Stereospecific recognition of pyochelin and enantio-pyochelin by the PchR proteins in fluorescent pseudomonads

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The siderophore pyochelin of *Pseudomonas aeruginosa* promotes growth under iron limitation and induces the expression of its biosynthesis genes via the transcriptional AraC/XylS-type regulator PchR. *Pseudomonas fluorescens* strain CHA0 makes the optical antipode of pyochelin termed enantio-pyochelin, which also promotes growth and induces the expression of its biosynthesis genes when iron is scarce. Growth promotion and signalling by pyochelin and enantio-pyochelin are highly stereospecific and are known to involve the pyochelin and enantio-pyochelin outer-membrane receptors FptA and FetA, respectively. Here we show that stereospecificity in signalling is also based on the stereospecificity of the homologous PchR proteins of *P. aeruginosa* and *P. fluorescens* towards their respective siderophore effectors. We found that PchR functioned in the heterologous species only if supplied with its native ligand and that the FptA and FetA receptors enhanced the efficiency of signalling. By constructing and expressing hybrid and truncated PchR regulators we showed that the weakly conserved N-terminal domain of PchR is responsible for siderophore specificity. Thus, both uptake and transcriptional regulation confer stereospecificity to pyochelin and enantio-pyochelin biosynthesis.

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INTRODUCTION

Iron is essential for the growth of most living organisms, including bacteria. Although an abundant element in nature, its bioavailability for bacteria growing in aerobic conditions is low due to poor solubility (about 1.4×10^{-9} M at pH 7.0 for ferric iron; Chipperfield & Ratledge, 2000). For pathogenic bacteria iron can be even scarcer due to it being bound to iron storage and transport proteins within mammalian hosts. To acquire sufficient iron for growth, bacteria have evolved a number of strategies, the most common of which is the production of low-molecular-mass iron-chelating molecules termed siderophores. Under iron limitation siderophores are secreted by the bacterium into the extracellular environment, where they chelate ferric iron and return it to the cell via specific receptor proteins in the cell membrane.

Pyochelin, 2-(2-*o*-hydroxyphenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid (Cox *et al.*, 1981), is a thiazoline siderophore produced by the opportunistic pathogen *Pseudomonas aeruginosa* PAO1 (ATCC 15692) and other closely related species (Sokol, 1986; Castignetti, 1997; Darling *et al.*, 1998). Pyochelin chelates ferric iron in solution with a 2:1 siderophore:ligand stoichiometry (Schlegel *et al.*, 2006; Tseng *et al.*, 2006) and a dissociation constant of 2.4×10^5 M⁻¹

in ethanol (Cox & Graham, 1979). This is low compared to the iron affinity of pyoverdine, the primary siderophore of *P. aeruginosa* (dissociation constant $\sim 10^{24}$ M⁻¹ at pH 7.0; Visca *et al.*, 2007), but the iron affinity of pyochelin in aqueous solution at physiological pH is unknown and may be higher. Pyochelin is a condensation product of salicylate and two cysteinyl residues and its biosynthesis in *P. aeruginosa* requires proteins encoded by the operons *pchDCBA* and *pchEFGHI* (Serino *et al.*, 1995, 1997; Reimmann *et al.*, 1998, 2001). Pyochelin contains three chiral centres (C4', C2'' and C4'') and exists in solution as a mixture of two interconvertible stereoisomers (Fig. 1): pyochelin I (4'R, 2''R, 4''R) and pyochelin II (4'R, 2''S, 4''R) (Ankenbauer *et al.*, 1988; Rinehart *et al.*, 1995) although only pyochelin I has been observed to bind ferric iron (Schlegel *et al.*, 2004, 2006).

Upon iron chelation, pyochelin is transported back into the cell via a specific outer-membrane receptor, FptA, in conjunction with an inner-membrane permease, FptX (Ankenbauer & Quan, 1994; Ó Cuiv *et al.*, 2004; Michel *et al.*, 2007). Pyochelin then upregulates its own biosynthesis and uptake genes by activating the AraC/XylS-family transcriptional regulator PchR. Activated PchR binds to a conserved sequence motif (PchR-box) in the promoter regions of *pchDCBA*, *pchEFGHI*, *fptABCX* and *pchR* and regulates transcription of these genes (Michel *et al.*, 2005, 2007). Unlike AraC, the prototype of this family (Schleif, 2003), PchR appears to have only one binding site in these

A supplementary table of primers and a supplementary figure are available with the online version of this paper.

Fig. 1. Structures and configurations of pyochelin and enantio-pyochelin. Pyochelin and enantio-pyochelin each contain three chiral carbons (C4', C2'' and C4'') and exist as a pair of spontaneously interconvertible stereoisomers. Epimerization upon binding of metal (M^{n+}) has only been shown for pyochelin (Schlegel *et al.*, 2004, 2006) but may also occur with enantio-pyochelin.

promoters and there is no evidence so far for DNA looping. Moreover, it is currently unknown whether PchR, like AraC, is functional as a dimer.

