by all fluorescent pseudomonads and, due to their high affinity for iron, pyoverdines are regarded as primary

siderophores of these bacteria (Cornelis 2010). In contrast, the affinity for iron is lower in pyochelin and other so-called secondary *Pseudomonas* siderophores, which, in addition to their role as iron carriers, often have other biological activities (Cornelis 2010).

We recently reported that the secondary siderophore of some *Pseudomonas fluorescens* strains is not Pch as expected from earlier studies (Castignetti 1997; Schmidli-Sacherer et al. 1997; Terano et al. 2002), but is in fact its optical antipode, which we have termed enantio-pyochelin (E-Pch; Youard et al. 2007). Interestingly, Pch and E-Pch are biologically active only in their producer species (Youard et al. 2007; Hoegy et al. 2009; Youard and Reimmann 2010), suggesting that iron uptake with these compounds is highly stereospecific.

Here we review the chemical structure, metal chelation and biosynthetic pathway of Pch and present our current knowledge on its enantiomer E-Pch. We compare the role of the two siderophores in iron uptake and transcriptional regulation in both *Pseudomonas* species and discuss the importance and biological implications of chirality in these processes.

The siderophores pyochelin and enantio-pyochelin

Pch was isolated in the late 1970s from culture broth of *Pseudomonas aeruginosa* ATCC15692 (strain PAO1) grown under iron limitation, and its chemical structure was determined as 2-(2-*o*-hydroxyphenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid (Cox and Graham 1979; Cox 1980; Cox et al. 1981; Liu and Shokrani 1978) (Fig. 1a). Pch is poorly soluble in water and has been reported to bind ferric iron with a stochiometry of 2:1 (Pch to Fe^{3+}) (Visca et al. 1992; Tseng et al. 2006) and a stability constant of 2×10^5 M^{-1}, determined in ethanol (Cox and Graham 1979). Pch binds, albeit with lower affinities, also other metals such as Ag^{+}, Al^{3+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Eu^{3+}, Ga^{3+}, Hg^{2+}, Mn^{2+}, Mo^{6+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, Tb^{3+}, Tl^{+}, and Zn^{2+}. However, besides

