Citrate-mediated iron uptake in *Pseudomonas aeruginosa*: involvement of the citrate-inducible FecA receptor and the FeoB ferrous iron transporter

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In an attempt to identify components of a ferric citrate uptake system in *Pseudomonas aeruginosa*, a mutant library of a siderophore-deficient strain (IA614) was constructed and screened for defects in citrate-promoted growth in an Fe-restricted medium. A mutant disrupted in gene PA3901, encoding a homologue of the outer-membrane ferric citrate receptor, FecA, of *Escherichia coli* (FecA_{E.c.}), was recovered and shown to be deficient in citrate-promoted growth and citrate-mediated Fe uptake. A mutant disrupted in gene PA4825, encoding a homologue of the MgtA/MgtB Mg²⁺ transporters in *Salmonella enterica*, was similarly deficient in citrate-promoted growth, though this was due to a citrate sensitivity of the mutant apparently resulting from citrate-promoted acquisition of Fe²⁺ and resultant oxidative stress. Consistent with citrate delivering Fe to cells as Fe²⁺, a *P. aeruginosa* mutant lacking the FeoB Fe²⁺ transporter homologue, PA4358, was compromised for citrate-promoted growth in Fe-restricted medium and showed markedly reduced citrate-mediated Fe uptake. Subsequent elimination of two Fe³⁺ transporter homologues, PA5216 and PA4687, in the *feoB* mutant failed to further compromise citrate-promoted growth or Fe uptake, though the additional loss of *pcoA*, encoding a periplasmic ferroxidase implicated in Fe²⁺ acquisition, completely abrogated citrate-mediated Fe uptake. Fe acquisition mediated by other siderophores (e.g. pyoverdine) was, however, unaffected in the quadruple knockout strain. These data indicate that Fe delivered to *P. aeruginosa* by citrate is released as Fe²⁺, probably in the periplasm, prior to its transport into cells via Fe transport components.

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INTRODUCTION

Iron is an essential nutrient whose acquisition by *Pseudomonas aeruginosa* is often facilitated by high-affinity iron-chelating molecules termed siderophores that, together with cell-surface receptors specific for the iron-siderophore complexes, serve to provide the organism with iron under the most nutritionally dilute conditions (Poole & McKay, 2003). *P. aeruginosa* produces two siderophores,

pyoverdine (Meyer & Hornsperger, 1978; Poole *et al.*, 1991; Poole & McKay, 2003; Visca *et al.*, 2007) and pyochelin (Ankenbauer *et al.*, 1988; Heinrichs *et al.*, 1991; Poole & McKay, 2003), though the organism is also able to utilize several siderophores produced by fungi and other bacteria (Cornelis *et al.*, 1987; Cuiv *et al.*, 2006, 2007; Llamas *et al.*, 2006, 2008; Meyer, 1992; Meyer *et al.*, 1999; Poole *et al.*, 1990), as well as additional molecules with Fe-chelating ability [e.g. citrate (Cox, 1980b; Harding & Royt, 1990)]. While outer-membrane receptors for most of these heterologous siderophores have been identified (Banin *et al.*, 2005; Cuiv *et al.*, 2006; Ghysels *et al.*, 2005; Llamas *et al.*, 2006, 2008; Poole *et al.*, 1990), additional (e.g. periplasmic or inner-membrane) transport components

Abbreviations: ECF, extracytoplasmic function; EDDA, ethylenediaminediacetic acid; EDDHA, ethylenediaminedi(*o*-hydroxyphenylacetic) acid.

Supplementary material with details of primers and cycling parameters for construction of deletion mutants is available with the online version of this paper.

are generally not evident from the genome sequence. This is in contrast to siderophore transport systems in *Escherichia coli* (Braun, 2003) and the pyochelin transport system in *P. aeruginosa* (Michel *et al.*, 2007), where siderophore-specific post-outer-membrane transporters have been identified. Homologues of the *fepBCG* genes associated with ferric enterobactin uptake across the periplasm and inner membrane of *E. coli* are, however, present in the *P. aeruginosa* genome (Poole & McKay, 2003) though an involvement in ferric enterobactin uptake has not been examined. Receptor genes for heterologous siderophores in *P. aeruginosa* are often only linked to genes encoding an extracytoplasmic function (ECF) sigma factor and cognate anti-sigma factor (Poole & McKay, 2003; Visca *et al.*, 2002) implicated in siderophore-dependent induction of receptor gene expression (Llamas *et al.*, 2006, 2008). This is presumably to ensure that these receptors are only synthesized when the cognate siderophore is available. *P. aeruginosa* may, thus, possess a common post-outer-membrane uptake system for Fe delivered to the organism via various heterologous siderophores and their receptors. Homologues of inner-membrane permeases for Fe^{3+} and Fe^{2+} have, for example, been described in *P. aeruginosa* (Poole & McKay, 2003), where they could function in trans-cytoplasmic membrane transport of Fe^{3+} or Fe^{2+} delivered to the periplasm by siderophores and their receptors.

A homologue of the *fecA* gene encoding a ferric citrate receptor in *E. coli*, PA3901, has been identified in *P. aeruginosa* (Poole & McKay, 2003; Visca *et al.*, 2002), and citrate-dependent binding of Fe to isolated outer membranes has been seen (Harding & Royt, 1990), consistent with the presence of a ferric citrate receptor in this organism. In *E. coli*, *fecA* and additional transport genes responsible for delivery of ferric citrate to the cytoplasm are specifically induced under Fe-limiting conditions in the presence of exogenous citrate (Braun, 2003). This is mediated by FecA in conjunction with the FecR sensor/anti-sigma factor and FecI ECF sigma factor, which collectively sense ferric citrate in the environment and activate *fec* transport gene expression (Braun & Mahren, 2005; Braun *et al.*, 2006; Visca *et al.*, 2002). *P. aeruginosa* *fecA* is linked to homologues of the *E. coli* *fecI* (PA3899) and *fecR* (PA3900) genes (Poole & McKay, 2003), though additional *fec* transport homologues are not identifiable in *P. aeruginosa*. While the citrate inducibility of PA3901 has not been examined, the ferric citrate-binding activity of isolated *P. aeruginosa* outer membranes is dependent upon growth in low-Fe citrate-containing medium (Harding & Royt, 1990), and PA3901/PA3900–3899 are inducible by Fe limitation (Ochsner *et al.*, 2002). Moreover, citrate-promoted biofilm formation in PA3901⁺ but not PA3901[−] *P. aeruginosa* (Banin *et al.*, 2005) is consistent with PA3901's annotated function as the ferric citrate receptor. The current study was undertaken to confirm FecA's presumed function in ferric citrate uptake and to identify constituents involved in post-outer-membrane transport of

Fe delivered to *P. aeruginosa* by citrate, as a model of heterologous siderophore uptake where siderophore-specific post-outer-membrane transport components are not evident.