Recently, we reported that the plant-beneficial bacterium *P. fluorescens* CHA0 produces the optical antipode of pyochelin termed enantio-pyochelin (Fig. 1; Youard *et al.*, 2007). Enantio-pyochelin was found to promote growth of *P. fluorescens* under iron limitation and to upregulate its own biosynthesis genes in a manner similar to that of pyochelin in *P. aeruginosa* (Youard *et al.*, 2007). However, enantio-pyochelin does not promote growth of *P. aeruginosa* and pyochelin has no such activity in *P. fluorescens* (Youard *et al.*, 2007). This stereospecificity correlates with the structural specificity of the pyochelin and enantio-pyochelin receptors. The pyochelin receptor FptA does not bind and transport iron-loaded enantio-pyochelin in *P. aeruginosa*, whereas the enantio-pyochelin receptor FetA does not bind and transport ferri-pyochelin in *P. fluorescens*. Swapping the pyochelin and the enantio-pyochelin uptake systems between the two bacterial species inverted their siderophore preference and allowed *P. aeruginosa* to utilize enantio-pyochelin as an iron source while *P. fluorescens* was enabled to grow with iron-loaded pyochelin (Hoegy *et al.*, 2009).

Stereospecificity is also observed in signalling with pyochelin and enantio-pyochelin. Addition of enantio-

pyochelin to *P. aeruginosa* cultures does not induce the expression of the pyochelin biosynthesis genes and addition of pyochelin to *P. fluorescens* cultures hardly induces the expression of the enantio-pyochelin biosynthesis genes (Youard *et al.*, 2007). It has not been clear, however, if this stereospecificity in signalling might be caused simply by the failure of FptA and FetA to internalize the siderophore of the other species, thereby preventing the activation of the PchR regulatory protein, or whether additionally the PchR protein might not be activated by the siderophore of the other species.

Here we show that stereospecificity in signalling is based, in addition, on the specificity of the PchR proteins of *P. aeruginosa* and *P. fluorescens* towards their siderophore ligand and we investigate how these proteins can distinguish between the two enantiomers. By constructing hybrid and truncated PchR proteins we obtained evidence for the N-terminal part of PchR being responsible for siderophore recognition.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown on nutrient agar and in nutrient yeast broth at 37 °C (*P. aeruginosa* and *Escherichia coli*) or 30 °C (*P. fluorescens*) (Stanisich & Holloway, 1972). For green fluorescent protein (GFP) reporter assays,

strains were cultivated in the complex medium GGP (Carmi *et al.*, 1994), in which iron is present but not immediately accessible, thus inducing the expression of siderophore biosynthesis genes. Antibiotics, when required, were added to the growth media at the following concentrations: ampicillin (Ap) 100 µg ml⁻¹, gentamicin (Gm) 10 µg ml⁻¹ and tetracycline (Tc) 25 µg ml⁻¹ for *E. coli*; Tc 125 µg ml⁻¹ and Gm 10 µg ml⁻¹ for *P. aeruginosa* and *P. fluorescens*; kanamycin (Km) 50 µg ml⁻¹ for *P. fluorescens*. To counterselect *E. coli* donor cells in gene replacement experiments, chloramphenicol (Cm) was used at a concentration of 10 µg ml⁻¹; mutant enrichment was performed with Tc at a final concentration of 20 µg ml⁻¹ and carbenicillin (for *P. aeruginosa*) or cycloserine (for *P. fluorescens*) at final concentrations of 2 mg ml⁻¹ and 50 mg ml⁻¹, respectively. Pyochelin and enantio-pyochelin were purified from culture supernatants of *P. aeruginosa* and *P. fluorescens*, respectively, as described previously (Youard *et al.*, 2007) and added to the growth medium at a final concentration of 20 µM. This concentration, which is at least 20-fold below the physiological concentration reached under iron starvation (Reimmann *et al.*, 1998 and our unpublished results), fully induces the expression of pyochelin and enantio-pyochelin biosynthesis genes in *P. aeruginosa* and *P. fluorescens*, respectively (Youard *et al.*, 2007) and restores growth under iron limitation in siderophore-negative mutants (Hoegy *et al.*, 2009). For IPTG-induced expression of proteins in GGP medium a concentration of 1 mM was used; for IPTG-induced expression of proteins on Chrome Azurol S (CAS) agar (Schwyn & Neilands, 1987), each plate was surface-spread with 100 µl of a 500 mM IPTG stock solution.

DNA manipulations and sequencing. Oligonucleotides used in this study are listed in Supplementary Table S1, available with the online version of this paper. Small- and large-scale preparations of plasmid DNA were made with the QIAprep Spin Miniprep kit (Qiagen) and Jetstar kit (Genomed), respectively. DNA fragments were purified from agarose gels using the MinElute and QIAquick Gel Extraction kits from Qiagen. DNA manipulations were performed according to standard procedures (Sambrook *et al.*, 2001). Transformation of *E. coli*, *P. aeruginosa* and *P. fluorescens* was carried out by electroporation (Farinha & Kropinski, 1990). All constructs involving PCR techniques were verified by sequence analysis. Sequencing was performed with the BigDye Terminator Cycle Sequencing kit and an ABI-PRISM 373 automatic sequencer (Applied Biosystems) or was carried out commercially by Microsynth AG. Sequence alignments were performed using the program BLAST 2 Sequences (Tatusova & Madden, 1999) available at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>).