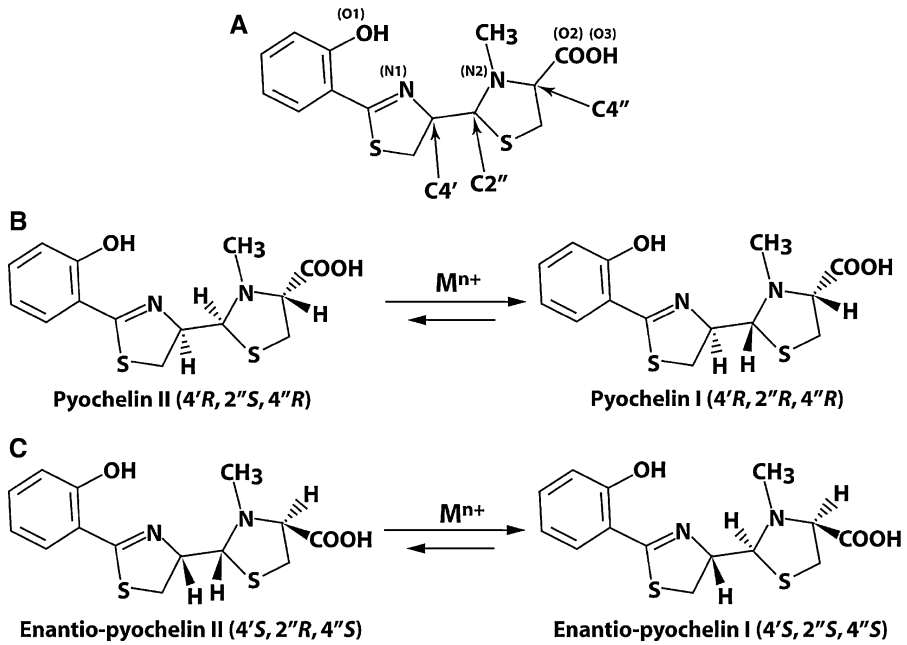

Fig. 1 Structural configurations of Pch and E-Pch isomers. Pch and E-Pch have three chiral carbons, C4′, C2″, and C4″ (**a**) and exist in nature as pairs of interconvertible diastereoisomers. Pch I and II (**b**) are made by *P. aeruginosa* PAO1 (Ankenbauer et al. 1988; Rinehart et al. 1995), whereas E-Pch I and II (**c**) are produced by the *P. fluorescens* strains Pf-5 and CHA0 (Youard et al. 2007). Note that the metal (M^{n+})-induced shift at C2″ has only been shown for Pch (Schlegel et al. 2004). In ferriPch complexes iron is tetradentately coordinated by one Pch molecule (O1, N1, N2, O2) and bidentately by a second Pch molecule (O1, N1 or N2, O3) or any other available ligand (Cobessi et al. 2005; Klumpp et al. 2005; Hayen and Volmer 2006; Schlegel et al. 2006; Tseng et al. 2006)

Fe^{3+}, only Co^{2+}, Ga^{3+}, Mo^{6+} and Ni^{2+} were transported to some extent by Pch in *P. aeruginosa* (Braud et al. 2009; Namiranian et al. 1997; Visca et al. 1992).

In the 2:1 Pch-iron complex, four of the six octahedral coordination sites of ferric iron are occupied by the phenolate and the carboxylate oxygen (O1 and O3, respectively) and by the two nitrogen atoms N1 and N2 of the first Pch molecule (Fig. 1a). The remaining two coordination sites can accomodate either O1 and N1 or N2 and O3 of the second Pch molecule (Tseng et al. 2006). However, Pch:Fe^{3+} (ferriPch) complexes with a 1:1 stoichiometry have been reported as well. In these complexes, the remaining two coordination sites of ferric iron were occupied by other available ligands (Klumpp et al. 2005; Cobessi et al. 2005; Hayen and Volmer 2006; Tseng et al. 2006; Schlegel et al. 2006). Thus, the nature of the biologically relevant ferriPch complex is not entirely clear and may depend on ligand concentrations in vivo.

Pch has three chiral centers (C4′, C2″, and C4″; Fig. 1a) and is extracted from *P. aeruginosa* PAO1 as a mixture of two interconvertible diastereoisomers whose absolute configuration was determined as 4′*R*, 2″*R*, 4″*R* (Pch I) and 4′*R*, 2″*S*, 4″*R* (Pch II) (Fig. 1b; Ankenbauer et al. 1988; Rinehart et al. 1995). In the presence of Fe^{3+} or Zn^{2+}, the *S* configuration at the chiral center C2″ is converted to the *R* configuration, inducing a shift from Pch II to the iron-binding diastereoisomer Pch I (Fig. 1b; Ino and Murabayashi 2001; Schlegel et al. 2004, 2006).

The two diastereoisomers isolated from the *Pseudomonas fluorescens* strains Pf-5 and CHA0 have a stereochemistry opposite to that of Pch I and II and were thus named E-Pch I (4′*S*, 2″*S*, and 4″*S*) and E-Pch II (4′*S*, 2″*R*, and 4″*S*) (Fig. 1c; Youard et al. 2007). By analogy with Pch, a metal-induced shift from E-Pch II to E-Pch I is expected to occur and it is assumed that E-PchI is the iron-binding diastereoisomer.

Biosynthesis

Pch and E-Pch are condensation products of salicylate and two molecules of cysteine, which are cyclized during the assembly and undergo a certain number of modifications (Fig. 2). The sole difference between Pch and E-Pch is the stereochemical configuration of the two cysteines incorporated. In Pch biosynthesis, it is the first L-cysteine that undergoes an epimerization reaction, while in E-Pch biosynthesis it is the second L-cysteine that is converted to its D-isoform. In the following we will briefly review the Pch biosynthesis pathway (Fig. 2a) and then highlight the differences and open questions with regard to E-Pch assembly (Fig. 2b).

