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In vitro-binding of the natural siderophore enantiomers pyochelin and enantiopyochelin to their AraC-type regulators PchR in *Pseudomonas*

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Abstract The enantiomeric siderophores pyochelin and enantiopyochelin of *Pseudomonas aeruginosa* and *Pseudomonas protegens* promote growth under iron limitation and activate transcription of their biosynthesis and uptake genes via the AraC-type regulator PchR. Here we investigated siderophore binding to PchR in vitro using fluorescence spectroscopy. A fusion of the N-terminal domain of *P. aeruginosa* PchR with maltose binding protein (MBP-PchR$'_{PAO}$) bound iron-loaded (ferri-) pyochelin with an affinity (K_d) of 41 ± 5 μM. By contrast, no binding occurred with ferri-enantiopyochelin. Stereospecificity of a similar fusion protein of the *P. protegens* PchR (MBP-PchR$'_{CHA0}$) was less pronounced. The K_d's of MBP-PchR$'_{CHA0}$ for ferri-enantiopyochelin and ferri-pyochelin were 24 ± 5 and 40 ± 7 μM, respectively. None of the proteins interacted with the iron-free siderophore enantiomers, suggesting that transcriptional activation by PchR occurs only when the respective siderophore actively procures iron to the cell.

Keywords Siderophore · *Pseudomonas* · Iron · Pyochelin · AraC-type regulator

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Introduction

As a cofactor of many redox-dependent enzymes, iron is essential for most organisms including bacteria. Despite its abundancy in nature, iron is not readily accessible for microorganisms as it forms poorly soluble ferric hydroxides in the oxic environment and is bound to iron transport and storage proteins in the mammalian host. To acquire iron, bacteria produce and release siderophores, which bind ferric iron avidly and transport it to the cytoplasm via specific outer membrane receptors and inner membrane permeases (Guerinot 1994; Wandersman and Delepelaire 2004).

Siderophore biosynthesis and uptake is tightly regulated to guarantee a sufficient iron acquisition and to prevent an iron overload, which would generate toxic hydroxyl radicals through the Fenton chemistry (Andrews et al. 2003). Under iron-replete conditions, siderophore biosynthesis and uptake genes are therefore repressed. In many bacteria this occurs by the Fur repressor, which, when complexed with ferrous iron, binds to conserved sequences in target promoters and prevents transcription (Escolar et al. 1999). When iron becomes limiting, Fur loses its cofactor and dissociates from its binding sites, thus allowing gene expression to occur. However, full expression of siderophore biosynthesis and/or uptake genes often involves additional regulators whose activities are controlled by the siderophores themselves. This positive regulation, also known as siderophore-mediated signaling (Lamont et al. 2002), assures that large amounts of a given

receptor are made only when the siderophore is present in the environment, and that only those siderophores are overproduced, which are actively procuring iron to the cell.

In *Pseudomonas aeruginosa* and *Pseudomonas protegens*, transcription of genes for the biosynthesis and uptake of the enantiomeric siderophores pyochelin and enantiopyochelin, respectively, require an AraC-type regulator named PchR. In the presence of the cognate siderophore, PchR recognizes a conserved PchR-box in target promoters and activates transcription (Youard et al. 2011). Previous genetic experiments suggested that pyochelin and enantiopyochelin activate their cognate PchR by binding to the proteins N-terminal part (Youard and Reimmann 2010). Here we demonstrate this binding with purified proteins in vitro. We show that binding is stereospecific and occurs only when the two siderophores are iron-loaded. The implication of the requirement for iron in this process is discussed.