Mutant constructions. Gene replacement mutants of *P. aeruginosa* and *P. fluorescens* were generated with suicide plasmids as described previously (Schnider *et al.*, 1995; Ye *et al.*, 1995; Laville *et al.*, 1998). All gene replacement mutants were checked by PCR. The suicide plasmid pME9253, used to generate an 897 bp deletion in the *pchR* gene of *P. fluorescens*, was constructed as follows. Two PCR fragments were generated from chromosomal DNA of *P. fluorescens* CHA0 using the primer pairs pchR1/pchR2 and pchR3/pchR4. The two fragments were then combined by overlap extension PCR using an overhang on the first fragment. The combined fragment, cleaved with *Hind*III and *Bam*HI, was cloned into the suicide vector pME3087 between the *Hind*III and *Bam*HI sites. Plasmid pME9253 was then mobilized from *E. coli* DH5α into the *P. fluorescens* strains CHA400 and CHA1084 using the helper plasmid pME497 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 to generate the mutants CHA1222 ($\Delta pchR$, *pvd*::Tn1733) and CHA1223 ($\Delta pchR$, $\Delta pchDHIEFKCBA$), respectively. The previously described suicide plasmids pME7531 (Hoegy *et al.*, 2009) and pME7152 (Michel *et al.*, 2007) were used in a similar way to delete the enantio-pyochelin receptor gene in *P. fluorescens* CHA0 and the

pyoverdine biosynthesis gene *pvdF* in *P. aeruginosa* PAO6274, generating the corresponding mutants CHA1086 ($\Delta fetA$) and PAO6402 (*pchR*:: ω , $\Delta pvdF$), respectively.

Cloning of *pchR*_{CHA0}. The *pchR* gene of *P. fluorescens* CHA0 and its complete upstream intergenic region were amplified from genomic DNA of *P. fluorescens* CHA0 by PCR using the primers pchR5 and pchR6. This fragment was cleaved with *Kpn*I and *Hind*III and cloned into the *Kpn*I- and *Hind*III-cut chromosomal integration vector pME3280a to generate plasmid pME9252.

Swapping the promoter and leader regions between *pchR*_{PAO1} and *pchR*_{CHA0}. Plasmid pME9254 containing *pchR* of *P. aeruginosa* fused to the upstream intergenic region (promoter and leader) of *P. fluorescens* *pchR* was constructed as follows. Two PCR fragments were generated from chromosomal DNA of *P. fluorescens* CHA0 and *P. aeruginosa* PAO1, respectively. Fragment 1 was amplified using the primers pchR7 and pchR8 and fragment 2 with primers pchR9 and pchR10. The two fragments were then combined by overlap extension PCR using an overhang on the first fragment and cleaved with *Hind*III and *Kpn*I. Finally, the cleaved fragment was inserted into pME3280a between the *Hind*III and *Kpn*I sites. Plasmid pME9255 containing *pchR* of *P. fluorescens* fused to the upstream intergenic region of *P. aeruginosa* *pchR* was constructed in a similar way. PCR fragment 1 was amplified from *P. aeruginosa* using the primers pchR11 and pchR12 and fragment 2 was amplified from *P. fluorescens* with primers pchR13 and pchR14. The two fragments were combined by overlap extension PCR using an overhang on the first fragment, cleaved with *Hind*III and *Kpn*I and ligated into pME3280a.

Construction of N-terminal PchR expression plasmids. To construct plasmid pME9264, nucleotides 1–609 of *pchR*_{PAO1} were amplified by PCR from chromosomal DNA of *P. aeruginosa* PAO1 using the primers pchR36a and pchR37. This fragment was cleaved with *Eco*RI and *Bam*HI and ligated into *Eco*RI- and *Bam*HI-cut pME6032. The resulting plasmid expresses amino acids 1–203 of PchR_{PAO1} fused to a C-terminal His₆ tag provided by primer pchR37. Plasmid pME9265 expressing amino acids 1–207 of PchR_{CHA0} fused to a C-terminal His₆ tag was obtained in a similar way. Nucleotides 1–621 of *pchR*_{CHA0} were PCR-amplified from *P. fluorescens* CHA0 using the primers pchR38a and pchR39. The resulting DNA fragment was then trimmed with *Eco*RI and *Bam*HI and cloned into pME6032.

Construction of hybrid *pchR* genes. Hybrid *pchR* genes were constructed by overlap PCR. DNA from *pchR*_{PAO1} and *pchR*_{CHA0} was fused at locations (determined by sequence alignment) that encoded identical amino acid sequences in both PchR proteins (see Fig. 5). For hybrid *pchRs* 1–3 two DNA fragments were amplified, one containing the upstream intergenic region of *pchR*_{CHA0} and a 5' section of the adjacent *pchR* gene (amplified from *P. fluorescens* CHA0 genomic DNA), and a second encoding a 3' section of *pchR*_{PAO1} (amplified from *P. aeruginosa* PAO1 genomic DNA) including the stop codon. The second fragment was amplified using a forward primer with a 5' overlap sequence identical to the 3' end of the first PCR fragment. The two PCR products were then combined with a second PCR cycle, cleaved with *Hind*III and *Spe*I and ligated into *Hind*III- and *Spe*I-cut pME3280a. Hybrid 1 (pME9263) was generated using primers pchR15, pchR34, pchR35 and pchR25, hybrid 2 (pME9258) using primers pchR15, pchR21, pchR22 and pchR25, and hybrid 3 (pME9260) using primers pchR15, pchR40, pchR41 and pchR25.

For hybrids 1a–3a the method was the same as described above except that pME9254 was used as the template (upstream intergenic region of *pchR*_{CHA0} fused to *pchR*_{PAO1}) for the first fragment and chromosomal DNA of *P. fluorescens* CHA0 was used as the template for the second. Hybrid 1a (pME9266) was constructed using primers pchR42, pchR54, pchR55 and pchR45, hybrid 2a (pME9269) using