The Pch biosynthesis genes of *P. aeruginosa* PAO1 are organized in two divergent operons, *pchDCBA* and *pchEFGHI* (Serino et al. 1997; Reimmann et al. 1998;

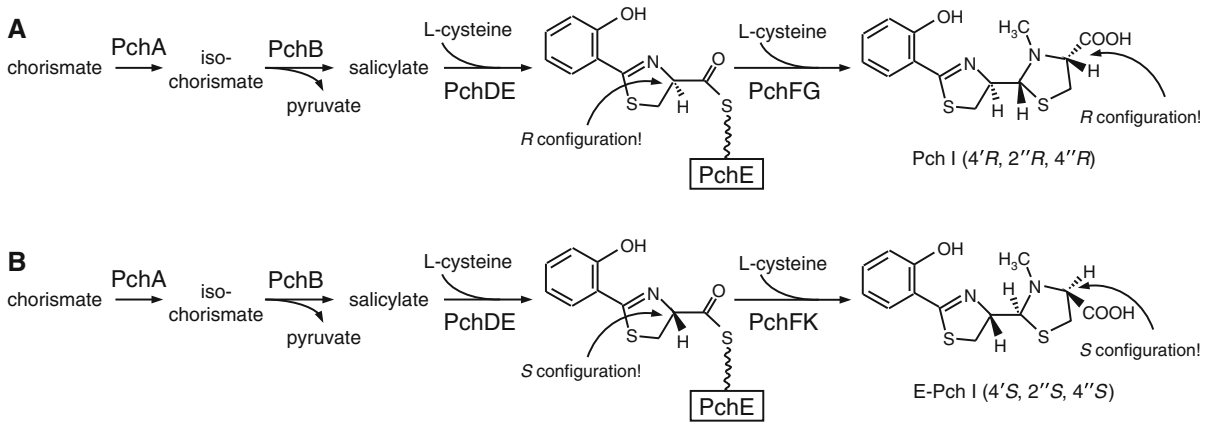

Fig. 2 Biosynthetic pathways generating Pch (**a**) in *P. aeruginosa* and E-Pch (**b**) in the *P. fluorescens* strains Pf-5 and CHA0. The Pch biosynthetic pathway has been studied with purified enzymes in vitro (see text for details) while the proposed pathway for E-Pch is essentially based on bioinformatic analysis. Note that the chiral center C2″ isomerizes spontaneously (Fig. 1) but for simplicity reasons, only the isomers I of the two siderophores are shown here

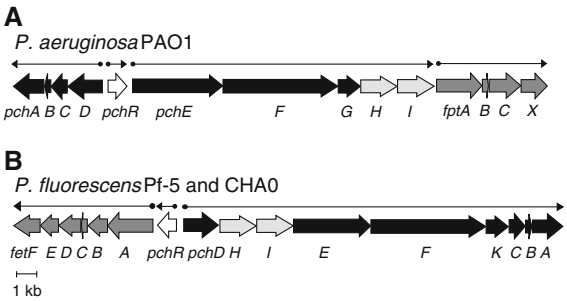

Fig. 3 Gene clusters for biosynthesis and transport of Pch in *P. aeruginosa* PAO1 (**a**) and for E-Pch in *P. fluorescens* Pf-5 and CHA0 (**b**), respectively. Genes for biosynthesis are in *black*, genes for siderophore uptake in *dark grey*, and the *pchR* regulatory genes are in *white*. The ABC transport genes *pchHI*, whose function in Pch transport is not clear, are shown in *light grey*. Transcriptional units (confirmed only for *pchDCBA* and *fptABCX* of *P. aeruginosa* [Serino et al. 1997; Michel et al. 2007]) are indicated by *black arrows* above the genes. The five genes located immediately downstream of the E-Pch receptor gene *fetA* (PFL_3498 in strain Pf-5) are named *fetBCDEF* here (PFL_3499 to PFL_3503 in strain Pf-5). Bioinformatics analysis predicts that FetCDE form a periplasmic binding protein-dependent ABC transporter potentially involved in E-Pch transport across the inner membrane. The figure is based on sequence data from Stover et al. (2000), Paulsen et al. (2005), Youard et al. (2007), and Hoegy et al. (2009)