METHODS

Bacterial strains and growth media. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultivated at 37 °C in Luria (L) broth as before (Nehme *et al.*, 2004) supplemented with antibiotics to maintain plasmids as needed [pEX18Tc, pRK415 and their derivatives: tetracycline (10 µg ml^{−1}, *E. coli*; 75 µg ml^{−1}, *P. aeruginosa*); pK18MobSacB and its derivatives: kanamycin (50 µg ml^{−1}, *E. coli*; 1500 µg ml^{−1}, *P. aeruginosa*); pUCP18 and its derivatives: ampicillin (100 µg ml^{−1}, *E. coli*) or carbenicillin (800 µg ml^{−1}, *P. aeruginosa*); pUT::mini-Tn5-*tet*: tetracycline (10 µg ml^{−1}, *E. coli*); pCR-Blunt II-TOPO: ampicillin (100 µg ml^{−1}, *E. coli*)]. The iron-limited succinate minimal media [here dubbed 'A' (Meyer & Abdallah, 1978) and 'B' (Poole *et al.*, 1990)] have been described previously.

DNA techniques. Standard protocols were used for restriction endonuclease digestions, ligations, transformation, preparation of electrocompetent cells and agarose gel electrophoresis, as described by Sambrook & Russell (2001). Purification of plasmids and PCR products was carried out using the QIAprep Spin Miniprep and QIAquick PCR Purification kits (Qiagen), respectively. Genomic DNA of *P. aeruginosa* was extracted according to the protocol of Barcak *et al.* (1991). *E. coli* cells were made competent using the method of Inoue *et al.* (1990). *P. aeruginosa* were made electrocompetent by the method described by Choi *et al.* (2005). DNA sequencing was performed by ACGT Corporation (Toronto, Ontario, Canada) with universal and custom oligonucleotides.

Plasmids. The PA3901 (*fecA*) and PA4825 (*mgtA*) genes were cloned into pUCP18 following their amplification (via PCR) from the chromosome of *P. aeruginosa* IA614 and initial cloning into plasmid pCR-Blunt II-TOPO (Invitrogen) to yield plasmids pBM11 and pBM13, respectively. PA3901 was amplified using the primer pair PA3901-For (5'-GAATTCGAATTCCACCTGAGCCTTTTCCCCAC-3'; tandem *EcoRI* sites underlined)/PA3901-Rev (5'-AAGCTTAAGCTTCTGGCGATCGTCCGCCGTTG-3'; tandem *HindIII* sites underlined) in a reaction that involved heating at 94 °C for 3 min followed by 30 cycles of 15 s at 94 °C, 30 s at 59 °C and 3 min at 68 °C, before finishing with 10 min at 68 °C. The blunt-ended PA3901-carrying PCR product was purified, cloned into pCR-Blunt II-TOPO according to a protocol provided by the manufacturer, excised from this plasmid via *EcoRI*/*HindIII* digestion and subsequently cloned into pUCP18. PA4825 was similarly cloned following amplification with the primer pair PA4825-For (5'-GGATCCGATCCACCGATCGCGAACATCGCG-3'; tandem *BamHI* sites underlined)/PA4825-Rev (5'-AAGCTTAAGCTTCTTGGCGACGCGCCGGGAAAG-3'; tandem *HindIII* sites underlined) in a reaction that included heating at 94 °C for 3 min followed by 30 cycles of 15 s at 94 °C, 30 s at 67.5 °C and 3 min at 68 °C, before finishing with 10 min at 68 °C. All reaction mixtures contained 50 ng *P. aeruginosa* IA614 chromosomal DNA, 30 pmol of each primer, 0.2 mM (each) dNTPs, and 1 × KOD buffer (Invitrogen), which was heated to 94 °C before addition of 2 units of KOD DNA polymerase. Cloned genes were sequenced to ensure that no mutations had been introduced during PCR. The PA4358 (*feoB*) gene was cloned into pRK415 following PCR amplification and initial cloning in pUC18. PA4358 was amplified using primer pair PA4358-For (5'-GACTAAGCTTAAGGACCTCGCCCTGCTGAC-3'; *HindIII* site underlined)/PA4358-Rev (5'-GACTGAATTCTGGCGGCTGAGCTGTTTCAG-3';

