Identification and characterization of *Pseudomonas* membrane transporters necessary for utilization of the siderophore pyridine-2,6-bis(thiocarboxylic acid) (PDTC)

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The compound pyridine-2,6-bis(thiocarboxylic acid) (PDTC) is known to be produced and excreted by three strains of *Pseudomonas*. Its reactivity includes the complete dechlorination of the environmental contaminant carbon tetrachloride. PDTC functions as a siderophore; however, roles as a ferric reductant and antimicrobial agent have also been proposed. PDTC function and regulation were further explored by characterizing the phenotypes of mutants in predicted membrane transporter genes. The functions of a predicted outer-membrane transporter (PdtK) and a predicted inner-membrane permease (PdtE) were examined in *Pseudomonas putida* DSM 3601. Uptake of iron from $^{55}\text{Fe(III)}$:PDTC, and bioutilization of PDTC in a chelated medium, were dependent upon PdtK and PdtE. Another strain of *P. putida* (KT2440), which lacks *pdt* orthologues, showed growth inhibition by PDTC that could be relieved by introducing a plasmid containing *pdtKCPE*. Transcriptional activation in response to exogenously added PDTC (25 μM) was unaltered by the *pdtK* or *pdtE* mutations; each mutant showed activation of a *pdt* transcriptional reporter, indistinguishable from an isogenic PDTC utilization-proficient strain. The data demonstrate that PdtK and PdtE constitute a bipartite outer-membrane/inner-membrane transport system for iron acquisition from Fe(III) :PDTC. Disruptions in this portion of the *P. putida* DSM 3601 *pdt* gene cluster do not abolish PDTC-dependent transcriptional signalling.

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INTRODUCTION

Pyridine-2,6-bis(thiocarboxylic acid) (PDTC) has been identified as the agent responsible for the complete dechlorination of carbon tetrachloride (CT) by certain strains of *Pseudomonas* (Lee *et al.*, 1999). *Pseudomonas putida* strains DSM 3601 and DSM 3602 and *Pseudomonas stutzeri* KC can excrete PDTC, and when *P. stutzeri* KC is injected into a CT-contaminated aquifer, enhanced CT removal is demonstrated (Dybas *et al.*, 2002), signifying their potential for bioremediation. The degradation of CT via PDTC does not provide the bacteria with a carbon or energy source, indicating that PDTC-producing organisms must compete with other organisms for metabolic resources at CT-contaminated sites. Understanding the physiology of PDTC production is critical for optimizing performance under field conditions.

Evidence indicates that PDTC can function as a siderophore, a ferric iron reductant, and as an antimicrobial agent

(Cortese *et al.*, 2002; Criddle *et al.*, 1990; Hersman *et al.*, 2000; Lewis *et al.*, 2004; Sebat *et al.*, 2001). Criddle *et al.* (1990) first described the iron-sensitive nature of CT-dechlorination activity, and hypothesized that the dechlorinating agent might be a siderophore. The identification of PDTC as the dechlorinating agent further supports that hypothesis, since it was already known to be a ferric iron chelator (Hildebrand *et al.*, 1983). Additional support comes from the sequencing of genes necessary for PDTC biosynthesis. The two sequenced *pdt* gene clusters (GenBank accession nos AF196567 and AY319946) have several characteristics of known siderophore gene clusters. A putative outer-membrane transporter PdtK is found in both the *P. stutzeri* KC and *P. putida* DSM 3601 *pdt* gene clusters. Ferric uptake regulator (Fur)-binding sites are predicted upstream of several of the *pdt* genes, and can explain iron-repressible transcription (Lewis *et al.*, 2000; Sepulveda-Torres *et al.*, 2002). The accumulated evidence in support of the siderophore role also includes a growth defect described for *pdt* mutants under conditions of decreased iron availability, and the uptake of ferric iron from $^{59}\text{Fe(III)}$:PDTC by cells of PDTC-producing cultures (Lewis *et al.*, 2004). Although other effects may be ascribed to PDTC (Sebat *et al.*, 2001), they are probably fortuitous

Abbreviations: Cm, chloramphenicol; CT, carbon tetrachloride; Fur, ferric uptake regulator; Gm, gentamicin; Km, kanamycin; o-p, 1,10-phenanthroline; PDTC, pyridine-2,6-bis(thiocarboxylic acid); Tc, tetracycline.

consequences of its structure/activity; PDTC biosynthesis and regulatory components are more likely to have evolved to fulfil a siderophore role.