Materials and methods

Overexpression and purification of PchR proteins fused to MBP

To overexpress the N-terminal parts of $PchR_{PAO}$ and $PchR_{CHA0}$ as fusions with MBP, the first 609 bp of $pchR_{PAO}$ and the first 621 bp of $pchR_{CHA0}$ were PCR-amplified from chromosomal DNA of *P. aeruginosa* PAO1 (ATCC 15692) and *P. protegens* CHA0 (Voisard et al. 1994) using the primer pairs npchR1(CCCGAATTCATGACCATCACCATCATTGCTC)/npchR2 (CCCAAGCTTATTAGGCGGCGTGGATGCGCTCGAC) and npchR3 (CCCGAATTCATGCTCAGCCCCAACGCTG)/npchR4 (GGGAAGCTTATTAGGCGGCATGGATGCGCTCGATG), respectively. PCR products were cleaved with EcoRI and HindIII and cloned into pMAL-c2E (New England Biolabs) to give pME9275 (overexpression of MBP-$PchR'_{PAO}$) and pME9276 (overexpression of MBP-$PchR'_{CHA0}$), respectively. For overexpression, *Escherichia coli* BL21(DE3) containing pME9275 and pME9276, respectively, was cultivated (200 ml in 1-l Erlenmeyer flasks) with shaking (180 rpm) at 37 °C in Rich medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl and 0.2 % glucose) containing ampicillin at 100 μg ml^{-1}. Cultures were induced with 0.3 mM IPTG and grown further at 37 °C for 2 h. Cells were spun down at 4,000×*g* for 10 min at 4 °C and resuspended in 10 ml of lysis buffer (20 mM Tris–HCl pH 7.5, 300 mM NaCl, 1 mM EDTA). The cell suspension was frozen in liquid nitrogen and stored at −80 °C. For purification (carried out at 4 °C), cells were thawed in an ice/water mixture, supplemented with DTT, PefaBloc® (Roche) and PMSF at 1 mM, and lysed by two passages through a French pressure cell (16,000 psi). After centrifugation (9,000*g* for 30 min), the supernatant was diluted fivefold with column buffer (20 mM Tris–HCl pH 7.5, 300 mM NaCl, 1 mM EDTA and 1 mM DTT), and loaded onto an amylose column (3 mL of amylose resin [New England Biolabs] packed into a 5 ml polypropylene column [1.5 × 7 cm, Qiagen] and washed with 5 mL of distilled water and 5 ml of lysis buffer) with a flow rate of 1 ml min^{-1}. The column was washed with 12 ml of column buffer (flow rate of 1 ml min^{-1}) and proteins were eluted with 5 ml column buffer supplemented with 10 mM maltose (flow rate of 0.5 ml min^{-1}). Fractions of 1 ml were collected. Those containing protein (identified by UV absorbance at 280 nm) were pooled and subsequently dialysed overnight at 4 °C in 1 l of 20 mM Tris–HCl pH 7.5, 300 mM NaCl, and 1 mM DTT, and again in 1 l of the same buffer the following day for 2 h. Proteins were then concentrated with centrifugal filters (ULTRACEL®, regenerated cellulose 10,000 MWCO, Millipore), and glycerol was added to a final concentration of 10 %. Proteins were frozen in liquid nitrogen and stored at −80 °C until further use.

Fluorescence assay of siderophore binding

Pyochelin and enantiopyochelin were purified as described previously (Youard et al. 2007) and stored as 8 mM solutions in methanol at −20 °C. Ferri-siderophores were generated by mixing siderophores with $FeCl_3$ in methanol in a molar ratio of 4:1. One day before fluorescence measurements, siderophores and ferri-siderophores were diluted to a final concentration of 0.25 mM in assay buffer (20 mM HEPES pH 7.5, 40 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA and 10 % glycerol) and stored at −20 °C. Fluorescence measurements were carried out at 25 °C with a PerkinElmer Luminescence Spectrometer (model LS55). Excitation was set at 280 nm and emission spectra were integrated over 280–520 nm, with a slit-width of 5 and 10 nm for excitation and emission, respectively.