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties*	Source or reference
<i>E. coli</i>		
SM10(λ_{pir})	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu; Km^r λ_{pir}</i>	Miller & Mekalanos (1988)
DH5 α	ϕ 80dlacZ Δ M15 <i>endA1 recA1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1 gyrA96 relA1 F⁻</i> Δ (<i>lacZYA-argF</i>)U169	Ausubel <i>et al.</i> (1992)
S17-1	<i>thi pro hsdR recA Tra⁺</i>	Simon <i>et al.</i> (1983)
<i>P. aeruginosa</i>		
IA614	Pvd-2 Pch ⁻ Sal ⁻ †	Ankenbauer & Cox (1988)
K2523	IA614 PA3901 (<i>fecA</i>)::mini-Tn5- <i>tet</i> ‡§	This study
K2525	IA614 PA4825 (<i>mgtA</i>)::mini-Tn5- <i>tet</i>	This study
K2931	IA614 Δ PA4358 (<i>feoB</i>)	This study
K2932	IA614 Δ <i>pcoA</i> Δ PA4358 (<i>feoB</i>)	This study
K2556	IA614 Δ PA4358 (<i>feoB</i>) Δ PA4687 Δ PA5216	This study
K2530	IA614 Δ <i>pcoA</i> Δ PA4358 (<i>feoB</i>) Δ PA4687 Δ PA5216	This study
Plasmids		
pUT::mini-Tn5- <i>tet</i>	Mini-Tn5- <i>tet</i> delivery vector; Ap ^r Tc ^r	de Lorenzo <i>et al.</i> (1990)
pK18MobSacB	Broad-host-range gene replacement vector; <i>sacB</i> Km ^r	Schäfer <i>et al.</i> (1994)
pBM001	pK18MobSacB:: Δ <i>pco</i>	This study
pBM003	pK18MobSacB:: Δ PA4687	This study
pBM004	pK18MobSacB:: Δ PA5216	This study
pEX18Tc	Gene-replacement vector; <i>sacB</i> Tc ^r	Hoang <i>et al.</i> (1998)
pCG003	pEX18Tc:: Δ 4358 (<i>feoB</i>)	This study
pUCP18	<i>P. aeruginosa</i> – <i>E. coli</i> shuttle cloning vector; Ap ^r /Cb ^r	Schweizer (1991)
pBM11	pUCP18::PA3901 (<i>fecA</i>)	This study
pBM13	pUCP18::PA4825 (<i>mgtA</i>)	This study
pRK415	<i>P. aeruginosa</i> – <i>E. coli</i> shuttle cloning vector; Tc ^r	Keen <i>et al.</i> (1988)
pCG002	pRK415::PA4358 (<i>feoB</i>)	This study

*Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Cb^r, carbenicillin resistance.

†Defective in pyoverdine, pyochelin and salicylate biosynthesis.

‡Gene disrupted by mini-Tn5-*tet* in the indicated insertion mutant derivatives of *P. aeruginosa* strain IA614 is identified using the designation given in the annotated genome sequence at <http://www.pseudomonas.com>.

§In instances where a PA gene has been annotated as a gene whose name and function have been established in another organism this gene name is given (in parentheses) along with the <http://www.pseudomonas.com> PA designation.

*Eco*RI site underlined) in a reaction mixture formulated as above except that Vent DNA polymerase and 1 × ThermoPol buffer (New England Biolabs) were employed and DMSO was included at 2% (v/v). The reaction mixture was heated for 3 min at 94 °C, followed by 30 cycles of 94 °C for 45 s, 63.9 °C for 45 s, 72 °C for 2 min, before finishing with 10 min at 72 °C. The PA4358-containing PCR product was digested with *Hind*III and *Eco*RI, and cloned into pUC18 for sequencing before being subcloned into pRK415 to yield pCG002.

Mini-Tn5-*tet* mutagenesis. The pyoverdine- and pyochelin-deficient *P. aeruginosa* strain, IA614, was mutagenized with mini-Tn5-*tet* (de Lorenzo *et al.*, 1990) following mobilization of mini-Tn5-*tet*-carrying plasmid pUT from *E. coli* SM10 (λ_{pir}) as described previously (Sobel *et al.*, 2005), with the IA614 mini-Tn5-*tet* insertion mutants selected on L-agar plates containing tetracycline (60 µg ml⁻¹) and imipenem (0.5 µg ml⁻¹; to counter-select donor *E. coli*). To screen for mutants with defects in citrate-mediated iron acquisition, two approaches were taken. Initially, the demonstration that the ethylenediaminedi(*o*-hydroxyphenylacetic) acid (EDDHA)-mediated (at 1.0 µg ml⁻¹) growth inhibition of *P. aeruginosa* strain IA614 on Fe-deficient succinate minimal medium A could be relieved by 10 mM citrate was used as an indicator of citrate-mediated iron acquisition, and mutants showing reduced or no citrate-stimulated

growth were recovered as possibly defective in citrate-mediated iron acquisition. Subsequently, following the demonstration that a mutant disrupted in the gene encoding a homologue of the *E. coli* ferric citrate receptor (*FecA*) was unable to grow on Fe-deficient succinate minimal medium B supplemented with 0.1 M citrate, potential ferric citrate uptake insertion mutants were identified by their failure to grow on citrate-containing Fe-deficient succinate minimal medium B agar plates. All mutants were tested for growth capability on Fe-deficient succinate minimal agar lacking citrate to ensure that they lacked only the ability to respond to/grow in the presence of citrate and were not, for example, generally defective in iron acquisition or, possibly, auxotrophic. Chromosomal DNA flanking the mini-Tn5-*tet* element in putative ferric citrate utilization mutants was cloned on *Pst*I fragments and sequenced as before (Cao *et al.*, 2004). The disrupted genes were then identified by BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST/>) searches of the available *P. aeruginosa* genome sequence (<http://www.pseudomonas.com>) (Stover *et al.*, 2000).

Growth assay. Overnight cultures of *P. aeruginosa* grown in iron-limited succinate minimal medium B were subcultured in the same medium substituted with EDDHA (1 µg ml⁻¹) with or without citrate (25 mM), pyoverdine (100 µg ml⁻¹; prepared as described in

Meyer *et al.*, 1998) or desferrioxamine B (Sigma; 80 μ M) to a final OD₆₀₀ of ~0.1. Cultures were incubated at 37 °C and OD₆₀₀ readings were taken every hour for 5–8 h. In experiments involving the *P. aeruginosa* quadruple deletion mutant K2530, bacteria were grown overnight in pyoverdine-supplemented (100 μ g ml⁻¹) minimal medium B, harvested and washed with an equal volume of minimal medium B (to remove traces of pyoverdine) prior to subculture. In some instances, overnight cultures were diluted 1:199 in the above media and growth assessed visually after 18 h incubation. Owing to EDDHA no longer being available commercially, later experiments were carried out using the related metal chelator, ethylenediamine-diacetic acid (EDDA) at 8 μ g ml⁻¹ in place of EDDHA.