Table 1. Bacterial strains and plasmids

Name	Relevant characteristics	Reference/source
<i>E. coli</i>		
DH5 α	<i>recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1</i> $\Delta(lacZYA-argF)U169$ (ϕ 80d <i>lacZ</i> AM15)	Sambrook & Russell (2001)
<i>P. aeruginosa</i>		
PAO1	Wild-type	ATCC 15692
PAO6274	<i>pchR::ω</i>	Reimmann <i>et al.</i> (1998)
PAO6331	$\Delta pchDCBA \Delta pchR \Delta pchEFGHI$	Reimmann <i>et al.</i> (2001)
PAO6382	$\Delta pvdF$	Hoegy <i>et al.</i> (2009)
PAO6402	<i>pchR::ω $\Delta pvdF$</i>	This study
PAO6428	$\Delta fptA$	Michel <i>et al.</i> (2007)
<i>P. fluorescens</i>		
CHA0	Wild-type	Voisard <i>et al.</i> (1994)
CHA400	<i>pvd::Tn1733, Km^r</i>	Keel <i>et al.</i> (1989)
CHA1084	$\Delta pchDHIEFKCBA$	Youard <i>et al.</i> (2007)
CHA1086	$\Delta fetA$	This study
CHA1222	$\Delta pchR pvd::Tn1733, Kmr$	This study
CHA1223	$\Delta pchR \Delta pchDHIEFKCBA$	This study
Plasmids		
pME497	Mobilizing plasmid, Ap ^r	Voisard <i>et al.</i> (1988)
pME3087	Suicide vector, ColE1 replicon, Tc ^r	Voisard <i>et al.</i> (1988)
pME3280a	Chromosomal integration vector, mini-Tn7, Ap ^r Gm ^r	Zuber <i>et al.</i> (2003)
pME6032	<i>lacI^q-P_{lac}</i> expression vector, Tc ^r	Heeb <i>et al.</i> (2002)
pME7152	pME3087 derivative carrying $\Delta pvdF$, Tc ^r	Michel <i>et al.</i> (2007)
pME7531	pME3087 derivative carrying $\Delta fetA$, Tc ^r	Hoegy <i>et al.</i> (2009)
pME7547	pME3280a derivative containing <i>pchR</i> _{PAO1} on mini-Tn7, Ap ^r Gm ^r	Michel <i>et al.</i> (2005)
pME7585	Translational fusion of <i>pchD</i> _{CHA0} to <i>gfp</i> in pPROBE-TT', Tc ^r	Youard <i>et al.</i> (2007)
pME7588	Translational fusion of <i>pchE</i> _{PAO1} to <i>gfp</i> in pPROBE-TT', Tc ^r	Youard <i>et al.</i> (2007)
pME9252	pME3280a derivative containing <i>pchR</i> _{CHA0} on mini-Tn7, Ap ^r Gm ^r	This study
pME9253	pME3087 derivative carrying $\Delta pchR$ _{CHA0} , Tc ^r	This study
pME9254	pME3280a derivative containing <i>pchR</i> _{PAO1} with promoter and leader region of <i>pchR</i> _{CHA0} on mini-Tn7, Ap ^r Gm ^r	This study
pME9255	pME3280a derivative containing <i>pchR</i> _{CHA0} with promoter and leader region of <i>pchR</i> _{PAO1} on mini-Tn7, Ap ^r Gm ^r	This study
pME9258	pME3280a derivative containing <i>pchR</i> hybrid 2 on mini-Tn7, Ap ^r Gm ^r	This study
pME9260	pME3280a derivative containing <i>pchR</i> hybrid 3 on mini-Tn7, Ap ^r Gm ^r	This study
pME9261	pME3280a derivative containing <i>pchR</i> hybrid 3a on mini-Tn7, Ap ^r Gm ^r	This study
pME9263	pME3280a derivative containing <i>pchR</i> hybrid 1 on mini-Tn7, Ap ^r Gm ^r	This study
pME9264	pME6032 derivative containing the first 609 bp of <i>pchR</i> _{PAO1} with a 3' histidine tag, Tc ^r	This study
pME9265	pME6032 derivative containing the first 621 bp of <i>pchR</i> _{CHA0} with a 3' histidine tag, Tc ^r	This study
pME9266	pME3280a derivative containing <i>pchR</i> hybrid 1a on mini-Tn7, Ap ^r Gm ^r	This study
pME9269	pME3280a derivative containing <i>pchR</i> hybrid 2a on mini-Tn7, Ap ^r Gm ^r	This study
pPROBE-TT'	pVS1/p15A shuttle vector for construction of <i>gfp</i> fusions, Tc ^r	Miller <i>et al.</i> (2000)
pUX-BF13	Plasmid carrying genes for Tn7 transposition, Ap ^r	Bao <i>et al.</i> (1991)

primers pchR42, pchR48, pchR49 and pchR45, and hybrid 3a (pME9261) using primers pchR42, pchR43, pchR44 and pchR45.

Wild-type and hybrid *pchR* genes were integrated as mini-Tn7-derivatives into the chromosome of *P. aeruginosa* and *P. fluorescens* as described previously, using plasmid pUX-BF13 containing the genes for Tn7 transposition (Zuber *et al.*, 2003).

