Identification and characterization of an iron-regulated gene, *chtA*, required for the utilization of the xenosiderophores aerobactin, rhizobactin 1021 and schizokinen by *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* utilizes several xenosiderophores under conditions of iron limitation, including the citrate hydroxamate siderophore aerobactin. Analysis of the *P. aeruginosa* genome sequence revealed the presence of two genes, *chtA* (PA4675) and PA1365, encoding proteins displaying significant similarity to the aerobactin outer-membrane receptor, *IutA*, of *Escherichia coli*. The *chtA* and PA1365 genes were mutated by insertional inactivation and it was demonstrated that ChtA is the outer-membrane receptor for aerobactin. ChtA also mediated the utilization of rhizobactin 1021 and schizokinen, which are structurally similar to aerobactin.

In contrast to the utilization of other xenosiderophores by *P. aeruginosa*, there was no apparent redundancy in the utilization of aerobactin, rhizobactin 1021 and schizokinen. The utilization of citrate hydroxamate siderophores by *P. aeruginosa* was demonstrated to be TonB1 dependent.

A Fur box was identified in the region directly upstream of *chtA* and it was demonstrated by the *in vivo* Fur titration assay that this region is capable of binding Fur and accordingly that expression of *chtA* is iron regulated. The PA1365 mutant was unaffected in the utilization of citrate hydroxamate siderophores.

**INTRODUCTION**

With few exceptions, all organisms display an absolute nutritional requirement for iron. For pathogenic bacteria, the host environment may be considered an iron-depleted environment, with all available iron located intracellularly or bound by high-affinity iron-binding proteins such as transferrin and lactoferrin (Litwin & Calderwood, 1993; Payne, 1993; Crosa, 1997). Under conditions of iron limitation, many bacteria produce low-molecular-mass high-affinity chelators termed siderophores that bind ferric iron and deliver it to the cell via specific outer-membrane receptors. Additionally, many bacterial pathogens also encode specific transport systems that allow for the assimilation of iron from host proteins such as haemoglobin, transferrin and lactoferrin (Ratledge & Dover, 2000; Genco & Dixon, 2001).

*Pseudomonas aeruginosa* is an opportunistic human pathogen that causes severe and often fatal infections and is the predominant cause of respiratory infections in individuals with cystic fibrosis. *P. aeruginosa* synthesizes two known siderophores, pyoverdine (Cox & Adams, 1985) and pyochelin (Cox, 1980), and has also been shown to be capable of utilizing a variety of xenosiderophores including enterobactin (Poole *et al.*, 1990) and desferrioxamine mesylate (Meyer, 1992). The ability of *P. aeruginosa* to utilize a wide variety of xenosiderophores possibly allows it to compete effectively with other micro-organisms for available iron and consequently to occupy extended ecological niches. However, despite the variety of xenosiderophores known to be transported by *P. aeruginosa*, in many cases their cognate outer-membrane receptors have not been identified.

Siderophore receptors function as gated channels in the outer membrane and display relatively tight specificity for their cognate siderophores. Siderophore-mediated iron transport across the outer membrane and into the periplasm is an energy-dependent process and the proton-motive force of the inner membrane is transduced to the outer membrane by the TonB protein in association with the cytoplasmic membrane proteins ExbB and ExbD. Together, these form a complex that transverses the periplasm and allows the interaction of TonB with siderophore-loaded receptors (Letaïn & Postle, 1997). TonB-dependent receptors have been shown to possess conserved sequences termed ‘TonB boxes’, which possibly function in the interaction of TonB with the receptor, resulting in a conformational change that allows the release of the ferric siderophore into the periplasm. Three TonB homologues have been identified and...
characterized in \emph{P. aeruginosa}. The \emph{tonB} gene encodes a protein, termed TonB1, which is similar to other TonB proteins with the exception of an extended N-terminal sequence that is required for function (Poole \emph{et al.}, 1996; Zhao & Poole, 2002). Mutants in \emph{tonB} are affected for siderophore-mediated iron acquisition and haem acquisition. A second \emph{tonB} gene, \emph{tonB2}, identified in \emph{P. aeruginosa} is linked to homologues of \emph{exbB} and \emph{exbD}; however, the \emph{TonB2} protein and the \emph{ExbB} and \emph{ExbD} proteins were found to be dispensable for iron acquisition (Zhao & Poole, 2000). A third \emph{tonB} gene, termed \emph{tonB3}, was found to be required for normal twitching motility and the assembly of extracellular pili (Huang \emph{et al.}, 2004). Little is known about the mechanisms of inner-membrane siderophore transport in \emph{P. aeruginosa}. In \emph{silico} analysis of the genome sequence indicates the presence of relatively few siderophore inner-membrane iron-transport systems with respect to the number of putative siderophore receptors (Köster, 2001; Poole & McKay, 2003; P. Ó Cuív & M. O’Connell, unpublished observation). Currently, the only inner-membrane siderophore transporter identified in \emph{P. aeruginosa} is the FptX permease, which has been implicated in pyochelin utilization (Ó Cuív \emph{et al.}, 2004).