Transport assay. *P. aeruginosa* cells were cultured to late exponential phase (OD₆₀₀ 1.6) in iron-limited succinate minimal medium B with or without 1 mM citrate, washed once in medium B without citrate and resuspended in the same medium at OD₆₀₀ 0.1. ⁵⁵Fe-citrate was prepared by incubating 24 pmol ⁵⁵FeCl₃ (PerkinElmer) and 50 μ mol citrate in 50 μ l at room temperature for 30 min before diluting 10-fold with iron-limited succinate minimal medium B. The uptake assay was initiated by adding 5 μ l of the diluted ⁵⁵Fe-citrate mixture to 1 ml cells (preincubated for 5 min at 37 °C) and was allowed to proceed for 15 min at 37 °C. Cells were then harvested by centrifugation (12 000 r.p.m., 2 min), washed three times with iron-limited succinate minimal medium B (1 ml) and resuspended vigorously in liquid scintillation cocktail (10 ml; Ultima Gold, PerkinElmer). Cell-associated radioactivity was measured using a model 1214 Rackbeta counter (LKB-Wallac). In some experiments, the uncoupler KCN (10 mM) was added to bacterial cells 5 min prior to addition of the ⁵⁵Fe-citrate mixture.

Site-directed deletion mutagenesis. To introduce deletions of one or more of the four annotated Fe²⁺/Fe³⁺ transport genes [*pco* (PA2065), PA4358, PA4687 and PA5216] into *P. aeruginosa*, deletion constructs were first prepared in plasmid pK18MobSacB (except for PA4358, which was engineered into pEX18Tc) by cloning PCR-amplified 1 kb DNA fragments corresponding to the regions upstream and downstream, respectively, of the sequences to be deleted. All PCR mixtures contained 50 ng *P. aeruginosa* IA614 chromosomal DNA, 30 pmol of forward and reverse primer, 0.2 mM (each) dNTPs, and 1 $\times$ ThermoPol buffer (New England Biolabs) in 50 μ l final volume. The upstream and downstream segments were amplified separately, cloned into pCR-Blunt II-TOPO using the Zero Blunt TOPO PCR cloning kit (Invitrogen), sequenced to verify that no mutations had been introduced during PCR, excised with appropriate restriction digestions and cloned sequentially into pK18MobSacB (or pEX18Tc) to yield the desired deletion construct (Δ *pco*, pBM001; Δ PA4358, pCG003; Δ PA4687, pBM003; Δ PA5216, pBM004). Deletion-containing pK18MobSacB or pEX18Tc derivatives were then mobilized into *P. aeruginosa* strain IA614 as described previously (Sobel *et al.*, 2003), and transconjugants carrying the plasmid in the chromosome were selected on L-agar plates containing kanamycin (1500 μ g ml⁻¹; pK18MobSacB) or tetracycline (75 μ g ml⁻¹; pEX18Tc) and imipenem (0.5 μ g ml⁻¹; to counter-select *E. coli*). Kanamycin- or tetracycline-resistant colonies were streaked onto L-agar plates containing 10% (w/v) sucrose, and sucrose-resistant colonies were screened for the presence of the respective deletions using colony PCR (Redly & Poole, 2003). Primers and cycling parameters are provided in the supplementary material available with the online version of this paper.

Outer-membrane proteins. Fractions containing outer-membrane proteins were prepared from 18 h cultures (50 ml) of *P. aeruginosa* grown in iron-deficient or iron-supplemented (100 μ M FeCl₃) medium B with or without citrate (1 or 25 mM) using a previously described protocol (Srikumar *et al.*, 1998). Briefly, cells were harvested by centrifugation, stored on ice for 30 min and then

processed as before to isolate cell envelopes. These were then extracted with 2% (v/v) Triton X-100–20 mM Tris/HCl (pH 8.0) and the outer-membrane proteins were recovered in the pellet fraction following centrifugation.

SDS-PAGE and immunoblotting. Outer-membrane preparations were electrophoretically separated on SDS-polyacrylamide [10% (w/v)] gels and electrotransferred onto Immobilon-P PVDF membrane (Millipore) as described previously (Srikumar *et al.*, 1998). Equal loading of protein in all wells was confirmed by rapid Coomassie staining of duplicate gels (Faguy *et al.*, 1996). Membranes were probed with 1:1999 dilution of polyclonal anti-*E. coli* FecA antiserum (provided by Dr V. Braun, Max Planck Institute for Developmental Biology, Tübingen, Germany).

Reverse-transcriptase (RT)-PCR. An overnight culture of *P. aeruginosa* IA614 grown in iron-deficient medium A was diluted 1:9 in the same medium with or without FeCl₃ (100 μ M) or citrate (1 mM) and grown for 2 h with shaking at 37 °C. Total cellular RNA was isolated using the Qiagen RNeasy Mini kit and RNase-free DNase (Qiagen) and a protocol provided by the manufacturer. The reverse transcription (RT)-PCR was performed with ~500 ng RNA using the Qiagen One Step RT-PCR kit, again according to a protocol provided by the manufacturer. Primer pairs *rpsL*-F (5'-GCAACTAT-CAACCAGCTG-3')/*rpsL*-R (5'-GCTGTGCTCTTGCAGGTTGTG-3') and *fecA*-F (5'-CACGACCTGGCATTGAACCTCG-3')/*fecA*-R (5'-CACGTTGTGG-GTGGTCTTCAGG-3') were used to assess expression of the *rpsL* and PA3901 (*fecA*) genes, respectively. Reaction mixtures were incubated for 30 min at 50 °C and 15 min at 95 °C, followed by 25–35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C, before finishing with 10 min at 72 °C.

RESULTS

Screening a *P. aeruginosa* mini-Tn5-*tet* insertion library for mutants deficient in citrate-mediated Fe acquisition

P. aeruginosa strain IA614 is deficient in endogenous siderophore production and unable to grow in Fe-limited minimal medium A supplemented with the Fe chelator EDDHA unless supplied with an exogenous 'siderophore' such as citrate (e.g. see Fig. 1A). Thus, citrate-promoted growth of strain IA614 in EDDHA-containing Fe-deficient minimal medium can be used as an indirect measure of citrate-promoted Fe acquisition. To identify genes involved in citrate-promoted Fe acquisition, a *P. aeruginosa* IA614 mini-Tn5-*tet* insertion mutant library was screened for lack of or reduced growth on Fe-deficient solid media supplemented with EDDHA and citrate. Of approximately 2700 mutants screened, two showed consistently reduced growth on the EDDHA/citrate plates but not on unsupplemented medium A plates. The mini-Tn5-*tet* element was inserted in the PA3901 gene encoding a homologue (63% identical) of the FecA outer-membrane ferric citrate receptor of *E. coli* in both mutants. One of these mutants, K2523, was subsequently confirmed as defective for growth in EDDHA- and citrate-supplemented Fe-deficient minimal medium, though it grew as well as its parent strain in unsupplemented Fe-limited medium (Fig. 1A). The mutant also grew well in EDDHA-supplemented Fe-limited