GFP reporter assays. *Pseudomonas* strains carrying translational *gfp* fusions were grown in 96-well black microtitre plates (Greiner bio-one) with a flat transparent bottom. Each well contained 200 μ l GGP

medium and was inoculated with 3 μ l of a bacterial culture grown overnight in the same medium. HPLC-purified pyochelin or enantio-pyochelin was dissolved in methanol to a concentration of 10 mM (determined by the CAS assay; Schwyn & Neilands, 1987) and added to the growth medium at the concentrations indicated. Microtitre plates were incubated at 37 °C (*P. aeruginosa*) or 30 °C (*P. fluorescens*) with orbital shaking at 500 r.p.m. At each given time point, growth (OD₆₀₀) and green fluorescence (excitation at 480 nm and emission at 520 nm) were measured from triplicate cultures using a Fluostar fluorescence microplate reader (BMG Lab Technologies). For each individual measurement the green fluorescence value was divided by the respective

Fig. 2. Siderophore production of *P. aeruginosa* and *P. fluorescens* *pchR* mutants complemented by homologous and heterologous *pchR* genes. The *pchR* genes of *P. aeruginosa* and *P. fluorescens* carried by pME7547 and pME9252, respectively, were tested for their ability to complement the pyoverdine-negative (*pvd*) *pchR* mutants PAO6402 and CHA1222. Pyochelin and enantio-pyochelin production can be visualized by the formation of an orange halo surrounding the colony. The pyoverdine-negative parent strains PAO6382 and CHA400 served as positive controls. The experiment was repeated twice with very similar results. Note that pyoverdine-negative strains were used to ensure that the haloes observed on CAS agar were caused by pyochelin or enantio-pyochelin.

OD₆₀₀ value to give the specific fluorescence of cells expressed as relative fluorescence units. The green fluorescence of cells carrying the empty vector pPROBE-TT' was determined for background fluorescence correction.

Test for siderophore production on CAS agar. CAS agar plates were prepared as described by Schwyn & Neilands (1987). CAS agar contains a blue-coloured iron dye complex (chrome azurol S–iron(III)–hexadecyltrimethylammonium bromide) that changes colour to orange upon formation of an iron–ligand complex and release of the dye. Strains were tested for siderophore production by dotting 2 µl of an overnight culture (grown in iron-limited GGP medium) onto CAS agar and incubating the plate overnight at the appropriate temperature. Siderophore-producing strains were identified by the presence of an orange halo surrounding the colony.

RESULTS

The *pchR* genes of *P. aeruginosa* and *P. fluorescens* do not complement *pchR* mutations in the other species

To see whether the PchR proteins are specific for pyochelin and enantio-pyochelin, respectively, we first tested if *pchR* of *P. aeruginosa* could complement a *pchR* mutation in *P. fluorescens* and vice versa (Fig. 2). As expected, no siderophore production was detected on CAS agar medium

with the pyoverdine-negative *pchR* mutant PAO6402, confirming that *pchR* is essential for pyochelin production in *P. aeruginosa* (Heinrichs & Poole, 1993). Pyochelin production in PAO6402 was entirely restored with *pchR* of *P. aeruginosa* (*pchR*_{PAO1}) carried by pME7547 and delivered in monocopy to the chromosome of the *pchR* mutant as a mini-Tn7 derivative (see Methods). By contrast, when *pchR* of *P. fluorescens* (*pchR*_{CHA0}) carried by pME9252 was integrated into the chromosome of PAO6402, no complementation occurred, indicating that PchR_{CHA0} does not function in *P. aeruginosa*. Likewise, *pchR*_{PAO1} was unable to complement the *pchR* mutation in the pyoverdine-negative *P. fluorescens* mutant CHA1222, whereas enantio-pyochelin production was restored in this mutant by *pchR*_{CHA0}. We conclude from these experiments that both *pchR* genes are species-specific.

Species-specificity of PchR could be caused (i) by *pchR* expression problems in the heterologous host, (ii) by sequence differences between the PchR-boxes of *P. aeruginosa* and *P. fluorescens*, or (iii) by a specificity of PchR for the native ligand. However, the PchR-box sequences of *P. aeruginosa* and *P. fluorescens* are almost identical (Michel *et al.*, 2005) and the *pchR* genes were expressed in the heterologous background (see below, Fig. 3). Therefore, it seemed likely that the PchR proteins were specific for their native ligand.

Fig. 3. Specificity of the *P. aeruginosa* and *P. fluorescens* PchR proteins for their native ligand. The pyochelin-negative *pchR* mutant of *P. aeruginosa* (PAO6331; a, b) and the enantio-pyochelin-negative *pchR* mutant of *P. fluorescens* (CHA1223; c, d) were complemented with *pchR*_{PAO1} ($\diamond$; pME7547), *pchR*_{CHA0} ($\blacktriangle$, pME9252) or transformed with the empty vector pME3280a ($\times$) and grown in GGP medium containing 20 μ M HPLC-purified pyochelin (Pch) (a, c) or enantio-pyochelin (E-Pch) (b, d). *P. aeruginosa* strains contained the reporter plasmid pME7588 with a *pchE*_{PAO1}'-'*gfp* translational fusion, *P. fluorescens* strains contained pME7585 with a *pchD*_{CHA0}'-'*gfp* translational fusion. Background fluorescence was assessed in both species with the empty vector pPROBE-TT'. Strains were grown in microtitre wells for 24 h, and green fluorescence and growth (OD₆₀₀) were measured from three parallel experiments. Fluorescence is expressed as relative fluorescence units $\pm$ standard deviation.