In \emph{Escherichia coli}, the Fur (Ferric uptake regulator) protein regulates the expression of iron-transport systems at the transcriptional level (Hantke, 1984, 2001). The \emph{fur} gene of \emph{P. aeruginosa} has been characterized and shown to be essential (Prince \emph{et al.}, 1993). The Fur protein has been shown to directly affect the transcription of several iron-acquisition systems, including both the \emph{phu} and \emph{has} haem-acquisition systems (Ochsner \emph{et al.}, 2000). The Fur protein also controls other regulators that are required for the expression of several virulence factors such as pyochelin, pyoverdine, exotoxin A and PrPL protease (Cunliffe \emph{et al.}, 1995; Miyazaki \emph{et al.}, 1995; Heinrichs & Poole, 1996; Ochsner \emph{et al.}, 1996; Reimann \emph{et al.}, 1998; Wilderman \emph{et al.}, 2001).

In a previous study, the ability of \emph{P. aeruginosa} to utilize the siderophore and important virulence factor aerobactin was described (Liu & Shokrani, 1978). Analysis by our group indicated that the aerobactin outer-membrane receptor IutA of \emph{E. coli} was capable of mediating the transport of the structurally similar citrate hydroxamate siderophores rhizobactin 1021 produced by \emph{Sinorhizobium meliloti} 2011 and schizokinen produced by \emph{Bacillus megaterium} ATCC 19213 (91-02) (Ó Cuív \emph{et al.}, 2004). Two putative proteins displaying significant similarity to IutA were identified in the \emph{P. aeruginosa} genome sequence. Here we report the construction of \emph{P. aeruginosa} mutants having insertions in the genes encoding the IutA homologues, and the identification of ChtA, the outer-membrane receptor for the citrate hydroxamate siderophores aerobactin, rhizobactin 1021 and schizokinen.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are described in Table 1. \emph{Pseudomonas aeruginosa}, \emph{Escherichia coli} and \emph{Bacillus megaterium} were cultured on LB medium (Sambrook \emph{et al.}, 1989) while \emph{Sinorhizobium meliloti} was cultured on TY medium (Birnboim & Dolin, 1979). Plasmid DNA was isolated from cultures of the alkaline lysis method (Birnboim & Dolin, 1979). PCR were carried out using the temperature-gradient block in a Thermo Hybaid PCR Express thermocycler. Restricted DNA fragments for subcloning were cut from ethidium-bromide-stained gels as required and purified using the Perfectprep gel clean-up kit as directed by the manufacturers (Roche). For Southern blot analysis, restricted genomic DNA was separated by agarose gel electrophoresis and transferred to nitrocellulose filters (Sambrook \emph{et al.}, 1989). Probes for the Southern blots were labelled and hybridized using the DIG DNA labelling and detection kit as directed by the manufacturers (Roche). All mutants constructed in this study were confirmed by Southern blot analysis.

Access to the \emph{P. aeruginosa} PAO1 genome sequence data was obtained at http://www.pseudomonas.com. Database searches were undertaken using the BLAST programs (Altschul \emph{et al.}, 1997) at the \emph{P. aeruginosa} genome site and the National Centre for Biototechnology Information (NCBI) at the National Library of Medicine. Multiple sequence alignments were performed using Multalin (Corpet, 1988) and Genedoc. Protein signal sequences were predicted using the SignalP program (Nielsen \emph{et al.}, 1997), while the PSORT program (Nakai & Kanehisa, 1991) was used to predict protein localization.

**Strain and plasmid construction.** To construct the mutant strain \emph{P. aeruginosa} PA4675km carrying the kanamycin-resistance cassette from pUC4K in the PA4675 gene, a cosmid, pMO010918, encoding the region of interest was obtained from the \emph{Pseudomonas} Genetic Stock Center. A 4 kb \emph{PvuII}–BamH1 site was deleted from pUC4K in the PA4675 gene, a cosmid, pMO010918, encoding the region of interest was obtained from the \emph{Pseudomonas} Genetic Stock Center. A 4 kb \emph{PvuII}–BamH1

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**DNA manipulations and in silico analysis.** Molecular biology techniques were performed using standard protocols (Sambrook \emph{et al.}, 1989). Total genomic DNA was prepared from \emph{P. aeruginosa} as described by Chen & Kuo (1993) but with the modification that the DNA was phenol extracted twice before the chloroform extraction. Cosmid DNA was prepared by the method of Little (1987) with the modification that the DNA was phenol extracted and precipitated with 2.5 vols ethanol on ice for 30 min. Plasmid DNA was isolated from cultures by the alkaline lysis method (Birnboim & Dolin, 1979). PCR were carried out using the temperature-gradient block in a Thermo Hybaid PCR Express thermocycler. Restricted DNA fragments for subcloning were cut from ethidium-bromide-stained gels as required and purified using the Perfectprep gel clean-up kit as directed by the manufacturers (Roche). All mutants constructed in this study were confirmed by Southern blot analysis.