Reimmann et al. 2001), which are clustered with the pyochelin uptake operon *fptABCX* (Ankenbauer and Quan 1994; Michel et al. 2007) and the regulatory gene *pchR* (Heinrichs and Poole 1993) (Fig. 3a). During the initial steps of Pch biosynthesis, chorismate is converted to salicylate via isochorismate (Fig. 2). These steps are carried out by the isochorismate synthase PchA (Serino et al. 1995; Gaille et al. 2003) and by the isochorismate pyruvate-lyase PchB (Serino et al. 1995; Gaille et al. 2002). The amino acid sequence of PchB is unrelated to pyruvate lyases of other shikimic acid metabolites, and its crystal structure reveals homology with chorismate mutases of the AroQα class (Zaitseva et al. 2006). Indeed, PchB is a bifunctional enzyme displaying, in addition, a chorismate mutase activity (Gaille et al. 2002; Künzler et al. 2005; DeClue et al. 2005). Conversion of chorismate to salicylate is a two-step process in Pch biosynthesis. In contrast, in the biosyntheses of other salicylate-derived siderophores such as yersiniabactin and mycobactin, salicylate is made from chorismate in a single step, which is carried out by Irp9 and MbtI, respectively (Pelludat et al. 2003; Kerbarh et al. 2005; Kerbarh et al. 2006; Harrison et al. 2006).

In the subsequent steps of Pch biosynthesis salicylate is linked to two molecules of L-cysteine by a thiotemplate mechanism involving the salicylate adenylating enzyme PchD, the two peptide synthetases PchE and PchF, and the reductase PchG (Serino et al. 1997; Reimmann et al. 1998, 2001; Quadri et al. 1999; Patel and Walsh 2001; Patel et al. 2003; Fig. 2a). Pch synthesis from salicylate and L-cysteine has been fully reconstituted with purified enzymes in vitro (Patel and Walsh 2001) and the individual reactions have been studied in detail (Quadri et al. 1999; Reimmann et al. 2001; Patel and Walsh 2001; Patel et al. 2003; for a recent review see also Thomas 2007). The main steps are briefly summarized here. The substrates salicylate and L-cysteine are adenylated by PchD and PchE, respectively, and loaded onto PchE via covalent thioester bonds that are provided by two posttranslationally added phosphopantheteinyl prostetic groups. The subsequent PchE-catalyzed condensation and cyclization reactions generate an enzyme-bound hydroxyphenyl-thiazoline intermediate. As shown in Fig. 2a, the chiral center C4′ of this intermediate has an *R* configuration, indicating that the thiazoline ring is derived from D-cysteine rather than L-cysteine. During ring formation, an epimerization reaction is carried out by a PchE-embedded epimerase domain, which converts the PchE-bound L-cysteinyl residue to its D-isoform (Reimmann et al. 1998; Patel et al. 2003). Meanwhile, a second L-cysteine molecule is adenylated by the peptide synthetase PchF and anchored, again via a phosphopantetheinyl linker, to a carrier domain in PchF. PchF then catalyzes the condensation of the PchE-bound hydroxyphenyl-thiazoline intermediate with L-cysteinyl-PchF and generates the second thiazoline ring (Quadri et al. 1999). The Pch backbone is now completed and the second thiazoline ring undergoes reduction and methylation before the final product is released from its thiotemplate. Reduction is carried out by the reductase PchG generating a thiazolidine ring. A methyltransferase domain in PchF then catalyzes N-methylation of this ring. The completed Pch is released from PchF by the protein's C-terminal thioesterase domain.

Three of the *pch* genes shown in Fig. 3a are not essential for the production of Pch. PchC encodes a type II thioesterase, which maximizes Pch production in *P. aeruginosa*, probably by removing wrongly charged substrates from the peptidyl carrier protein

domains of PchE and PchF (Reimmann et al. 2004). The *pchH* and *pchI* genes encode ABC transporters, whose potential function in Pch transport may be redundant, as a deletion of these genes did not reduce the amount of Pch found in culture supernatants of *P. aeruginosa* (Reimmann et al. 2001).

The biosynthesis of E-Pch in *P. fluorescens* has not yet been studied biochemically, but is probably quite similar to the Pch pathway in *P. aeruginosa*. A closely related gene cluster is present in the chromosome of the *P. fluorescens* strains Pf-5 and CHA0, although the arrangement of the individual genes is different from that in *P. aeruginosa* and there is no gene with obvious sequence homology to *pchG* (Paulsen et al. 2005; Youard et al. 2007; Fig. 3b). Bioinformatics predict that reduction of the second thiazoline ring in E-Pch biosynthesis could be catalyzed by PchK, a putative thiazolinyl reductase encoded downstream of *pchF* in *P. fluorescens* (Fig. 3b; H.J. Imker and C.T Walsh, personal communication).