Data were collected with a speed of 200 nm min^{-1}. A sample (598 µl) containing 10 µg ml^{-1} of purified protein in assay buffer was incubated with 10 mM DTT (final concentration) at 25 °C for 10 min before recording fluorescence emission spectra. For each of 6 subsequent fluorescence measurements, 1 µl of (ferri-)siderophore (at 0.25 mM) was added and the sample was incubated for 5 min at 25 °C. Each series of measurements was repeated at least 3 times. MBP-PchR proteins have maximal fluorescence emission intensity at 330 nm. For determination of K_d values, the ratios between initial fluorescence at 330 nm and fluorescence after addition of (ferri-)siderophores were plotted against the concentration of (ferri-)siderophore. Fluorescence emission at 420 nm of different concentrations of (ferri-)siderophores was recorded in the absence of MBP-PchR proteins to serve as a standard curve for the calculation of (ferri-)siderophore concentration in the binding assay.

Determination of K_d

To calculate the K_d values, the Stern–Volmer equation (Eq.) for static fluorescence quenching was applied (Stern and Volmer 1919):

$$F + Q \leftrightarrow FQ \quad (1)$$

F is the fluorophore, represented by a functional dimer of the respective MBP-PchR protein, and Q is the quencher, *i.e.* the (ferri-)siderophore. According to the law of mass action, the Stern–Volmer constant K_{SV} can be derived from Eq. (1). The Stern–Volmer constant K_{sv} also corresponds to the association constant K_a:

$$K_{sv} = K_a = [FQ]/[F][Q] \quad (2)$$

[FQ] represents the concentration of the fluorophore-quencher complex, [F] the concentration of free fluorophore and [Q] the concentration of the quencher. The total fluorophore concentration $[F]_0$ is composed of the concentration of free fluorophore and fluorophore-quencher complex:

$$[F]_0 = [F] + [FQ] \quad (3)$$

Substituting [FQ] derived from Eq. (3) into Eq. (2), the association constant K_a can be described as following:

$$K_a = [F]_0/[F][Q] - 1/[Q] \quad (4)$$

Eq. (4) is rewritten as following:

$$[F]_0/[F] = 1 + K_a[Q] \quad (5)$$

As the intensity of fluorescence is proportional to the concentration of the fluorophore, Eq. (5) can be rewritten as following:

$$[F]_0/[F] = F_0/F = 1 + K_a[Q] \quad (6)$$

By plotting the relative fluorescence intensity against the concentration of the free quencher, the association constant K_a can be obtained. The dissociation constant K_d corresponds to the rate of the reverse reaction, therefore:

$$K_d = 1/K_a \quad (7)$$

Analytical methods

Protein concentration were determined using a BCA (bicinchoninic acid) protein assay kit with reagents purchased from Pierce and bovine serum albumin as a standard (Smith et al. 1985). For SDS PAGE, NuPAGE® Tris–Acetate 3–8 % gels (Invitrogen) were used following the instructions of the supplier. Continuous native PAGE was done with a 6 % gel at pH 7.4 according to standard protocols of the Mini-PROTEAN® system from BioRad (McLellan 1982). Commercially purchased bovine serum albumin (Sigma) was used as molecular mass marker.

Results

Purification of the siderophore binding domains of $PchR_{PAO}$ and $PchR_{CHA0}$

AraC-type proteins are notoriously difficult to purify in a soluble form, but their insolubility can be partially overcome by removing the protein's C-terminal DNA binding domain (Schleif 2003; Weldon et al. 2007). We therefore overexpressed only the N-terminal siderophore recognition domains of the two PchR homologs (Youard and Reimmann 2010) and purified the proteins as fusions with MBP (see Fig. 1a for overexpression and purification of MBP-$PchR'_{PAO}$). We found that both proteins were remarkably stable and could be stored at 4 °C over a long period with very little degradation. However, native PAGE analysis showed that they formed multiple oligomers, which remained stable even in the presence of Tween 20

(0.25 %), Triton X-100 (2.5 mM) or octyl glucoside (0.6 %) (data not shown). Treatment of the protein preparations with 10 mM DTT finally yielded relatively homogenous dimer populations (see Fig. 1b for MBP-PchR′$_{PAO}$) suitable for binding studies. As many AraC-type proteins are active as dimers, no further attempt was made to obtain PchR monomers. Therefore, all MBP-PchR preparations were routinely incubated with 10 mM DTT for 10 min at RT immediately before starting the in vitro binding reactions.