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Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source*</th>
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<tr>
<td><strong>P. aeruginosa</strong></td>
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<td>PA01</td>
<td>Wild-type</td>
<td>Ankenbauer <em>et al.</em> (1986)</td>
</tr>
<tr>
<td>CDC5</td>
<td>pvd-2</td>
<td>Heinrichs &amp; Poole (1996)</td>
</tr>
<tr>
<td>DH119</td>
<td>CDC5, pchR</td>
<td>This study</td>
</tr>
<tr>
<td>PA4675km</td>
<td>DH119, chtA::Km'</td>
<td>This study</td>
</tr>
<tr>
<td>PA1365km</td>
<td>DH119, PA1365::Km'</td>
<td>This study</td>
</tr>
<tr>
<td>IA1</td>
<td>PA01, does not produce pyoverdine or pyochelin</td>
<td>Ankenbauer <em>et al.</em> (1985)</td>
</tr>
<tr>
<td>K1040</td>
<td>PAO6609, ΔTonB::TcHg</td>
<td>Zhao <em>et al.</em> (1998)</td>
</tr>
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<td><strong>E. coli</strong></td>
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<td></td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>recA1 hsdR17 (N&lt;sup&gt;-&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt;) supE44 lac [F' proAB&lt;sup&gt;+&lt;/sup&gt; lacI21, lacZAM15::Tn10(TetR)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>INV&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F', recA1 hsdR17 (N&lt;sup&gt;-&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt;) supE44 &lt;del&gt;80&lt;/del&gt;lacZAM15 Δ(lacZYA-argF)U169</td>
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<td>MC4100</td>
<td>F&lt;sup&gt;-&lt;/sup&gt; araD139 Δ(argF–lac)U169 rpsL150 relA1 flbB5301 depC1 ptsF25 rbsR</td>
<td>Casadaban (1976)</td>
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<td>H1717</td>
<td>araD139 rpsL150 Δ(argF–lac)U169 relA1 flbB5301 depC1 ptsF25 rbsR araB lhuF::2placMu; host strain for FURTA</td>
<td>Stojiljkovic <em>et al.</em> (1994)</td>
</tr>
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<td><strong>S. meliloti</strong></td>
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</tr>
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<td>2011</td>
<td>Wild-type, str</td>
<td>Lynch <em>et al.</em> (2001)</td>
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<tr>
<td>2011rhhA62</td>
<td>rhhA::Tn5lac, rif</td>
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<td><strong>B. megaterium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 19213 (91-02)</td>
<td>Produces schizokinen</td>
<td>NCIMB</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pMO010918</td>
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<td>PGSC</td>
</tr>
<tr>
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<td>Cosmid carrying the region encoding PA1365, mob Te'</td>
<td>PGSC</td>
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<td>pMO012405</td>
<td>Cosmid carrying the region encoding fptA; mob Te'</td>
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<td>pRK600</td>
<td>Cm', pRK2013 Nm::Tn9, provides transfer functions</td>
<td>Finan <em>et al.</em> (1986)</td>
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<td>pJQ200sk</td>
<td>Cm' mob sacB</td>
<td>Quandt &amp; Hynes (1993)</td>
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<td>pBBR1MCS-5</td>
<td>Broad-host-range cloning vector, Cm' mob lacZ</td>
<td>Kovach <em>et al.</em> (1995)</td>
</tr>
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<td>pPROBE-GT</td>
<td>Broad-host-range promoter-probe vector, Cm' mob gfp</td>
<td>Miller <em>et al.</em> (2000)</td>
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<td>pCR2.1</td>
<td>PCR cloning vector, Ap' Km' lacZ</td>
<td>Invitrogen</td>
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<td>pRG13</td>
<td>Cm', pACYC184 carrying the region encoding iucABCD from pColV-K311</td>
<td>Gross <em>et al.</em> (1985)</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Ap', source of Km' cassette</td>
<td>Amersham PharmaNova</td>
</tr>
<tr>
<td>pUC18</td>
<td>Ap' lacZ</td>
<td>Amersham PharmaNova</td>
</tr>
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<td>pFur7, 8</td>
<td>pUC18 carrying the Fur box consensus sequence</td>
<td>Heinrichs <em>et al.</em> (1999)</td>
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<td>pUC 0-8 B/P</td>
<td>pUC18 carrying a 0-8 kb BamHI–PstI insert</td>
<td>This study</td>
</tr>
<tr>
<td>pUC 1-4 S/E</td>
<td>pUC18 carrying a 1-4 kb SalI–EcoRI insert</td>
<td>This study</td>
</tr>
<tr>
<td>pOC3-4K X/Xm</td>
<td>pJQ200ks carrying 3-4 kb XhoI–XmaI fragment with the Km' cassette from pUC4K cloned into the BamHI site of chtA</td>
<td>This study</td>
</tr>
<tr>
<td>pOC4-0K A/B</td>
<td>pJQ200ks carrying 4-0 kb Apal–BamHI fragment with the Km' cassette from pUC4K cloned into the XhoI site of PA1365</td>
<td>This study</td>
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<tr>
<td>pPOC7</td>
<td>pBBR1MCS-5 with chtA cloned as a XhoI–EcoRI fragment</td>
<td>This study</td>
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<tr>
<td>pPOC9</td>
<td>pBBR1MCS-5 with iutA cloned as an Apal–HindIII fragment</td>
<td>This study</td>
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<tr>
<td>pPROBE-ChtA</td>
<td>pPROBE-GT with chtA and its promoter cloned as a HindIII–EcoRI fragment</td>
<td>This study</td>
</tr>
</tbody>
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*NCIMB, National Collection of Industrial, Food and Marine Bacteria, Aberdeen, Scotland, UK; PGSE, Pseudomonas Genetic Stock Center (http://www.ecu.edu/pseudomonas/).

Fragment was subcloned from the cosmid into the suicide vector pJQ200sk, removing the vector-borne XhoI site and leaving a unique XhoI site within the gene. The kanamycin-resistance cassette from pUC4K was inserted as a SalI fragment into this unique site, generating pOCA4-0K A/B. The PA1365 mutant was constructed in a similar manner to *P. aeruginosa* PA4675km.