How is the *S* configuration at the chiral centers C4′ and C4″ of E-Pch generated? Apparently, the *P. fluorescens* PchE does not have an epimerase domain (Youard et al. 2007). The first L-cysteine incorporated by this enzyme will therefore remain in its original L-conformation, generating an *S* stereochemistry at C4′ (Fig. 2b). The origin of the *S* stereochemistry at C4″ in E-Pch is not known. When the $pchDCBA_{PAO1}$ genes are co-expressed together with $pchEFK_{CHA0}$, E-Pch is produced in *P. aeruginosa*, indicating that an epimerase function is encoded either by $pchF_{CHA0}$ or by $pchK_{CHA0}$ (N. Wenner and C. Reimmann, unpublished results).

Iron uptake

The protein machineries transporting ferriPch and ferriE-Pch are not related. The ferriPch complex is recognized at the cell surface of *P. aeruginosa* and transported into the periplasm by a specific outer membrane transporter, FptA (Ankenbauer and Quan,

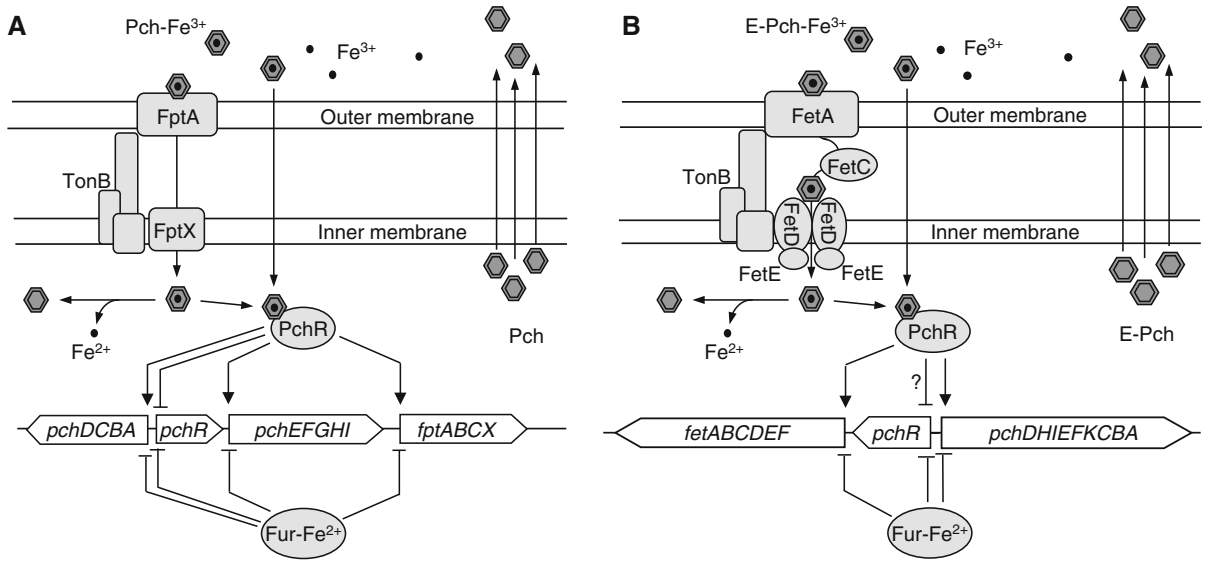

Fig. 4 Iron uptake with Pch and E-Pch and its transcriptional regulation. In *P. aeruginosa* (**a**), ferriPch crosses the outer membrane via the receptor FptA and the inner membrane via the permease FptX. FerriPch then activates the PchR regulator, which in turn induces the expression of *pchDCBA*, *pchEFGHI*, and *fptABCX*, and represses its own gene. Iron uptake and signalling with E-Pch in *P. fluorescens* (**b**) is expected to proceed in a similar way, except that after FetA-dependent transport through the outer membrane, the iron-siderophore complex may cross the inner membrane via a classical ABC transport system encoded by genes of the *fetABCDEF* operon. Note that in the absence of FptA and FetA, respectively, activation of PchR by its cognate siderophore is reduced but not abolished (Youard and Reimmann 2010), implying that the siderophores can enter the cells also by an alternative, low-efficiency uptake pathway. Under iron-replete conditions, the Fur repressor blocks transcription of *pchDCBA*, *pchR*, *pchEFGHI*, and *fptABCX* in *P. aeruginosa*, and a similar repression of *pchDHIEFKCBA*, *pchR*, and *fetABCDEF* probably occurs in *P. fluorescens* (see text)