Fig. 1. FecA-dependent citrate-mediated Fe acquisition in *P. aeruginosa*. (A) Citrate-promoted growth of *P. aeruginosa* in Fe-restricted minimal medium. *P. aeruginosa* IA614 (FecA⁺; solid symbols) and K2523 (FecA⁻; open symbols) were subcultured in Fe-deficient succinate minimal medium without supplementation (●, ○) or supplemented with EDDHA (1 μg ml⁻¹) (■, □) or EDDHA (1 μg ml⁻¹) and citrate (25 mM) (▲, △) and growth was measured as a function of time. The data are representative of a minimum of three independent experiments. (B) Citrate-mediated Fe uptake in *P. aeruginosa* IA614 (FecA⁺) and K2523 (FecA⁻) grown in Fe-deficient succinate minimal medium with (+Cit) or without (-Cit) citrate (1 mM). Data are reported as pmol ⁵⁵Fe accumulated after 15 min incubation of 1 ml *P. aeruginosa* cells in medium containing citrate (50 μmol) and ⁵⁵FeCl₃ (24 pmol) with or without prior treatment with KCN (10 mM) as indicated. The mean ± SD of three independent experiments is shown.

medium containing pyoverdine (100 μg ml⁻¹) or FeCl₃ (100 μM) (data not shown) indicating that it was specifically deficient in citrate-promoted growth. In transport assays, the parent strain, IA614, showed enhanced citrate-promoted Fe acquisition when cultured in Fe-limited medium supplemented with citrate as compared to citrate-free medium (Fig. 1B), in parallel with the increased *fecA* expression (Fig. 2A) and FecA production (Fig. 2B) in citrate-supplemented Fe-limited medium. Citrate-promoted Fe acquisition was drastically reduced in KCN-treated strain IA614 grown with or without citrate and virtually no enhanced Fe acquisition

Fig. 2. Citrate-inducible expression of *fecA* and FecA in *P. aeruginosa*. *P. aeruginosa* IA614 (AI/AII and BI/BII) and its FecA⁻ derivative K2523 (BIII) were cultivated in Fe-deficient (-Fe) and Fe-sufficient (+Fe) succinate minimal medium with (+Cit) or without (-Cit) citrate (1 mM, panels A and panel BI; 25 mM, panels BII and BIII). (A) Expression of *fecA* (AI) and *rpsL* (AII) was assessed using semiquantitative RT-PCR. The *rpsL* reaction served as an internal control that ensured equal amounts of RNA were employed in all of the RT-PCRs shown. The PCR portion of the reactions was carried out for 27 (top panel in AI and AII) or 28 (bottom panel in AI and AII) cycles. (B) Production of FecA was assessed using Western immunoblotting of outer-membrane protein fractions developed with antibody to *E. coli* FecA. The lack of cross-reactivity of this antibody with outer-membrane protein fractions of the FecA⁻ strain K2523 (panel III) confirms its specificity for FecA in *P. aeruginosa*.

was seen in citrate-grown (vs citrate-free) cells (Fig. 1B), consistent with the observed citrate-promoted Fe acquisition being an active process. In contrast to strain IA614, the *fecA* (PA3901) mutant showed no increase in citrate-mediated Fe acquisition when grown in citrate-supplemented Fe-limited medium (Fig. 1B), consistent with the strain being deficient in citrate-inducible ferric citrate uptake and with the lack of FecA (Fig. 2B). Finally, the cloned *fecA* (PA3901) gene restored growth of strain K2523 in EDDHA- and citrate-supplemented Fe-deficient minimal medium (Fig. 3A) and citrate-promoted Fe

Fig. 3. Complementation of the citrate-promoted growth and Fe transport defects of FecA⁻ *P. aeruginosa* with cloned *fecA*. (A) *P. aeruginosa* K2523 (FecA⁻) (▲), K2523 carrying the *fecA* plasmid pBM11 (△) and *P. aeruginosa* IA614 (FecA⁺) (▼) were subcultured in succinate minimal medium supplemented with EDDHA (1 µg ml⁻¹) and citrate (25 mM) and growth was measured as a function of time. Growth of strain K2523 in Fe-deficient succinate minimal medium without supplementation (●) or supplemented with EDDHA (1 µg ml⁻¹) alone (■) is also shown. The data are representative of a minimum of three independent experiments. (B) Citrate-mediated Fe uptake in *P. aeruginosa* K2523 (FecA⁻), its pBM11-harbouring derivative (K2523-C), and IA614 (FecA⁺) grown in Fe-deficient succinate minimal medium with citrate (1 mM). Data are reported as pmol ⁵⁵Fe accumulated after 15 min incubation of 1 ml *P. aeruginosa* cells in medium containing citrate (50 µmol) and ⁵⁵FeCl₃ (24 pmol). The mean ± SD of three independent experiments is shown.

acquisition in citrate-grown cells (Fig. 3B). PA3901 is, thus, confirmed as the ferric citrate receptor in *P. aeruginosa* and is hereafter described as FecA.