For the genetic complementation experiments, the genes of interest were PCR amplified with their own ribosome-binding sites and cloned into the broad-host-range mobilizable plasmid pBBR1MCS-5, placing them under the control of the lac promoter. The plasmid pPOC7, expressing chtA, was constructed by PCR amplifying the gene with primers that introduced unique XhoI and EcoRI sites into the PCR-amplified product (forward primer Pr<sup>+</sup> 5'-CTG AAC ACA GCA ACT CGG AAA TCT CCT CAT GCT CTC CC-3', reverse primer Pr<sup>-</sup> 5'-GAA TTC TCA GAA TTC CAC GCT ATA GGT CAG GCT GT-3'). The PCR product was subsequently cloned into XhoI- and EcoRI-restricted pBBR1MCS-5. The plasmid pPOC9, expressing IutA, was constructed...
by PCR amplifying the gene with primers that introduced unique Apal and HindIII restriction sites into the PCR-amplified product (forward primer P₁ 5′-GGG CCC TTC CGC CCG TAT GGA GGA ATA ATG ATG-3′; reverse primer P₂ 5′-AAG CTT TCA GAA CAG CAC AGA GTA GTT CAG ACC-3′). The PCR product was subsequently cloned into Apal- and HindIII-restricted pBBR1MCS-5.

The FURTA was employed to analyse the promoter region of chtA for the presence of a functional Fur-binding site. Plasmids were introduced into E. coli H1717 and transformants were patched onto MacConkey agar supplemented with FeCl₃ and ampicillin. The pUC 0-8 B/P plasmid, carrying the chtA promoter region and the 5′ end of chtA, was constructed by subcloning a 0-8 kb BamHII-PstI fragment from pMO010918 into pUC18. The pUC 1-4 S/E plasmid, carrying the region extending from the 5′ end of fptA to the 3′ end of pelI and thus the fptA promoter, was constructed by subcloning a 1-4 kb SalI–EcoRI fragment from pMO012405 into pUC18. This plasmid was used as a control to confirm the ability of the FURTA to detect P. aeruginosa Fur boxes. pUC18 was used as a vector control while pFur7, 8, carrying the E. coli consensus Fur box, was used as a positive control.

For the analysis of chtA expression, a gfp transcriptional fusion was constructed. An artificial operon was constructed by cloning chtA with its promoter upstream of gfp in the broad-host-range mobilizable promoter probe vector pPROBE-GT. The vector carries an optimally placed ribosome-binding site for gfp, so that both chtA and gfp are independently translated. The chtA promoter and gene were PCR amplified with primers that introduced HindIII and EcoRI restriction sites into the PCR product (forward primer P₁ 5′-AAG CTT GGT CAA TTT GTA GCC AGC ATG AAT CCG-3′; reverse primer P₂ 5′-GAA TTC TCA GAA CTC CAC GCT ATA CGT CAG CGT GT-3′). The PCR product was ligated into the pCR2.1 vector and subsequently cloned into HindIII- and EcoRI-restricted pPROBE-GT, generating pPROBE-ChtA. The chtA promoter region was sequence verified by comparison with the P. aeruginosa PAO1 genome sequence.

Siderophore utilization biosaass. For growth-stimulation assays, P. aeruginosa was grown in LB broth until early stationary phase and then 200 μl was inoculated into molten LB agar containing 2,2′-dipyridyl at a concentration of 1 mM and antibiotics as appropriate. Wells were punched into the solidified agar and 50 μl volumes of the test solutions were added. The plates were incubated at 37 °C, after which they were examined for growth stimulation. For semi-quantitative analysis, the diameter of the zone of growth stimulation was measured typically after 18–24 h incubation. Results are the mean of three independent experiments where each experiment consisted of at least three independent plates. Growth stimulation assays involving E. coli were similarly performed, except that 2,2′-dipyridyl was used at a final concentration of 300 μM. Where necessary, IPTG was added to a final concentration of 1 mM.

Concentrated supernatants containing siderophores were prepared by growing the culture of interest in broth under iron-depleted conditions to induce siderophore production. The cell-free culture supernatant was subsequently concentrated in a vacuum dryer and filter-sterilized. E. coli and B. megaterium strains were grown in LB with 2,2′-dipyridyl at a final concentration of 300 μM, while P. aeruginosa was grown in LB with 2,2′-dipyridyl at a final concentration of 500 μM. S. meliloti was grown in TY with 2,2′-dipyridyl at a final concentration of 200 μM. Aerobactin was prepared from E. coli H1443 carrying pRG13. The aerB mutation in E. coli H1443 abolishes the production of enterobactin, while aerobactin biosynthesis is encoded by iucABCDD on pRG13. To confirm that growth promotion was due to enterobactin, concentrated culture supernatant was also prepared from E. coli H1443 grown under the same conditions. Concentrated supernatant containing rhizobactin 1021 was prepared from S. meliloti 2011. Concentrated culture supernatant was also prepared from S. meliloti 2011 rhaA62, which does not produce rhizobactin 1021, grown under similar conditions. Schizokinen was prepared from B. megaterium ATCC 19213 (91-02).

Concentrated culture supernatants containing pyoverdine and pyochelin were prepared from P. aeruginosa PAO1 and CDC5 respectively. Concentrated culture supernatant was also prepared from P. aeruginosa IA1, which does not produce pyochelin or pyoverdine, grown under similar conditions. Enterobactin was prepared from E. coli MC4100 grown in LB with 2,2′-dipyridyl at a final concentration of 300 μM. Stock solutions of desferrioxamine mesylate (Sigma), ferrichrome (Sigma) and monoferric dicitrate (ferricitrate) were prepared at concentrations of 1 mM, 0.5 mM and 10 mM respectively in ultrapure water. Purified deferrated aerobactin was purchased from EMC Microcollections (Tübingen, Germany) and prepared as a 1 mM stock solution in ultrapure water.