1994; Fig. 4a). The structure of this protein (Cobessi et al. 2005) is typical for this class of transporters: a transmembrane 22-β-stranded barrel occluded by an N-terminal domain forming the cork. Binding of ferriPch to FptA occurs with high affinity ($K_d = 2.5 \pm 1.1$ nM; Hoegy et al. 2009) and, consistent with the hydrophobicity of the siderophore, the ferriPch binding pocket is mainly composed of hydrophobic and aromatic residues. Once translocated to the periplasm, ferriPch crosses the inner membrane via FptX, a member of a novel family of permeases that function as single-subunit siderophore transporters (Ó Cuív et al. 2004; Fig. 4a). Iron release from Pch is expected to occur by a reductive process, although no experiments have been performed along these lines. The *fptA* and *fptX* genes form an operon together with *fptB* and *fptC* (Michel et al. 2007; Fig. 3a). The function of these latter genes is not clear, as their deletion does not affect the utilization of ferriPch as an iron source in *P. aeruginosa* (Michel et al. 2007).

Uptake of ferriE-Pch in *P. fluorescens* requires the FetA receptor (Youard et al. 2007; Fig. 4b) which binds ferriE-Pch with high affinity ($K_d = 3.7 \pm 2.1$ nM) and functionally resembles FptA (Hoegy et al. 2009). FetA is encoded by the first gene of the putative E-Pch uptake operon *fetABCDEF* (Fig. 3b). Bioinformatics suggest that *fetB* encodes a PepSY-associated membrane protein whose function is not known. The *fetCDE* genes code for a classical ATP-binding cassette (ABC) transport system comprising a periplasmic binding protein (FetC), a permease (FetD), and an ATP-binding protein (FetE). We suspect that this ABC transporter is responsible for ferriE-Pch translocation across the inner membrane (Fig. 4b). The last gene of the operon, *fetF*, encodes a transporter of the major facilitator superfamily (MFS). Could this transporter play a role in E-Pch recycling after iron release?

Regulation

Under iron-replete conditions, the biosynthesis of Pch and the uptake system for ferriPch are repressed by the ferric uptake regulator Fur, which, when complexed with ferrous iron, binds to a conserved sequence element (Fur-box) in the promoter regions of iron-regulated genes and blocks their transcription (Escolar et al. 1999). Fur-boxes are present in the promoters of the *pchDCBA*, *pchEFGHI*, *pchR* and *fptABCX* genes and expression studies have demonstrated Fur-dependent repression of these genes in iron-replete conditions (Heinrichs and Poole 1993; Ochsner et al. 1995; Ankenbauer and Quan 1994; Serino et al. 1997; Reimmann et al. 1998; Fig. 4a). When iron becomes limiting, Fur dissociates from the Fur-boxes allowing a basal level of gene expression to occur. Full expression of *pchDCBA*, *pchEFGHI*, and *fptABCX* requires the transcriptional activator PchR (Heinrichs and Poole 1993, 1996; Reimmann et al. 1998). Pch (probably in its iron-loaded form) acts as an effector molecule of PchR and allows this regulator to bind a 32-bp sequence motif (PchR-box) in promoter regions of these genes (Fig. 4a; Michel et al. 2005). Binding of PchR $\sim$ Pch to the PchR-box in the *pchD* promoter also represses transcription of *pchR* itself, as this PchR binding site is located downstream of the *pchR* transcription start (Michel et al. 2005).

Regulation in *P. fluorescens* is probably similar (Fig. 4b). Well-conserved Fur-boxes are present in the promoter regions of *pchDHIEFKCBA*, *pchR*, and *fetABCDEF* (Paulsen et al. 2005), suggesting that these genes are repressed under iron replete conditions. Moreover, potential PchR-boxes are found upstream of *fetABCDEF* and in the *pchR-pchDHIEFKCBA* intergenic region (Michel et al. 2005; Paulsen et al. 2005). Indeed, *pchDHIEFKCBA* expression was shown to require PchR and E-Pch (Youard et al. 2007; Youard and Reimmann 2010). As the transcription start sites of *pchR* and *pchDHIEFKCBA* have not yet been determined, it cannot be predicted if binding of PchR $\sim$ E-Pch to the PchR-box upstream of *pchD* will result in concomitant repression of *pchR* (Fig. 4b).