PchR of *P. aeruginosa* binds iron-loaded but not iron-free pyochelin in vitro

We first studied siderophore binding of MBP-PchR′$_{PAO}$. When exicited at 280 nm, the protein emits intrinsic fluorescence with a maximal intensity at 330 nm. Upon addition of ferri-pyochelin, fluorescence was quenched (Fig. 2). Taking advantage of this quenching, the protein's affinity for its natural ligand was determined by plotting the relative fluorescence intensity (F_0/F) of MBP-PchR′$_{PAO}$ against the concentration of ferri-pyochelin (Fig. 3a). Data were fit to a linear regression from which a K_d value of 41 ± 5 μM was calculated. When MBP-PchR′$_{PAO}$ was incubated with iron-free pyochelin, the relative fluorescence intensity was equal or smaller than 1, showing that there was no quenching, and hence no binding between MBP-PchR′$_{PAO}$ and the iron-free siderophore (Fig. 3a).

Ferri-siderophore binding to *P. aeruginosa* PchR is highly stereospecific

In vivo, transcriptional activation by PchR$_{PAO}$ is stimulated by pyochelin but not by the *P. protegens* siderophore enantiopyochelin (Youard et al. 2007; Youard and Reimmann 2010). We evaluated whether MBP-PchR′$_{PAO}$ would display a similar ligand specificity in vitro and found that this was indeed the case. As shown in Fig. 3b, no fluorescence quenching was observed when the protein was incubated with different concentrations of ferri-enantiopyochelin, indicating that MBP-PchR′$_{PAO}$ does not bind the optical antipode of its natural ligand.

(Ferri)-siderophore binding to the PchR protein of *P. protegens*

Similar binding studies were carried out with MBP-PchR′$_{CHA0}$. Fluorescence assays showed that MBP-PchR′$_{CHA0}$ bound ferri-enantiopyochelin with an affinity (K_d) of 24 ± 10 μM (Fig. 3c) while no binding occurred with the iron-free siderophore (data not shown). Interestingly, MBP-′PchR$_{CHA0}$ was also