Evidence for citrate delivery of Fe²⁺ to *P. aeruginosa*

While the recovery of the *fecA* gene validated the previous screen, it was not unexpected and provided no insights as regards post-outer-membrane constituents for ferric citrate uptake in *P. aeruginosa*. Still, with an available mutant

defective in citrate-mediated Fe acquisition it was possible to develop a second screen more robust than the first, which relied on the observed inability of a mutant deficient in citrate-mediated Fe acquisition to grow in the presence of excess citrate. Using a second Fe-limited minimal medium (medium B) it was observed, for example, that the addition of 0.1 M citrate completely inhibited growth of FecA⁻ strain K2353 but not its FecA⁺ parent, strain IA614 (data not shown), presumably because all available Fe is complexed to citrate at such high concentrations, making it unavailable to a mutant deficient in ferric citrate uptake. To obtain mutants compromised for citrate-mediated Fe acquisition, then, 6000 mini-Tn5-*tet* insertion mutants of strain IA614 were subsequently screened for lack of growth on 0.1 M citrate-supplemented Fe-limited minimal medium B. Of several non-growing mutants recovered, two grew on unsupplemented medium B (i.e. were not auxotrophs). One mutant was again disrupted in the *fecA* gene while the second, strain K2525, had the mini-Tn5-*tet* element inserted into the PA4825 gene encoding a homologue of the MgtA and MgtB Mg²⁺ transporters of *Salmonella enterica* serovar Typhimurium (52 % and 69 % identical, respectively). The growth deficiency of strain K2525 in EDDHA- and citrate-supplemented medium B was reversed by the cloned *mgtA* (PA4825) gene (Fig. 4), indicating that it resulted from disruption of *mgtA* (PA4825). A link to citrate-mediated Fe acquisition was not obvious, however, suggesting that the growth deficiency of the *mgtA* (PA4825) mutant might be explainable by a sensitivity to citrate. In agreement with this, pyoverdine- (100 µg ml⁻¹) or FeCl₃- (100 µM) mediated growth promotion of strain K2525 in EDDHA-containing Fe-limited medium B was abrogated in the presence but not the absence of citrate (0.1 M) (assessed visually after 18 h; data not shown). This contrasted with the FecA⁻ K2523 mutant, where pyoverdine- and FeCl₃-mediated growth promotion in EDDHA-containing medium was not abrogated by citrate (data not shown). Intriguingly, mutants of *S. enterica* defective in both *mgtA* and *mgtB* show enhanced Fe accumulation and increased Fe²⁺-dependent oxidative killing, the loss of these transporters apparently affecting the expression or activity of other metal transporters capable of accommodating Fe (Chamnongpol & Groisman, 2002). These results were consistent with loss of the Mg²⁺ transporters ultimately promoting Fe²⁺ acquisition and suggest, in light of the citrate sensitivity of an *mgtA* mutant in *P. aeruginosa*, that Fe delivered to *P. aeruginosa* by citrate may ultimately be entering the cell as Fe²⁺.

Contribution of the FeoB Fe²⁺ transporter to citrate-promoted Fe acquisition

Should citrate ultimately provide Fe to cells as Fe²⁺, then a mutant deficient in Fe²⁺ transport across the cytoplasmic membrane should be defective in citrate-promoted Fe acquisition. A cytoplasmic membrane Fe²⁺ transporter, FeoB, has been described in *E. coli* (Hantke, 1987; Kammler *et al.*, 1993) and other Gram-negative bacteria (Katoh *et al.*, 2001; Naikare *et al.*, 2006; Robey & Cianciotto, 2002;

Fig. 4. Defect in citrate-promoted growth of a *P. aeruginosa* PA4825 (*mgtA*) mutant in Fe-restricted medium. *P. aeruginosa* K2525 (*MgtA*⁺) was subcultured in Fe-deficient succinate minimal medium without supplementation (●) or supplemented with EDDHA (1 $\mu\text{g ml}^{-1}$) (■) or EDDHA (1 $\mu\text{g ml}^{-1}$) and citrate (25 mM) (▲) and growth was measured as a function of time. Growth of K2525 carrying the *mgtA* plasmid pBM13 in this medium is also shown (▼). The data are representative of a minimum of three independent experiments.

Velayudhan *et al.*, 2000; Wyckoff *et al.*, 2006) and a homologue has been identified in *P. aeruginosa* (PA4358; 67.5 % identical to *E. coli* FeoB). To assess a role for *feoB* (PA4358) in citrate-mediated Fe acquisition, the gene was deleted in strain IA614 and the impact on citrate-promoted growth in Fe-limited medium supplemented with the Fe chelator EDDA (Fig. 5A, top panel) and citrate-promoted Fe uptake (Fig. 5B) was assessed. Loss of *feoB* (PA4358) markedly reduced growth in EDDA and citrate-supplemented medium as well as citrate-promoted Fe uptake (Fig. 5, compare IA614 and K2931), consistent with the involvement of this Fe²⁺ transporter in citrate-mediated Fe acquisition. The cloned *feoB* (PA4358) gene restored citrate-promoted growth of strain K2931 in EDDA-supplemented minimal medium [Fig. 6A, compare growth of K2931 carrying pCG002 (pRK415::PA4358) vs K2931 carrying pRK415], though plasmid-bearing K2931 strains grew much slower than their plasmid-free counterparts (Fig. 6A). Cloned *feoB* also restored citrate-mediated Fe acquisition in strain K2931 (Fig. 6B). As in EDDHA-containing Fe-limited medium (Fig. 1A), citrate failed to promote growth of the *fecA* mutant K2523 in EDDA-containing medium (data not shown), confirming the need for *FecA* for citrate-promoted Fe acquisition.

Fig. 5. FeoB-dependent citrate-mediated Fe acquisition in *P. aeruginosa*. (A) Defect in citrate-promoted growth of *P. aeruginosa* PA4358 (*feoB*) mutants in Fe-restricted medium. *P. aeruginosa* IA614 (parent strain; ●), K2931 (Δ *feoB*; ■), K2932 (Δ *feoB* Δ *pcoA*; ○), K2556 (Δ *feoB* Δ PA4687 Δ PA5216; □) and K2530 (Δ *feoB* Δ *pcoA* Δ PA4687 Δ PA5216; ◇) were subcultured in succinate minimal medium supplemented with EDDA (8 $\mu\text{g ml}^{-1}$) and either citrate (25 mM) (Cit; top panel) or pyoverdine (100 $\mu\text{g ml}^{-1}$) (Pvd; bottom panel) and growth was monitored as a function of time. Growth of IA614 in succinate minimal medium supplemented with EDDA (8 $\mu\text{g ml}^{-1}$) only (◆) is shown (top panel) as a representative control highlighting lack of growth in the absence of siderophore. (B) Citrate-mediated Fe uptake in *P. aeruginosa* IA614 and its FeoB[−] derivatives K2931 (Δ *feoB*), K2556 (Δ *feoB* Δ PA4687 Δ PA5216) and K2932 (Δ *feoB* Δ *pcoA*) grown in Fe-deficient succinate minimal medium with citrate (1 mM). Data are reported as pmol ⁵⁵Fe accumulated after 15 min incubation of 1 ml *P. aeruginosa* cells in medium containing citrate (50 μmol) and ⁵⁵FeCl₃ (24 pmol) and have been adjusted to eliminate uptake seen in cells grown without citrate. The mean $\pm$ SD of three independent experiments is shown.

