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.

**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 fepB,C,G 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 Fe³⁺ and Fe²⁺ have, for example, been described 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 electro-competent 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 PA4825-For (5'-GGATCCGATCGCGAACATCGCG-3') and PA3901-For (5'-GACTGAAATTCTCCACCGTGGCATTGGTTCCTCCCGAC-3'), respectively. Genomic 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 Inoue et al. (1991). *E. coli* cells were made competent using the method of Inoue et al. (1990). *P. aeruginosa* were made electro-competent by the method described by Choi et al. (2005). DNA sequencing was performed by ACGT Corporation (Toronto, Ontario, Canada) with universal and custom oligonucleotides.

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## Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties*</th>
<th>Source or reference</th>
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<td><strong>E. coli</strong></td>
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<tr>
<td>SM10 (Δpox)</td>
<td>thi-1 thr leu tonK lacY supE recA::RP4-2-Tc::Mu; Km (^\dagger)</td>
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<td>DH5α</td>
<td>φ80d lacZAM15 endA1 recA1 hsdR17 (rK mK) supE44 thi-1 gyrA96 relA1 F’ Δ(lacZYA–argF)U169</td>
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<td>S17-1</td>
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<td></td>
<td></td>
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<td>IA614</td>
<td>Pvd-2 Pch-1 Sal+</td>
<td>Ankenbauer &amp; Cox (1988)</td>
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<td>K2523</td>
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<td>de Lorenzo et al. (1990)</td>
</tr>
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<td>pK18MobSacB::ΔPA4687</td>
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*Ap’, ampicillin resistance; Tc’, tetracycline resistance; Km’, kanamycin resistance; Cb’, 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.

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EcoRI site underlined) in a reaction mixture formulated as above except that Vent DNA polymerase and 1 x 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 HindIII and EcoRI, 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 (Δpox) 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 PstI 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 µM) to a final OD_{600} of ~0.1. Cultures were incubated at 37 °C and OD_{600} 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 µg ml\(^{-1}\)) minimal medium, 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-diaceic acid (EDDA) at 8 µg ml\(^{-1}\) in place of EDDHA.

**Transport assay.** P. aeruginosa cells were cultured to late exponential phase (OD_{600}, 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 OD_{600} 0.1. 55Fe-citrate was or without 1 mM citrate, washed once in medium B without citrate and resuspended in the same medium at OD_{600} 0.1. 55Fe-citrate mixture was initiated by adding 5 µl 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 Fe\(^{2+}/\text{Fe}^{3+}\) transport genes [po (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 × ThermoPol buffer (New England Biolabs) in 50 µl 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 plasmid 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 µg ml\(^{-1}\); pKMobSacB) or tetracycline (75 µg ml\(^{-1}\); pEX18Tc) and imipenem (0.5 µg 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 (Reddy & 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 µM FeCl\(_3\)) 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 1:199 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 FeCl\(_3\) (100 µM) 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-CAACCCACGTG-3’)-rpsL-R (5’-GCTTGGCTTTGCAATTGTG-3’) and fecA-F (5’-ACGACCTGGATTAACCTTG-3’)-fecA-R (5’-GCTTGGTTGG-GTGTCTTAC-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 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

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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-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⁺; solid symbols) and K2523 (FecA⁻; open symbols) 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.

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.

Fig. 2. Citrate-inducible expression of fecA and FecA in P. aeruginosa. (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.
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 Fe$^{2+}$ 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 K2553 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 Mg$^{2+}$ 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 FeCl$_{3}$- (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 FeCl$_{3}$-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 Fe$^{2+}$-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 Mg$^{2+}$ transporters ultimately promoting Fe$^{2+}$ 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 Fe$^{2+}$.

**Contribution of the FeoB Fe$^{2+}$ transporter to citrate-promoted Fe acquisition**

Should citrate ultimately provide Fe to cells as Fe$^{2+}$, then a mutant deficient in Fe$^{2+}$ transport across the cytoplasmic membrane should be defective in citrate-promoted Fe acquisition. A cytoplasmic membrane Fe$^{2+}$ 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;
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 Fe\(^{2+}\) 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. 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 \(\mu\)g ml\(^{-1}\)) (■) or EDDHA (1 \(\mu\)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.](http://mic.sgmjournals.org)

![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 ΔpcoA*; ○), K2556 (Δ*feoB ΔPA4687 ΔPA5216*; □) and K2530 (Δ*feoB ΔpcoA ΔPA4687 ΔPA5216*; ◊) were subcultured in succinate minimal medium supplemented with EDDA (8 \(\mu\)g ml\(^{-1}\)) and either citrate (25 mM) (Cit; top panel) or pyoverdine (100 \(\mu\)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 \(\mu\)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 ΔPA4687 ΔPA5216*) and K2932 (Δ*feoB ΔpcoA*) grown in Fe-deficient succinate minimal medium with citrate (1 mM). Data are reported as pmol \(^{55}\)Fe accumulated after 15 min incubation of 1 ml *P. aeruginosa* cells in medium containing citrate (50 \(\mu\)mol) and \(^{55}\)FeCl\(_3\) (24 pmol) and have been adjusted to eliminate uptake seen in cells grown without citrate. The mean ± so of three independent experiments is shown.](http://mic.sgmjournals.org)
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$^{3+}$ 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) ΔPA4687 ΔPA5216 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 Fe$^{2+}$/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

![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 $^{55}$Fe accumulated after 15 min incubation of 1 ml *P. aeruginosa* cells in medium containing citrate (50 μmol) and $^{55}$FeCl$_3$ (24 pmol). The mean ± SD of three independent experiments is shown.](image)

**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$^{3+}$ 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) ΔPA4687 ΔPA5216 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 Fe$^{2+}$/Fe$^{3+}$ transport components were not important for Fe transport mediated by these other siderophores.

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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 fecB 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 (Naikere et al., 2006), Legionella pneumophila (Robey & Cianciotto, 2002) and Synechocystis sp. strain PCC 6803 (Katoh 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 fecB 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 fecB 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 fecB 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 fecB 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 fecB 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 Fe^{2+}/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 Fe$^{2+}$ 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.

**ACKNOWLEDGEMENTS**

The authors thank V. Braun for providing polyclonal antiserum to *E. coli* Fe6A and Christelle Gruffaz for technical assistance. This was work supported by operating grants from the Canadian Institutes of Health Research (to K. P. and to A. S.).

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Edited by: P. Cornelis