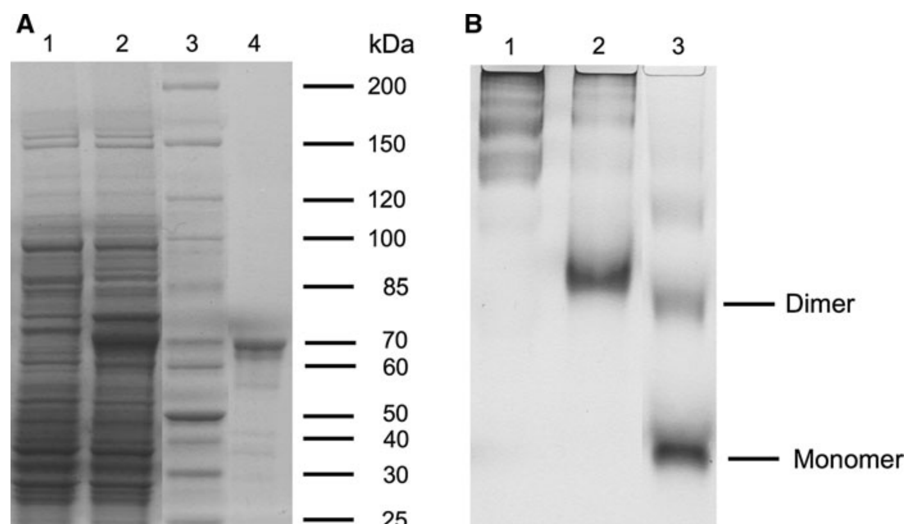

Fig. 1 Overexpression and purification of MBP-PchR′$_{PAO}$. **a** SDS-PAGE. Lane 1, crude extract from non-induced cells; lane 2, crude extract from cells induced with 0.3 mM IPTG; lane 3, molecular size marker, lane 4, purified MBP-PchR′$_{PAO}$. **b** Native PAGE at pH 7.5. Lane 1, oligomers of purified MBP-PchR′$_{PAO}$; lane 2, homogenous dimer population of MBP-PchR′$_{PAO}$ after 15 min incubation at RT with 10 mM DTT; lane 3, monomer and dimer populations of bovine serum albumin

able to bind ferri-pyochelin, but the affinity ($K_d = 40 \pm 7$ μM) was lower than for its natural ligand. We thus conclude that the stereospecificity for ligand recognition is very stringent for MBP-PchR′$_{PAO}$ and more relaxed for MBP-PchR′$_{CHA0}$.

Discussion

PchR belongs to the large family of AraC/XylS-type transcriptional regulators, which are involved in the control of carbon metabolism, stress response and pathogenesis (Gallegos et al. 1997; Martin and Rosner 2001). Many of the proteins in this family, including PchR, are organized in two domains, a non-conserved, N-terminal domain (about 200 amino acids) involved in effector recognition and dimerization, and a highly conserved, C-terminal domain (about 100 amino acids) for DNA binding. To study the interaction of PchR with its potential ligands, we overexpressed and purified its N-terminal domain as a fusion with MBP since this strategy was shown to increase protein solubility (Sun et al. 2011). Soluble fusion proteins were obtained, but they showed a strong tendency to form multiple oligomers. Incubation with high concentrations of DTT finally generated a relatively homogenous dimer population (see Fig. 1b for MBP-PchR′$_{PAO}$), which could be used for binding studies. Although not demonstrated experimentally, it is very likely that PchR, like numerous other AraC/XylS proteins, is active as a dimer in vivo as well (Yang et al. 2011).

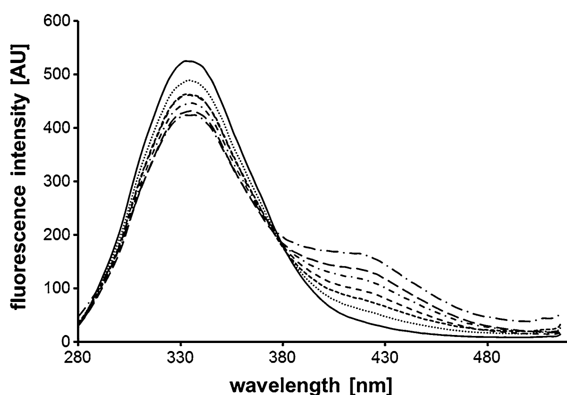

Fig. 2 Fluorescence emission spectra of MBP-PchR′$_{PAO}$ in the presence of different ferri-pyochelin concentrations. MBP-PchR′$_{PAO}$ was incubated with 10 mM DTT at 25 °C for 10 min and has maximal fluorescence emission at 330 nm (*solid line*). Its fluorescence was quenched with addition of 0.4 μM (*dotted line*), 0.8 μM (*short-dashed line*), 1.25 μM (*dashed line*), 1.7 μM (*dash-dot line*), 2.1 μM (*long-dashed line*), 2.5 μM (*long dash-dot line*) ferri-pyochelin. Ferri-pyochelin has maximal fluorescence emission at 420 nm

Fluorescence spectroscopy measurements showed that MBP-PchR′$_{PAO}$ and MBP-PchR′$_{CHA0}$ bound ferri-pyochelin and ferri-enantiopyochelin, respectively (Fig. 3). These results thus confirm and extend previous in vivo experiments, which showed that the N-terminal domains of the two PchR homologs are important for ligand recognition (Youard and Reimmann 2010). In vitro binding assays also confirmed the stereospecificity of the two proteins observed in vivo. We found that MBP-PchR′$_{PAO}$ bound ferri-pyochelin but not ferri-enantiopyochelin, while the stereospecificity of MBP-PchR′$_{CHA0}$ was more relaxed as the protein bound both iron-loaded enantiomers. The biological significance of these differences are not clear at present.