Specificity of PchR for pyochelin or enantio-pyochelin

We compared the ability of pyochelin and enantio-pyochelin to activate PchR_{PAO1} and PchR_{CHA0} in both *Pseudomonas* species. Experiments were carried out with the *pchR* mutant strains PAO6331 and CHA1223, which were grown in iron-limited GGP medium supplemented with HPLC-purified pyochelin or enantio-pyochelin. These strains are also pyochelin- and enantio-pyochelin-negative, respectively, to ensure that the only siderophore able to interact with PchR would be the one supplied to the growth medium. In agreement with previous findings (Michel *et al.*, 2005), monocopy insertion of *pchR*_{PAO1} into the chromosome of PAO6331 enabled expression of the pyochelin biosynthesis gene *pchE*_{PAO1} when the strain was grown in medium supplemented with pyochelin (Fig. 3a). Addition of enantio-pyochelin to the growth medium did not promote *pchE*_{PAO1} expression (Fig. 3b). Conversely, *pchR*_{CHA0} promoted *pchE*_{PAO1} expression in PAO6331

when grown with enantio-pyochelin (Fig. 3b) while pyochelin had only a very small effect (Fig. 3a). Stereospecificity of the two PchR proteins was confirmed by experiments carried out in *P. fluorescens* using the enantio-pyochelin biosynthesis gene *pchD*_{CHA0} as a reporter. As shown in Fig. 3(c, d) *pchR*_{PAO1} was able to promote the expression of *pchD*_{CHA0} only in the presence of pyochelin, whereas activation of PchR_{CHA0} occurred with enantio-pyochelin. Again, PchR_{CHA0} was slightly activated by pyochelin as well, thus confirming our previous findings, which suggested that PchR_{CHA0} could have a somewhat more relaxed specificity for its ligand than PchR_{PAO1} (Youard *et al.*, 2007).

We conclude from these data that (i) both *pchR* genes are expressed in *P. aeruginosa* and *P. fluorescens*, (ii) both PchR proteins are able to recognize the PchR-box in the promoter regions of *pchE*_{PAO1} and *pchD*_{CHA0}, and (iii) PchR_{PAO1} is specific for pyochelin while PchR_{CHA0} prefers enantio-pyochelin. Note that expression levels and kinetics

of the reporter were not identical in the two species, especially not for enantio-pyochelin (Fig. 3b, d), probably because of differences in growth conditions (37 °C for *P. aeruginosa* vs 30 °C for *P. fluorescens*), siderophore uptake (receptor-dependent and receptor-independent, see below), PchR-box recognition (different reporter genes were used in the two species), or *pchR* expression levels.

Stereospecificity of the receptor proteins FptA and FetA is not a prerequisite for stereospecificity in signalling

The pyochelin and enantio-pyochelin receptors FptA and FetA are specific for binding and transport of their respective ferri-siderophores (Ankenbauer & Quan, 1994; Hoegy *et al.*, 2009), implying that for interaction with PchR (Fig. 3) both siderophores must also be able to enter the cells via another less specific mechanism. This was confirmed by comparing the expression of the pyochelin and enantio-pyochelin biosynthesis genes in the receptor mutants PAO6428 ($\Delta fptA$) and CHA1086 ($\Delta fetA$) to that in the wild-type strains PAO1 and CHA0 (Fig. 4). In both mutants, expression of the siderophore biosynthesis genes was delayed, but not abolished, compared to the wild-type strains and addition of pyochelin and enantio-pyochelin, respectively, to the growth medium increased expression in the wild-type and in the receptor mutants (Fig. 4). Moreover, deletion of the receptor genes in pyochelin- and enantio-pyochelin-negative mutants, respectively, did not abolish induction of *pchE*_{PAO1} by pyochelin in *P. aeruginosa* and of *pchD*_{CHA0} by enantio-pyochelin in *P.*

fluorescens, but reduced the dose-response compared to their corresponding receptor-positive parent (data not shown). Thus, the pyochelin/enantio-pyochelin receptors FptA/FetA increase signalling efficiency but they are not essential for signalling because sufficient amounts of both siderophores can bypass the specific receptors and enter the cell through a secondary, as yet unknown mechanism.

Siderophore specificity of PchR is conferred by the N-terminal part of the protein

Alignment of the deduced amino acid sequences of PchR_{PAO1} and PchR_{CHA0} showed that the primary sequences of the two regulators are very similar, with most variation occurring in the N-terminal ~20 residues and highest similarity in the C-terminal ~100 residues (Fig. 5a). On the basis of homology to other members of the AraC/XylS family of proteins (Gallegos *et al.*, 1997) the C-terminal region of PchR is presumed to form the DNA-binding domain, which could allow binding of PchR to the PchR-box in the promoter of pyochelin- and enantio-pyochelin-regulated genes. The N-terminal two-thirds of PchR are expected to form the siderophore recognition domain and may also be involved in multimerization, as shown for other members of the AraC/XylS family (Ruiz *et al.*, 2003 and references therein).

To investigate if the PchR N-terminal region is responsible for siderophore binding and stereospecificity we constructed a series of *pchR* hybrids encoding PchR proteins with different parts of PchR_{PAO1} and PchR_{CHA0}. Using the alignment of PchR_{PAO1} and PchR_{CHA0} we selected three

Fig. 4. The pyochelin/enantio-pyochelin receptors FptA/FetA contribute to, but are not essential for, siderophore signalling. (a) The *P. aeruginosa* wild-type (wt) strain PAO1 and its receptor-negative mutant PAO6428 ($\Delta fptA$) containing either the reporter plasmid pME7588 with a *pchE*_{PAO1}'-'*gfp* translational fusion or the empty vector pPROBE-TT' (control for background fluorescence) were grown in microtitre wells, and green fluorescence and growth (OD₆₀₀) were measured from three parallel experiments. Strains were grown in GGP medium (◇ for PAO1; ○ for PAO6428) or GGP medium amended with 20 μM HPLC-purified pyochelin (Pch) (◆ for PAO1+Pch; ● for PAO6428+Pch). Fluorescence is expressed as relative fluorescence units ± standard deviation. (b) The same experiment was performed with the *P. fluorescens* wild-type strain (wt) CHA0 and its receptor-negative derivative CHA1086 ($\Delta fetA$) grown in GGP medium (◇ for CHA0; ○ for CHA1086) or GGP medium with 20 μM enantio-pyochelin (E-Pch) (◆ for CHA0+E-Pch; ● for CHA1086+E-Pch). Strains carried pME7588 with *pchD*_{CHA0}'-'*gfp* as a reporter or pPROBE-TT' for measuring background fluorescence.