Stereospecificity

Iron uptake and transcriptional regulation with Pch and E-Pch are stereospecific processes in both *Pseudomonas* species (Youard et al. 2007; Hoegy et al. 2009; Youard and Reimmann 2010). Stereospecificity of iron uptake firstly involves the siderophore receptors FptA and FetA which are not closely related (Fig. 5a) and have opposite binding enantioselectivities. Hoegy et al. (2009) have shown that the Pch receptor FptA does not bind and transport ferriE-Pch, while the E-Pch receptor FetA is unable to interact with ferriPch. Docking experiments using

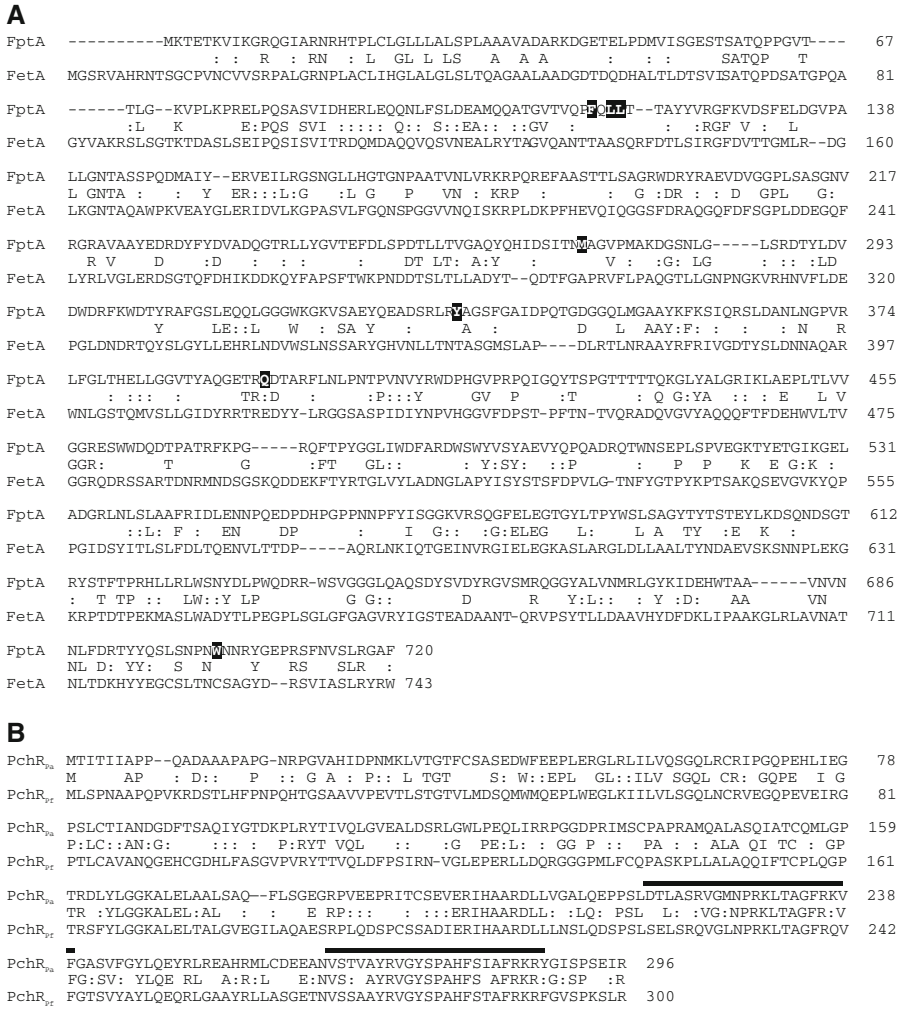

Fig. 5 Amino acid sequence alignment of the outer membrane receptors FptA and FetA (**a**) and of the two regulatory proteins $PchR_{Pa}$ (*P. aeruginosa*) and $PchR_{Pf}$ (*P. fluorescens*) (**b**) performed with ClustalW2. Note that FptA and FetA are not closely related (25% identical amino acids) and that the highlighted residues F114, L116, L117, M271, Y334, Q395 and W702 which form the ferriPch binding pocket in FptA (Cobessi et al. 2005) are not conserved in the FetA receptor. In the PchR proteins, siderophore specificity maps to the N-terminal part (amino acids ~20 to ~170; Youard and Reimmann 2010) while the C-terminal domain is involved in DNA binding (the two helix-turn-helix DNA binding motifs are highlighted by *black bars* above the sequence alignment)

the FptA structure (Cobessi et al. 2005) indicate that stereospecific siderophore recognition is based on the configuration of the chiral centers C2″ and C4″ while the configuration at C4′ seems less important (Hoegy et al. 2009).