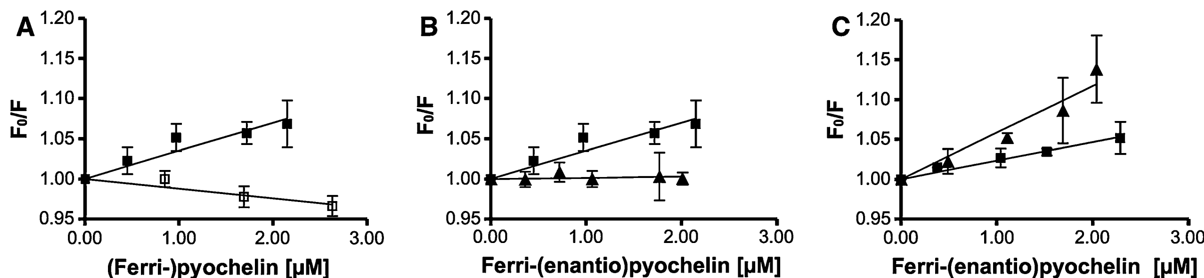

Fig. 3 Stern-Volmer plot for siderophore binding affinity determination of MBP-PchR′ proteins. Relative fluorescence intensity (F_0/F) of MBP-PchR′$_{PAO}$ **a** and **b** and MBP-′PchR$_{CHA0}$ **c** at 330 nm in the prescence of ferri-pyochelin (*filled square*), pyochelin (*open square*) and ferri-enantiopyochelin (*filled triangle*) were plotted against (ferri-)siderophore concentrations. Data were fit with a linear regression. $F_0/F \leq 1$ indicates that no binding between MBP-PchR′ proteins and (ferri-)siderophores occurred

The binding affinities measured here should be regarded as relative affinities rather than absolute values due to several technical constraints. As fluorescence of MBP-PchR was quenched by free iron as well (data not shown), a siderophore to iron ratio of 4:1 was used. Although this ratio guaranteed that no free iron was present in the binding assays, it led to a population of iron-free siderophores, which did not bind to MBP-PchR. A second constraint was the tendency of MBP-PchR to form stable oligomers, which had to be converted into dimers using DTT at very high concentrations. The binding specificities of these dimers reflected the stereospecificities the two PchR proteins have in vivo. Nevertheless, it is not clear what percentage of the protein preparations is active under these experimental conditions.

Previous band-shift experiments showed that pyochelin promoted PchR binding to its DNA target only when iron was present in the assay (Michel et al. 2005). Here we found that MBP-PchR$'_{PAO/CHA0}$ bound iron-loaded but not iron-free pyochelin/enantiopyochelin (Fig. 3a and data not shown), thus confirming that only the ferri-siderophore can act as a PchR effector. Due to the reducing nature of the cytoplasm and the high amount of reductant used during in vitro binding assays, we speculate that it is the ferrous form of iron (Fe^{2+}) that is present in the complex with pyochelin/enantiopyochelin when binding to PchR occurs.

Why should PchR be activated by the iron-loaded rather than by the iron-free siderophore? Under severe iron limitaton, iron is acquired in *P. aeruginosa* predominantly with pyoverdine, which has a much stronger affinity for iron than pyochelin (Brandel et al. 2012). Although basal levels of pyochelin are made under these conditions, PchR will not be activated, as no iron-loaded pyochelin is available. As a consequence, no upregulation of the pyochelin iron uptake system will occur. By contrast, when iron is only moderately limited, Fur represses pyoverdine production very strongly, while pyochelin synthesis remains relatively high (Dumas et al. 2013). Iron is therefore incorporated via pyochelin, and the formation of iron-loaded pyochelin will subsequently upregulate pyochelin biosynthesis and uptake genes. In conclusion, we believe that the requirement for iron prevents an unnecessary upregulation of the pyochelin-mediated iron uptake system under conditions when other iron uptake systems are much more effective.