Fig. 5. Construction and activity of wild-type and hybrid *pchR* genes. (a) The deduced protein sequences of *P. aeruginosa* PchR_{PAO1} and *P. fluorescens* PchR_{CHA0} were aligned using the NCBI BLAST 2 Sequences algorithm (Tatusova & Madden, 1999). The two helix–turn–helix DNA-binding motifs characteristic of members of the AraC/XylS family (Gallegos *et al.*, 1997) are indicated by black bars above the sequence alignment. The three highlighted regions were selected for the construction of PchR hybrids. (b) The ability of wild-type and hybrid *pchR* genes to restore pyochelin and enantio-pyochelin production in the *pchR* mutant strains PAO6402 and CHA1222 was assessed on CAS agar and is indicated by stars, ranging from no stars for no complementation and no siderophore production to five stars for full complementation and wild-type siderophore production. The upstream promoter and leader regions of each gene are represented by a triangle and the *pchR* genes are represented by the adjacent rectangle. Sequences of *P. aeruginosa* PAO1 origin are in light grey; sequences from *P. fluorescens* CHA0 are in dark grey. The coordinates of PchR_{PAO1} and PchR_{CHA0} amino acids correspond to those shown in (a). For the original CAS agar images on which this figure is based see Supplementary Fig. S1.

regions of identical sequences to create the hybrids. Regions 1 and 2 are predicted to be within the siderophore-binding domain of PchR while region 3 is at the start of the predicted DNA-binding domain (Fig. 5a). Wild-type and hybrid *pchR* genes were integrated in monocopy into the pyoverdine-negative *pchR* mutants PAO6402 and CHA1222 and scored for their ability to complement the *pchR* mutation and restore production of

pyochelin and enantio-pyochelin, respectively. We first tested if swapping the promoter/leader sequences of *pchR*_{PAO1} and *pchR*_{CHA0} would affect the ability of the two genes to complement *pchR* mutations in *P. aeruginosa* and *P. fluorescens*, respectively. As shown in Fig. 5(b) and in Supplementary Fig. S1, pyochelin production was restored in PAO6402 equally well with pME7547 and pME9254, indicating that the *pchR*_{PAO1} gene is fully

functional in *P. aeruginosa* when linked to the promoter and leader sequences of *pchR*_{CHA0}. In contrast, enantio-pyochelin production in CHA1222 with pME9255 was low compared to pME9252 (Fig. 5b and Supplementary Fig. S1). For unknown reasons, combining the *pchR*_{PAO1} upstream region with the coding sequence of *pchR*_{CHA0} seems to prevent normal *pchR* expression in *P. fluorescens*. To circumvent this problem, we constructed all hybrid *pchR* genes with the promoter and leader region of *pchR*_{CHA0}.

Hybrids 1 and 1a (Fig. 5b) were constructed by an exchange at the conserved glycine residue located at positions 23 and 26, respectively, in PchR_{PAO1} and PchR_{CHA0} (Fig. 5a). Hybrid 1 was less effective in complementing PAO6402 than wild-type *pchR*_{PAO1} and non-functional in CHA1222. Hybrid 1a, in contrast, was almost fully functional in CHA1222 but unable to complement the *pchR* mutation in PAO6402. Thus, the first ~20 residues of PchR do not seem to be involved in conferring siderophore specificity to PchR but are functionally important, at least for PchR_{PAO1}.

Hybrids 2 and 2a were constructed by an exchange at the conserved sequence YLGKALEL (Fig. 5a). Hybrid 2 was non-functional in PAO6402 but restored a minor production of enantio-pyochelin in CHA1222, whereas hybrid 2a was non-functional in CHA1222 but partially active in PAO6402 (Fig. 5b; Supplementary Fig. S1). Hybrids 3 and 3a were constructed by an exchange at a conserved sequence NPRKLTAGFR within the predicted C-terminal domain of PchR (Fig. 5a) and complementation data resembled those obtained with hybrids 2 and 2a. Hybrid 3 partially complemented the *pchR* mutation in CHA1222, but was not functional in PAO6402, whereas hybrid 3a was unable to complement CHA1222 but showed partial activity in PAO6402 (Fig. 5b; Supplementary Fig. S1).

Together the results obtained with hybrids 2, 2a, 3 and 3a show that the specificity of PchR_{PAO1} for pyochelin and that of PchR_{CHA0} for enantio-pyochelin can be swapped by replacing the N-terminal domain of PchR with that of the other species. More precisely, these data suggest that the residues responsible for siderophore recognition lie between the conserved glycine (G23 in PchR_{PAO1}, G26 in PchR_{CHA0}) and the conserved sequence YLGKALEL (164–172 in PchR_{PAO1}, 166–174 in PchR_{CHA0}).

Overexpression of the PchR N-terminal domain reduces siderophore production

Overexpression of PchR derivatives lacking the C-terminal DNA-binding domain from an IPTG-inducible *tac* promoter interfered with siderophore production. This was seen in strain PAO6382, where pyochelin production was strongly reduced by overexpressing the first 203 amino acids of PchR_{PAO1} (N-PchR_{PAO1}), whereas overexpression of the first 207 amino acids of PchR_{CHA0} (N-PchR_{CHA0}) had no effect (Fig. 6). Likewise, overexpression of N-

PchR_{CHA0}, but not that of N-PchR_{PAO1}, reduced the production of enantio-pyochelin in CHA400 (Fig. 6). These results show that overexpression of the N-terminal part of either PchR protein inhibits the production of the corresponding siderophore, but does not interfere with siderophore production in the other species.