**Analysis of GFP expression levels.** Cultures of P. aeruginosa PA675km carrying pPROBE-GT or pPROBE-ChtA were grown overnight at 37 °C and inoculated to a starting OD₆₀₀ of 0.01 in 20 ml pre-warmed LB containing gentamicin at a concentration of 50 μg ml⁻¹. The LB was treated with FeCl₃ (1 mM) or 2,2′-dipyridyl (500 μM) to model iron-replete and iron-depleted conditions respectively. Aerobactin was added to a final concentration of 2 μM as appropriate. This level of aerobactin was sufficient to provide a clear growth promotion effect. The culture was grown to an OD₆₀₀ of approximately 1.0–1.5 and the culture fluorescence was measured on a Perkin Elmer Luminescence Spectrometer LS 50 B with an excitation wavelength of 490 nm and a slit width of 5 nm, and an emission wavelength of 510 nm and a slit width of 15 nm. The fluorescence readings were adjusted to take account of the background fluorescence of the cell-free culture supernatant and were normalized to an OD₆₀₀ of 1.0. Fluorescence values and standard deviations were calculated from three separate experiments where each experiment consisted of three individual cultures that were analysed in duplicate and averaged. A two-sample t test was applied to determine significant differences using readings from the nine flasks that were representative of the three independent experiments performed per condition. Culture fluorescence due to GFP was found to increase in a linear manner with culture OD₆₀₀ over the experimental conditions examined.

**RESULTS**

**P. aeruginosa is capable of utilizing structurally similar citrate hydroxamate siderophores**

Rhizobactin 1021 is an asymmetric citrate hydroxamate siderophore and is the only siderophore known to be produced by the soil bacterium S. meliloti 2011. In a previous study, we cloned and characterized the RhtA outer-membrane receptor from S. meliloti 2011, and determined that it functioned in the utilization of rhizobactin 1021 (Lynch et al., 2001). BLASTp analysis of the RhtA receptor sequence indicated that the protein displayed significant similarity to the aerobactin outer-membrane receptor, IutA, of E. coli. Rhizobactin 1021 is structurally similar to several citrate hydroxamate-type siderophores, including aerobactin, which is produced by some members of the Enterobacteriaceae, and schizokinen, which is produced by B. megaterium ATCC 19213 (91-02) (Fig. 1). Similarly to aerobactin, the utilization of rhizobactin 1021 and schizokinen by E. coli is dependent on the outer-membrane receptor IutA (Ó Cuív et al., 2004). Following publication of the P.
P. aeruginosa genome sequence (Stover et al., 2000), a putative TonB-dependent receptor, PA4675, displaying significant similarity to IutA was identified. BLASTP analysis at the P. aeruginosa Genome Project database revealed that PA4675 displays 46% identity to IutA. An additional putative TonB-dependent receptor, PA1365, displaying 29% identity to IutA was also identified, although this receptor displays over 50% identity with the alcaligin E receptor, AleB, from Ralstonia metallidurans (Gils et al., 1996).

The ability of P. aeruginosa to utilize aerobactin has previously been reported (Liu & Shokrani, 1978), and therefore the ability of P. aeruginosa to utilize rhizobactin 1021 and schizokinen was examined. A P. aeruginosa strain defective in pyoverdine production and pyochelin production and utilization was used for the analysis. The P. aeruginosa DH119 strain displays limited growth on iron-depleted media, making it suitable as an indicator strain for xenosiderophore utilization. Analysis by the siderophore utilization bioassay indicated that P. aeruginosa DH119 was capable of utilizing rhizobactin 1021 and schizokinen present in concentrated culture supernatant, to overcome the conditions of iron limitation (Fig. 2, Table 2). No growth was observed when P. aeruginosa DH119 was grown in the presence of concentrated culture supernatant lacking either siderophore (data not shown).

Roles of PA4675 and PA1365 in siderophore utilization

To investigate the possible roles of PA4675 and PA1365 in siderophore utilization, mutants were constructed by the insertion of a kanamycin-resistance cassette into the chromosome by allelic exchange. A kanamycin-resistance cassette was inserted into the PA4675 gene of P. aeruginosa DH119, generating P. aeruginosa PA4675km. Similarly, a kanamycin-resistance cassette was also introduced into the PA1365 gene of P. aeruginosa DH119, generating P. aeruginosa PA1365km. The ability of the P. aeruginosa PA4675km and PA1365km strains to utilize aerobactin, rhizobactin 1021 and schizokinen was compared to that of the P. aeruginosa DH119 parent strain. Analysis by the siderophore utilization bioassay indicated that P. aeruginosa PA4675km, in contrast to the parent P. aeruginosa DH119, did not utilize any of the siderophores tested (Fig. 2, Table 2). The PA4675 gene was thus designated chtA (citrate hydroxamate transport). When P. aeruginosa PA4675km was complemented with pPOC7, carrying the chtA gene, the ability of the mutant to utilize aerobactin, rhizobactin 1021 and schizokinen was restored (Fig. 2, Table 2). P. aeruginosa PA1365km was unaffected for the utilization of aerobactin, rhizobactin 1021 and schizokinen (Table 2).