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References

Andrews SC, Robinson AK, Rodríguez-Quiñones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237

Brandel J, Humbert N, Elhabiri M, Schalk IJ, Mislin GL, Albrecht-Gary AM (2012) Pyochelin, a sideophore of *Pseudomonas aeruginosa*: physicochemical characterization of the iron(III), copper(II) and zinc(II) complexes. Dalton Trans 41:2820–2834

Dumas Z, Ross-Gillespie A, Kümmerli R (2013) Switching between apparently redundant iron-uptake mechanisms benefits bacteria in changeable environments. Proc R Soc B 280:20131055

Escolar L, Perez-Martin J, de Lorenzo V (1999) Opening the iron box: transcriptional metalloregulation by the Fur protein. J Bacteriol 181:6223–6229

Gallegos M-T, Schleif R, Bairoch A, Hofmann K, Ramos JL (1997) AraC/XylS family of transcriptional regulators. Microbiol Mol Biol Rev 61:393–410

Guerinot ML (1994) Microbioal iron transport. Annu Rev Microbiol 48:743–772

Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML (2002) Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 99:7072–7077

Martin RG, Rosner JL (2001) The AraC transcriptional activators. Curr Opin Microbiol 4:132–137

McLellan T (1982) Electrophoresis buffers for polyacrylamide gels at various pH. Anal Biochem 126:94–99

Michel L, González N, Jagdeep S, Nguyen-Ngoc T, Reimmann C (2005) PchR-box recognition by the AraC-type regulator PchR of *Pseudomonas aeruginosa* requires the siderophore pyochelin as an effector. Mol Microbiol 58:495–509

Schleif R (2003) AraC protein: a love-hate relationship. Bioassays 25:274–282

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85

Stern O, Volmer M (1919) Über die Abklingzeit der Fluoreszenz. Physik Zeitschr 20:183–188

Sun P, Tropea JE, Waugh DS (2011) Enhancing the solubility of recombinant proteins in *Escherichia coli* by using hexahistidine-tagged maltose-binding protein as a fusion partner. Methods Mol Biol 705:259–274

Voisard C, Bull C, 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: O'Gara F, Dowling D, Boesten B (eds) Molecular ecology of rhizosphere microorganisms. VCH Publishers, Weinheim, pp 67–89

Wandersman C, Delepelaire P (2004) Bacterial iron sources: from siderophores to hemophores. Annu Rev Microbiol 58:611–647

Weldon JE, Rodgers ME, Larkin C, Schleif RE (2007) Structure and properties of a truely apo form of AraC dimerization domain. Proteins 66:646–654

Yang J, Tauschek M, Robins-Browne BM (2011) Control of bacterial virulence by AraC-like regulators that respond to chemical signals. Trends in Microbiol 19:128–135

Youard ZA, Reimmann C (2010) Stereospecific recognition of pyochelin and enantio-pyochelin by the PchR proteins in fluorescent pseudomonads. Microbiology 156:1772–1782

Youard ZA, Mislin GLA, Majcherczyk PA, Schalk IJ, Reimmann C (2007) *Pseudomonas fluorescens* CHA0 produces enantio-pyochelin, the optical antipode of the *Pseudomonas aeruginosa* siderophore pyochelin. J Biol Chem 282:35546–35553

Youard ZA, Wenner N, Reimmann C (2011) Iron acquisition with the natural siderophore enantiomers pyochelin and enantiopyochelin in *Pseudomonas* species. Biometals 24:513–522