DISCUSSION

Stereospecificity in pyochelin/enantio-pyochelin signalling was thought to be caused by the reported stereospecificity of the FptA/FetA outer-membrane receptors (Hoegy *et al.*, 2009), which would prevent the heterologous siderophore from entering the cell and activating the PchR regulatory protein if no other siderophore uptake system were present. In this study we have shown, however, that stereospecificity in pyochelin/enantio-pyochelin signalling is additionally conferred by the transcriptional regulator PchR. Through the construction of hybrid *P. aeruginosa*/*P. fluorescens* PchR proteins we have demonstrated that the region important for recognition of pyochelin/enantio-pyochelin is located in the N-terminal part of PchR. Moreover, overexpressed N-terminal parts of PchR_{PAO1} and PchR_{CHA0} specifically inhibited the production of their native siderophores.

It appears that stereospecificity of PchR_{CHA0} is less stringent than that of PchR_{PAO1}, as the *P. fluorescens* protein was activated not only by enantio-pyochelin but to a minor extent also by pyochelin. In contrast, the activity of the *P. aeruginosa* protein was strictly dependent on pyochelin (Fig. 3; Youard *et al.*, 2007). It is not known,

Fig. 6. Inhibition of siderophore production by N-terminal PchR proteins. Pyoverdine-negative strains of *P. aeruginosa* (PAO6382) and *P. fluorescens* (CHA400) containing the N-terminal PchR expression plasmids pME9264 (for overexpression of N-PchR_{PAO1}) and pME9265 (for overexpression of N-PchR_{CHA0}) were grown overnight in GGP medium or GGP medium supplemented with 1 mM IPTG and tested for siderophore production on IPTG-surface-spread or IPTG-free CAS agar plates. Production of pyochelin/enantio-pyochelin can be visualized by the presence of an orange halo surrounding the colony. The experiment was repeated twice with very similar results.

however, if the expression levels of the two proteins are comparable in the two species, making a strong statement on this issue premature. Siderophore binding studies with purified PchR proteins *in vitro* will address this point in the future.

As shown in Fig. 3, the PchR proteins of *P. aeruginosa* and *P. fluorescens* were functional as regulators in the heterologous species when their native siderophore was supplied in the growth medium. Given that the *P. aeruginosa* outer-membrane receptor FptA is specific for pyochelin and the *P. fluorescens* receptor FetA is specific for enantio-pyochelin (Hoegy *et al.*, 2009), this implies that both siderophores are able to enter cells via another route at concentrations that are sufficient to permit PchR activation. Indeed, previous experiments performed in *P. aeruginosa* (Michel *et al.*, 2007) and the results shown in Fig. 4 confirm that mutations in the pyochelin and enantio-pyochelin receptor genes *fptA* and *fetA*, respectively, delay, but do not abolish, signalling in *P. aeruginosa* and *P. fluorescens*. By contrast, deletion of the pyochelin and enantio-pyochelin receptors prevents *Pseudomonas* from utilizing pyochelin and enantio-pyochelin for iron uptake (Michel *et al.*, 2007; Youard *et al.*, 2007). Therefore, it appears that pyochelin/enantio-pyochelin signalling requires a lower extracellular siderophore concentration than does growth promotion by pyochelin/enantio-pyochelin. Similar findings were reported for the siderophore yersiniabactin in *Yersinia pestis*, where transcriptional activation of the yersiniabactin biosynthesis genes by the PchR-related AraC-type regulator YbtA required a 500-fold lower siderophore concentration than was required for growth stimulation. Interestingly, however, mutations in the *Y. pestis* siderophore uptake genes did not reduce or delay transcriptional activation of the yersiniabactin genes, suggesting that receptor bypass of the siderophore in *Y. pestis* is even more efficient than in *Pseudomonas* and allows full activation of the YbtA regulator (Perry *et al.*, 2003).

Swapping the first ~170 residues of PchR changed the siderophore specificity of PchR_{PAO1} and PchR_{CHA0} (Fig. 5, hybrids 2 and 2a) while the ~20 N-terminal amino acids were not important (Fig. 5, hybrids 1 and 1a). These results indicate that key residues forming the siderophore-binding pocket of PchR lie in the region between residues ~20 and ~170. Except for hybrid 1a, none of the hybrids completely restored pyochelin or enantio-pyochelin production (Fig. 5b and Supplementary Fig. S1). This may not be too surprising, as the assay system used (siderophore production on CAS agar) does not directly measure siderophore binding to wild-type and hybrid PchR proteins but integrates the whole response on siderophore biosynthesis and export, meaning that minor changes in PchR activity or stability can have a large impact.

Overexpression of the N-terminal domains of PchR_{PAO1} and PchR_{CHA0} inhibited the function of their corresponding full-length proteins. At this stage it is uncertain whether inhibition is caused by binding and sequestering of the

respective siderophore, by physical interaction with the corresponding full-length PchR protein, or by a combination of both mechanisms. An interaction of N-PchR with PchR would support the idea that PchR functions as a dimer or multimer whereas siderophore titration seems less likely because siderophore production was still quite high, as evidenced by the orange halo produced on CAS agar (Fig. 6).

In conclusion, we have shown that PchR plays a crucial role in the stereospecificity of pyochelin and enantio-pyochelin signalling in *Pseudomonas* spp. and that the N-terminal part of PchR is responsible for siderophore recognition.

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