A possible stereospecificity of the inner membrane transporters has not been investigated so far but it seems that siderophore translocation across the cytoplasmic membrane is generally less specific than transport through the outer membrane (Köster 2001). It is interesting to note however that the two bacterial species employ transporters belonging to different families, i.e. a single-subunit siderophore transporter for ferriPch uptake in *P. aeruginosa* and a periplasmic protein-dependent ABC transporter for ferriE-Pch transport in *P. fluorescens*.

Stereospecificity in transcriptional regulation with Pch and E-Pch is conferred by the regulatory proteins PchR (Youard and Reimmann 2010). In *P. aeruginosa*, PchR requires Pch as an effector and no activation occurs with E-Pch (Michel et al. 2005; Youard et al. 2007; Youard and Reimmann 2010). Likewise, the *P. fluorescens* PchR protein is activated by E-Pch but not by Pch, although the specificity seems somewhat less stringent than that of *P. aeruginosa* PchR (Youard et al. 2007; Youard and Reimmann 2010). How do the PchR homologues distinguish between the two enantiomers? The amino acid sequences at the C-terminal DNA binding domains of both PchR homologues are similar, while their N-terminal domains are less conserved and could thus be involved in siderophore recognition

(Fig. 5b). Indeed, an analysis of hybrid PchR proteins suggests that an N-terminal domain of about 150 amino acids confers siderophore specificity (Youard and Reimmann 2010).

Concluding remarks and outstanding questions

The naturally occurring siderophore enantiomer pair Pch and E-Pch offers a unique opportunity to study stereospecificity in iron uptake at several levels. With regard to ferrisiderophore transport across the outer membrane, it will be fascinating to determine the crystal structure of FetA and to compare it with the published structure of the FptA receptor (Cobessi et al. 2005). This will show how siderophore binding pockets have evolved to accommodate ligands of opposite chirality. As mentioned above, in ferriPch and ferriE-Pch translocation across the inner membrane, the importance of siderophore chirality is unknown and it is not clear why mechanistically different transporters are utilized. Swapping the genes for these transporters between *P. aeruginosa* and *P. fluorescens* will allow us to assess a potential stereospecificity in this process.

Regarding siderophore-dependent activation of PchR, there is now good evidence that both siderophores interact, in a stereospecific manner, with the N-terminal part of their cognate PchR protein. In vitro binding studies could confirm this, and, moreover, such experiments could clarify if PchR binds the iron-loaded form of the siderophore as proposed earlier (Michel et al. 2005).

The biosynthetic pathway for E-Pch is not yet fully understood. (i) It is not clear which enzyme epimerizes the second cysteine moiety. A candidate is the methyltransferase domain of PchF, which may have a dual methyltransferase/epimerase function (H.J. Imker and C.T. Walsh, personal communication). (ii) The reductase activity of PchK remains to be demonstrated biochemically. To solve these issues, E-Pch synthesis needs to be reconstituted with purified proteins in vitro, and pathway intermediates and end products need to be identified.

What is the biological role and significance of siderophore enantiomers? Stereochemical variations of siderophores can be regarded as a relatively rare form of defense against siderophore piracy. To our knowledge, the only other known example of siderophore enantiomers is rhizoferrin, which is made as *R,R*-rhizoferrin by the fungus *Rhizopus* and other members of the Zygomycetes (Drechsel et al. 1992; Thieken and Winkelmann 1992), and as *S,S*-rhizoferrin by the soil bacterium *Ralstonia pickettii* (Münzinger et al. 1999). Compared with the immense structural variety of pyoverdines made by fluorescent pseudomonads (Meyer 2000; Budzikiewicz 2004), the possibilities of generating diversity through siderophore enantiomers are more limited. Given that secondary siderophores of pseudomonads are often endowed with additional biological activities important in antibiosis, plant defense, inflammation, or biodegradation (Cornelis 2010), it may be that Pch enantiomers could have novel biological activities to be discovered.

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