Fig. 6. Complementation of the citrate-promoted growth and Fe transport defects of *FeoB*[−] *P. aeruginosa* with cloned *feoB*. (A) *P. aeruginosa* K2931 (*FeoB*[−]; ■), K2931 carrying plasmid pCG002 (pRK415::*feoB*; ▼) or pRK415 without insert (▲), and *P. aeruginosa* IA614 (*FeoB*⁺; ●) were subcultured in succinate minimal medium supplemented with EDDA (8 µg ml^{−1}) and citrate (25 mM) and growth was measured as a function of time. Growth of IA614 in succinate minimal medium supplemented with EDDA (8 µg ml^{−1}) only (◆) is shown as a representative control highlighting lack of growth in the absence of citrate. The data are representative of a minimum of three independent experiments. (B) Citrate-mediated Fe uptake in *P. aeruginosa* K2931 (*FeoB*[−]), its pCG002- (K2931C) and pRK415- (K2931P) harbouring derivatives, and *P. aeruginosa* IA614 (*FeoB*⁺) grown in Fe-deficient succinate minimal medium with citrate (1 mM). Data are reported as pmol ⁵⁵Fe accumulated after 15 min incubation of 1 ml *P. aeruginosa* cells in medium containing citrate (50 µmol) and ⁵⁵FeCl₃ (24 pmol). The mean ± SD of three independent experiments is shown.

Contribution of the PcoA ferroxidase to citrate-promoted Fe acquisition

Residual growth and Fe transport are clearly evident in the Δ *feoB* (PA4358) mutant K2931, suggesting that additional Fe transporters can function in citrate-promoted Fe acquisition. Two operons encoding homologues of Fe^{3+} transporters in other bacteria have been annotated in *P. aeruginosa*, PA5216–5217 (homologue of the HitABC system in *Haemophilus influenzae*) (Sanders *et al.*, 1994)

and PA4687–4688 (homologue of the SfuABC and FbpABC systems in *Serratia marcescens* and *Neisseria* spp., respectively) (Adhikari *et al.*, 1996; Angerer *et al.*, 1992). Disruption of PA5216 (45 % identical to HitA) and PA4687 (30 % identical to SfuB) in K2931, yielding the triple knockout strain K2556, did not further compromise citrate-promoted growth in EDDA-containing medium (Fig. 5A, top panel) or citrate-mediated Fe uptake (Fig. 5B). These putative Fe^{3+} transport systems are, therefore, not involved in citrate-promoted Fe acquisition. Recently, a periplasmic multicopper oxidase with demonstrated ferroxidase activity, PcoA, has been shown to play a role in Fe^{2+} transport in *P. aeruginosa*, apparently functioning to oxidize Fe^{2+} to Fe^{3+} for subsequent transport via Fe^{3+} transporters (Huston *et al.*, 2002). A mutant strain, K2932, lacking both *feoB* (PA4358) and *pcoA* was also not more compromised for growth in EDDA-containing minimal medium (Fig. 5A, top panel) though intriguingly it was completely deficient in citrate-mediated Fe uptake (Fig. 5B). Elimination of *pcoA* from K2556 to produce a Δ *pcoA* Δ *feoB* (PA4358) Δ PA4687 Δ PA5216 quadruple deletion mutant did, however, completely abrogate citrate-promoted growth in EDDA-containing medium (Fig. 5A, top panel). None of the mutants showed any defect in pyoverdine- (Fig. 5A, bottom panel) or ferrioxamine B- (data not shown) promoted growth in EDDA-supplemented minimal medium, indicating that these $\text{Fe}^{2+}/\text{Fe}^{3+}$ transport components were not important for Fe transport mediated by these other siderophores.

DISCUSSION

Citrate promotion of Fe acquisition in *P. aeruginosa* has been known for some time, though when first reported it was seen to be independent of citrate in the growth medium and was, paradoxically, lowest in cells grown with citrate (Cox, 1980b). Still, a study by Harding & Royt (1990) clearly showed that a citrate-inducible ferric citrate uptake mechanism is present in *P. aeruginosa* grown under Fe-limiting conditions. Moreover, these authors confirmed that a citrate-inducible ferric citrate-binding activity was present in the outer membrane of this organism (Harding & Royt, 1990) reminiscent of the FecA ferric citrate receptor of *E. coli* (Braun, 2003). While a *fecA* homologue has been identified in *P. aeruginosa* (PA3901) (Ochsner *et al.*, 2002) and ferric citrate-promoted biofilm formation by *P. aeruginosa* requires this *fecA* gene (Banin *et al.*, 2005), consistent with PA3901 functioning as a ferric citrate receptor, the results presented here represent the first confirmation that PA3901 does indeed function as a receptor for ferric citrate. As in *E. coli* (Braun, 2003), and in agreement with the results of Harding & Royt (1990), *P. aeruginosa* FecA is found in the outer membrane of Fe-limited cells grown in the presence of citrate. In *E. coli*, the citrate-inducibility of *fecA* and the other ferric citrate transport components under Fe-limiting conditions is dependent upon the *fecIR* genes, encoding an ECF sigma

factor and its cognate anti-sigma factor (Braun & Mahren, 2005; Braun *et al.*, 2006; Visca *et al.*, 2002). *fecIR* homologues have been described in *P. aeruginosa* (PA3899, PA3900), where they are linked to *fecA* on the chromosome and have been shown to be strongly induced under Fe-limiting conditions (Ochsner *et al.*, 2002), consistent with a role in mediating the citrate inducibility of *fecA* under conditions of Fe limitation. While some induction of *P. aeruginosa* *FecA* in citrate-free Fe-limited medium was apparent in immunoblots, in agreement with earlier results showing modest Fe-limitation-inducibility of PA3901 (Ochsner *et al.*, 2002), maximal expression was citrate-dependent.