To eliminate the possibility that the P. aeruginosa PA4675km phenotype was due to a pleiotropic effect on siderophore utilization, the ability of P. aeruginosa PA4675km to utilize various catechol- and hydroxamate-type xenosiderophores was examined. The capacity of P. aeruginosa PA4675km to utilize the catechol-type siderophore enterobactin and the hydroxamate-type siderophores desferrioxamine mesylate and ferrichrome was similar to that of the parent strain P. aeruginosa DH119 (Table 2). The ability of P. aeruginosa PA4675km to utilize ferricitrate was also unaffected. P. aeruginosa PA4675km was also examined for its ability to utilize the endogenous siderophore pyoverdine. P. aeruginosa PA4675km was shown to be unaffected in its ability to utilize siderophore prepared from P. aeruginosa PAO1. P. aeruginosa PAO1 produces two endogenous siderophores, pyoverdine and pyochelin. To confirm that it was pyoverdine and not pyochelin that was being utilized, the ability of P. aeruginosa PA4675km to utilize siderophore prepared from P. aeruginosa CDC5, which only produces pyochelin, was examined. Analogously to the parental strain, P. aeruginosa DH119, P. aeruginosa PA4675km was unable to utilize pyochelin. The results indicated that the insertion in chtA did not result in a pleiotropic effect on siderophore utilization.

P. aeruginosa displays receptor redundancy in the utilization of several siderophores including desferrioxamine B, type I siderophore...
pyoverdine and enterobactin (Dean et al., 1996; Ghysels et al., 2004; Banin et al., 2005). Incubation of siderophore bioassays for periods of up to 48 h resulted in the same pattern of utilization, indicating that there is no receptor redundancy in the utilization of citrate hydroxamate siderophores by *P. aeruginosa*.

**In silico analysis of ChtA**

The ChtA sequence was used to perform a search of the GenBank database using the BLASTP program. Several IutA-like proteins in the TonB-dependent outer-membrane receptor family were identified that displayed significant similarity to ChtA. The highest percentage identity was to the putative receptor Paer03002374 from *P. aeruginosa* UCBPP-PA14 (95%), followed by IutA from *Klebsiella pneumoniae* CG43 (48%), IutA from *E. coli* Nissle (47%), PP2193 from *Pseudomonas putida* KT2440 (43%) and PFL_3154 from *Pseudomonas fluorescens* Pf-5 (41%).

A putative Shine–Dalgarno sequence (CGGA) is located upstream of the predicted *chtA* translational start codon. Directly downstream of *chtA*, an inverted repeat was identified that possibly functions as a transcriptional termination sequence. A potential 28 amino acid signal sequence typical for outer-membrane proteins was identified at the N-terminal of the predicted protein. Cleavage between the ALA-AD30 residues would result in a mature protein with a molecular mass of 78 kDa. The ChtA protein contains sequence motifs conserved among TonB-dependent receptors and designated regions I, II and III (Bitter et al., 1991).

Region I, typically referred to as the ‘TonB box’ is generally located at the N-terminus and has the consensus sequence (D/E)TXXVXA(A/S) (Bitter et al., 1991; Ankenbauer & Quan, 1994). A weak ‘TonB box’-like motif was identified at amino acid residues 24–31 of the mature protein. Region II is typically located at the C-terminus and a signature sequence completely matching the consensus sequence (F/I/L/M/V)XXX(I/L/V)XNLX(D/N)(K/R)XY was identified at position 692–704 of ChtA. Region III is typically located 100 amino acids from region I, and a motif corresponding to the consensus sequence R(V/I)(D/E)(I/V)(I/V/L)(K/R)-GXX(G/S/A)XXXGXXXXG(G/A)X(V/I) was identified at position 131–152 of ChtA. The location of ChtA by PSORT analysis is predicted to be the outer membrane and in conjunction with the presence of the ‘TonB motifs’, this further suggests that ChtA is a TonB-dependent outer-membrane receptor.

**Activity analysis of ChtA**

Three TonB homologues, TonB1, TonB2 and TonB3, have been identified in the *P. aeruginosa* genome sequence and all three homologues have been experimentally characterized (Poole et al., 1996; Takase et al., 2000; Zhao & Poole, 2000, 2002; Huang et al., 2004). Siderophore-mediated iron transport in *P. aeruginosa* is primarily dependent on the activity of the TonB1 protein. Investigation of the ability of the *tonB1* mutant, *P. aeruginosa* K1040, to utilize aerobactin,
rhizobactin 1021 and schizokinen revealed that these siderophores were not utilized, indicating that siderophore-mediated iron transport via the ChtA outer-membrane receptor is TonB1 dependent (Table 2).

The ChtA protein displays significant similarity to IutA, the outer-membrane receptor for citrate hydroxamate-type siderophores in E. coli. The ability of ChtA to functionally substitute IutA in E. coli strain H1443 was examined. This strain is defective in enterobactin production and also lacks IutA, and is thus suitable for use as an indicator strain. The pPOC7 plasmid was introduced into E. coli H1443 and the ability of the strain to utilize aerobactin, rhizobactin 1021 and schizokinen was examined. Analysis indicated that the ability to utilize the siderophores was not conferred upon the strain. In a reciprocal experiment, the pPOC9 plasmid carrying iutA was introduced into P. aeruginosa PA4675km and the ability of the strain to utilize aerobactin, rhizobactin 1021 and schizokinen was examined; all of the siderophores tested were utilized. It was not possible to identify ChtA in outer-membrane profiles of P. aeruginosa DH119, possibly due to the presence of co-migrating proteins (data not shown); however, the ability of IutA to functionally substitute ChtA provides further evidence that ChtA is an outer-membrane receptor. As expected, the introduction of pPOC9 into E. coli H1443 conferred upon the strain the ability to utilize aerobactin, rhizobactin 1021 and schizokinen as analysed by the siderophore utilization bioassay.

**Regulation of chtA expression**

The genomic region directly upstream of chtA was examined to identify motifs commonly found in iron-regulated genes of P. aeruginosa. A putative Fur box consensus sequence was identified, having 14 out of 19 matches with the consensus E. coli Fur box, although the characteristic palindrome is not well conserved, suggesting that this sequence may have limited Fur-binding ability. The Fur-binding capability of the chtA Fur box was examined using the in vivo FURTA in the E. coli reporter strain H1717 (Stojiljkovic et al., 1994). E. coli H1717 carries a fluF–lacZ fusion and under iron-replete conditions expression of the fusion is repressed and colonies appear white when analysed on MacConkey agar. The introduction of a functional Fur-binding site on a multicopy plasmid, however, results in a titration of the intracellular Fur levels and concomitant expression of the fluF–lacZ fusion; colonies thus appear red when analysed on MacConkey agar. The FURTA has previously been successfully applied to pseudomonads, including P. aeruginosa (Leoni et al., 1996). Examination demonstrated that a plasmid carrying the chtA promoter region and associated Fur box (pUC 0.8 B/P) exhibited moderate Fur-binding activity as compared with the positive control plasmids, pFur7, carrying the consensus E. coli Fur box and pUC 1.4 S/E carrying the fptA promoter region and associated Fur box (17/19 matches to the E. coli consensus sequence), which displayed strong Fur-binding activity as expected. In contrast, pUC18 did not display any Fur-binding activity.

In order to determine the effect of iron on the expression of chtA, a chtA–gfp transcriptional fusion, with the chtA promoter region and entire chtA gene, was constructed (pPROBE-ChtA) and introduced into P. aeruginosa PA4675km. Analysis of chtA–gfp expression indicated that the fusion was expressed slightly above background fluorescence under iron-replete conditions (1 mM FeCl$_3$; 1.9 ± 1.7 GFP fluorescence units) and that expression was increased approximately 25-fold under iron-depleted conditions (500 μM 2,2'-dipyridyl; 48.0 ± 4.1 units). Addition of 2 μM exogenous aerobactin to iron-depleted cultures resulted in a further increase of approximately 16% in culture fluorescence and this increase was determined to be statistically significant (55.7 ± 5.6 units). As expected, no fluorescence was detected in cells containing empty vector under the conditions tested. Fluorescence microscopy was employed to confirm expression of the chtA–gfp fusion under iron-replete and -depleted conditions (data not shown).

**DISCUSSION**

P. aeruginosa is an opportunistic human pathogen that can cause severe and often fatal infections in the immunocompromised host. The ability to acquire iron is essential for the growth and proliferation of P. aeruginosa, and iron stress leads to specific patterns of gene induction and repression (Ochsner et al., 2002; Palma et al., 2003). P. aeruginosa, in common with other fluorescent pseudomonads, encodes a plethora of putative TonB-dependent outer-membrane receptors (Cornelis & Matthijs, 2002). Consistent with this, P. aeruginosa is known to utilize a wide variety of xenosiderophores.

It was previously reported that P. aeruginosa is capable of utilizing the citrate hydroxamate siderophore aerobactin. Aerobactin is structurally similar to rhizobactin 1021 and schizokinen. Here we have shown that all three siderophores are utilized via the ChtA receptor. ChtA displays significant similarity to the citrate hydroxamate siderophore receptor, IutA, of E. coli; however, introduction of a plasmid carrying chtA into an E. coli strain defective in the ability to utilize aerobactin, rhizobactin 1021 and schizokinen did not result in the restoration of the ability to utilize any of the siderophores. The transport of the citrate hydroxamate siderophores via ChtA in P. aeruginosa is dependent on the activity of the TonB1 protein. TonB1, in contrast to E. coli TonB, contains an N-terminal extension that has been shown to be essential for activity in both P. aeruginosa and E. coli (Zhao & Poole, 2002). It is possible that the E. coli TonB protein cannot interact with ChtA to mediate transport of citrate hydroxamate siderophores. Alternatively, the release of the siderophores into the periplasm may require an interaction with an additional protein, possibly a periplasmic binding protein that is encoded in the P. aeruginosa genome. However, mutants defective in the production of the periplasmic binding protein PhuD in E. coli accumulate aerobactin in the periplasm, suggesting that an interaction with IutA is not
necessary for release of the siderophore into the periplasm of this organism (Wooldridge et al., 1992).

The ability of ChtA to transport all three citrate hydroxamate siderophores implies that it can recognize and accommodate the different siderophore structures. It is likely that ChtA recognizes the core citrate hydroxamate siderophore structure involved in the chelation of the ferric ion, thus enabling a broad range of structurally similar siderophores to be utilized. Currently, there is much interest in the exploitation of siderophore transport as a means of developing novel methods for drug delivery, through the use of siderophore–drug conjugates. In this context, it will be of interest to determine how the structural variation between each siderophore affects their binding affinity with ChtA. Similarly, the structural variations may also have implications for substrate specificity and thus the transport efficiency of each siderophore via ChtA. Such analyses may provide important information on receptor–ligand interactions and allow for a more rational design of siderophore–drug conjugates.