In the absence of obvious genes for a cytoplasmic membrane ferric citrate permease, the observation that a *feoB* mutant is compromised for citrate-promoted Fe acquisition provides important insights into the mechanism of citrate-mediated Fe uptake in *P. aeruginosa*. It is now well established that *FeoB*, first described as a cytoplasmic membrane Fe^{2+} transporter in *E. coli* (Hantke, 1987; Kammler *et al.*, 1993), contributes to Fe^{2+} uptake in a variety of micro-organisms including *Helicobacter pylori* (Velayudhan *et al.*, 2000), *Vibrio cholerae* (Wyckoff *et al.*, 2006), *Campylobacter jejuni* (Naikare *et al.*, 2006), *Legionella pneumophila* (Robey & Cianciotto, 2002) and *Synechocystis* sp. strain PCC 6803 (Kato *et al.*, 2001). As such, *FeoB*'s demonstrated involvement in citrate-mediated Fe acquisition clearly indicates that Fe^{3+} delivered to *P. aeruginosa* by citrate (via *FecA* since citrate-dependent Fe uptake and growth are compromised in a *fecA* mutant) must be reduced to Fe^{2+} prior to its transport into the cytosol. This explains the apparent lack of ferric citrate-specific cytoplasmic membrane transport components in *P. aeruginosa* and underscores a need for a reductive mechanism for release of Fe from citrate, presumably operational in the periplasm. The failure of two transposon mutant screens to identify a reductase as a component of citrate-mediated Fe acquisition in *P. aeruginosa* suggests that this component may be essential, though we cannot rule out that it was missed owing to inherent limitations of the screen itself. While periplasmic ferric citrate reductase activity has been reported in other organisms [*L. pneumophila* (Poch & Johnson, 1993) and *Vibrio vulnificus* (Mazoy *et al.*, 1999)], the only ferric citrate reductase activity reported to date in *P. aeruginosa* was found in the cytoplasm (Cox, 1980a). Assay conditions and/or sample preparation in this study may not, however, have been optimal for the proposed periplasmic reductase.

Citrate-promoted Fe acquisition has been demonstrated in *H. pylori* where it, too, is markedly reduced in a *feoB* mutant (Velayudhan *et al.*, 2000), consistent with citrate ultimately providing Fe to *H. pylori* cells as Fe^{2+} . In *Leptospira biflexa*, growth inhibition seen in Fe-limited medium was reportedly reversed by ferric citrate in wild-type cells but not *fecA* or *feoB* mutants, though no data were shown (Louvel *et al.*, 2005). As in *P. aeruginosa*, this

suggested that citrate-promoted Fe acquisition in *L. biflexa* proceeds via *FecA* and *FeoB*. Interestingly, *L. biflexa* also appears to lack *fecBCDE* homologues associated with ferric citrate uptake post-outer membrane.

A model whereby Fe is released from citrate in the periplasm is supported by the observation that additional loss of *pcoA* in a *feoB* mutant fully compromised citrate-mediated Fe uptake, given the periplasmic ferroxidase activity of *PcoA* and its demonstrated involvement in Fe^{2+} acquisition in *P. aeruginosa* (Huston *et al.*, 2002). Presumably, a reductive mechanism releases Fe from citrate and the resultant Fe^{2+} can then be transported via *FeoB* or oxidized to Fe^{3+} for transport via a cytoplasmic membrane Fe^{3+} transporter. That loss of the presumptive cytoplasmic membrane Fe^{3+} transporters PA5216–5217 and PA4687–4688 did not further compromise citrate-mediated Fe acquisition in a *feoB* mutant suggests that there must then be other systems for Fe^{3+} uptake across the cytoplasmic membrane. While *FeoB* and *PcoA* may be the major components for post-*FecA* transport of Fe delivered to cells by citrate, the observation that a *pcoA feoB* double mutant still demonstrated some citrate-dependent growth in Fe-restricted medium may be explained by an additional perhaps non-specific reductase activity that allows for sufficient Fe^{3+} for modest growth even though its transport was not detectable in a 15 min uptake assay. Certainly, loss of all four of *FeoB*, *PcoA*, PA5216–5217 and PA4687–4688 completely abrogated citrate-dependent growth and, indeed, prevented growth in low-Fe minimal medium, where exogenous addition of pyoverdine was required (data not shown).

In contrast to results with citrate, loss of the $\text{Fe}^{2+}/\text{Fe}^{3+}$ transport genes had no adverse effect on pyoverdine- or ferrioxamine B-dependent growth of *P. aeruginosa* in Fe-restricted medium. As such, either Fe is not released into the periplasm from these siderophores and/or siderophore-specific transporters function in the post-outer-membrane receptor delivery of Fe provided by these siderophores. A cytoplasmic-membrane-associated ferrioxamine B transporter, *FoxB*, has, in fact, been described in *P. aeruginosa* (Cuiv *et al.*, 2007). While there are a few candidate genes for cytoplasmic membrane ferric pyoverdine transporters, mutations in these do not compromise pyoverdine-mediated Fe acquisition (Ochsner *et al.*, 2002; Ravel & Cornelis, 2003) and, indeed, several studies indicate that Fe delivered to *P. aeruginosa* via pyoverdine, as with citrate, is released in the periplasm (Greenwald *et al.*, 2007; Mielczarek *et al.*, 1990; Royt, 1990) via a reductive mechanism (Greenwald *et al.*, 2007; Royt, 1990). Still, the observation that mutants lacking *FeoB* or, indeed, all of the other presumptive post-outer-membrane Fe transporters are competent for pyoverdine-promoted Fe acquisition suggests that Fe released from pyoverdine is probably transported via components specific for the pyoverdine system.

In conclusion, the results of this study confirm the involvement of the outer-membrane *FecA* receptor and

the cytoplasmic membrane FeoB Fe²⁺ transporter in citrate-mediated Fe uptake in *P. aeruginosa*. This system may serve as a model for Fe uptake promoted by heterologous siderophores in cases where siderophore-specific outer-membrane receptor genes are known but genes for siderophore-specific post-outer-membrane transport are lacking.

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