The utilization of enterobactin by P. aeruginosa has been shown to be redundant, with mutants in the enterobactin receptor pfeA being capable of low-level enterobactin transport (Dean et al., 1996). Recently, a second receptor for enterobactin, pirA, was identified in P. aeruginosa and a double pfeA pirA mutant was shown to be defective in enterobactin utilization (Ghysels et al., 2005). The utilization of type I pyoverdine by P. aeruginosa has also been shown to be redundant, with fpvB encoding a second receptor for this siderophore (Ghysels et al., 2004), and thus suggesting that redundancy in siderophore utilization is a general phenomenon in P. aeruginosa. In contrast, the utilization of citrate hydroxamate siderophores is dependent on ChtA alone, and P. aeruginosa does not appear to display redundancy in the utilization of these siderophores.

By the FURTA, it was shown that the chtA promoter is capable of moderately binding Fur, suggesting that control of chtA expression is sensitive to intracellular iron levels. In E. coli, iron-regulated genes are expressed in a differential manner depending on the level of iron stress (Klebba et al., 1982). Differential expression may be explained by the degenerate nature of Fur boxes and the consequent variation in the affinity of Fur for these binding sites. The utilization of xenosiderophores, in contrast to the production of endogenous siderophores, may be considered an energy-efficient strategy, as the bacterium does not expend limited cell resources in producing siderophore but can scavenge those produced by other organisms. An associated benefit is that it prevents competitors from scavenging any endogenous siderophores that might otherwise be produced. A characteristic of P. aeruginosa is an ability in the presence of a siderophore to specifically upregulate its cognate receptor. While exogenous aerobactin was shown to increase expression of chtA by approximately 16%, the degree of induction is in contrast to that reported for the enterobactin receptor (pfeA), the pyoverdine receptor (fpvA) and the pyochelin receptor (ftpA), where a dramatic increase in expression (approx. 300%, 600% and 41 000%, respectively) at the transcriptional level is observed in the presence of the cognate siderophore (Dean et al., 1996; Beare et al., 2003; Rédy & Poole, 2003; Michel et al., 2005). This suggests that aerobactin does not induce the expression of chtA. Analysis of the genomic region encoding chtA did not reveal the presence of any putative regulators of chtA. Examination of the ChtA sequence likewise did not reveal the presence of an N-terminal extension characteristic of receptors that are regulated by sigma/anti-sigma or two-component regulatory systems. The introduction of pPROBE-ChtA into the heterologous host S. meliloti resulted in expression of the chtA–gfp fusion (data not shown), further suggesting the absence of a specific regulator for chtA expression. It cannot be discounted that chtA is regulated post-transcriptionally and this remains to be examined.

The inner-membrane transport system for citrate hydroxamate siderophores does not appear to be encoded in the vicinity of chtA and further study will be required to determine how citrate hydroxamate siderophores traverse the inner membrane. In E. coli, the transport of citrate hydroxamate siderophores across the inner membrane is mediated by the FhuCDB transport system. P. aeruginosa does not appear to encode a FhuCDB system although, interestingly, this organism is still capable of utilizing ferrichrome. In S. meliloti 2011 the utilization of rhizobactin 1021 and schizobactin is dependent on the RhtX permease. A homologue of RhtX, termed FptX, has been characterized in P. aeruginosa; however, this protein functions in pyochelin utilization and does not participate in citrate hydroxamate siderophore utilization (Ó Cuív et al., 2004; P. Ó Cuív & M. O’Connell, unpublished results).

The ability of P. aeruginosa to utilize xenosiderophores broadens the available sources of iron, and allows the bacterium to occupy extended ecological niches. In this context, the ability of P. aeruginosa to utilize citrate hydroxamate siderophores is of interest. Previously it was reported that P. aeruginosa and P. putida are capable of utilizing aerobactin (Liu & Shokrani, 1978; Loper & Henkels, 1999) while a genetically uncharacterized halophilic strain, Pseudomonas sp. X40, was shown to produce and utilize aerobactin (Buyer et al., 1991). The pseudomonads are primarily soil bacteria and are known to transport several xenosiderophores produced by other soil micro-organisms. The ability of P. aeruginosa to utilize rhizobactin 1021 is intriguing in this context, suggesting a possible interaction between it and S. meliloti 2011 in the rhizosphere. In contrast, S. meliloti 2011 cannot utilize pyoverdine or pyochelin produced by P. aeruginosa PAO1 (P. Ó Cuív & M. O’Connell, unpublished results). A more detailed analysis of siderophore cross-utilization by micro-organisms found in the rhizosphere may provide an insight into community interactions and competition in this environment.

In this study we have described the identification of the outer-membrane receptor for citrate hydroxamate siderophores in...
P. aeruginosa. Homologues of ChTA are readily identifiable in the genomes of other Pseudomonas spp.; however, further study will be required to determine if the utilization of citrate hydroxamate siderophores is a common phenomenon amongst these species. Iron availability contributes to the competitiveness of P. aeruginosa, and the identification and characterization of ChTA reveals another route by which this iron requirement can be satisfied.

ACKNOWLEDGEMENTS

This publication has emanated from research conducted with the financial support of Science Foundation Ireland and Enterprise Ireland. P. O’C. was the recipient of an Orla Benson Research Award while P. C. was supported by the National Centre for Sensor Research. We thank colleagues who sent strains and plasmids, Kieran Nolan for drawing the chemical structures and Michael Parkinson for providing assistance with the statistical analysis.

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