Several siderophore-mediated iron-acquisition systems of Gram-negative bacteria have been characterized at the molecular level, including transport (Ferguson & Deisenhofer, 2002) and regulatory processes (Visca *et al.*, 2002). Siderophore transport typically occurs through the function of TonB-dependent outer-membrane transporters, cytoplasmic-membrane-spanning ABC-type transporters, and periplasmic-binding proteins. Complex regulation has evolved for siderophore biosynthesis. This is apparently to prevent the wasteful expenditure of resources, when soluble iron is abundant, or when the siderophore diffuses away from the cell without the productive return of iron. Intracellular iron down-regulates the production of the siderophore at the transcriptional level by means of the Fur protein. In the presence of the cognate siderophore, some outer-membrane siderophore transporters are known to participate in activating the transcription of their respective genes, as well as that of the associated siderophore biosynthetic genes.

Signal transduction systems known to regulate siderophore biosynthetic genes in pseudomonads fall into two categories: those that involve membrane-spanning signal-transducing receptors, and those that involve an AraC-family transcriptional regulator (Poole & McKay, 2003). The second mechanism is less well understood but includes many known siderophore systems of Gram-negative bacteria, including pyochelin (Heinrichs & Poole, 1993), rhizobactin (Lynch *et al.*, 2001), quinolobactin (Matthijs *et al.*, 2004), alcaligin (Brickman *et al.*, 2001) and a utilization system for enterobactin (Anderson & Armstrong, 2004). This type of regulatory system is exemplified by the well-characterized pyochelin system of *Pseudomonas aeruginosa*. Pyochelin (*pch*) gene clusters encode the AraC-type transcriptional activator PchR (Michel *et al.*, 2005). A model to explain activation of *pch* biosynthetic and receptor gene transcription posits that ferripyochelin is an essential effector, interacting with PchR to bring about transcriptional activation (Heinrichs & Poole, 1996; Poole & McKay, 2003). Support for this model comes from studies demonstrating that PchR binds specific DNA sequences in a pyochelin-dependent manner (Michel *et al.*, 2005). An implicit component of the pyochelin effector model is the import of the ferrisiderophore into the cytoplasm to allow interaction with a DNA-binding protein.

Like the pyochelin system, transcription of genes within the *pdt* gene cluster of *P. putida* DSM 3601 is upregulated in response to exogenous PDTC (Lewis *et al.*, 2004). Additionally, the *pdt* gene clusters described to date contain an *araC* homologue (Lewis *et al.*, 2000) (GenBank AY319946). In this study, we have examined the predicted outer-membrane transporters encoded by the *pdtK* genes from *P. stutzeri* KC and *P. putida* DSM 3601, as well as the cytoplasmic membrane transporter encoded by *pdtE*. These gene products

were expected to be essential for the siderophore function of PDTC, and regulation of PDTC production, by virtue of their transport activity. Characterization of mutants of *P. putida* DSM 3601 lacking the predicted transporters was undertaken in order to test these proposed roles.

METHODS

Growth media and culture conditions. Tryptic soy broth and Luria broth were used to maintain cultures of pseudomonads and *Escherichia coli*, respectively. *Pseudomonas* spp. were incubated at 30 °C. Small cultures (5 ml) of pseudomonads were incubated on a roller drum. Larger cultures (50 ml) were grown in 250 ml Erlenmeyer flasks on a rotary shaker. PIPES-buffered minimal medium (PM) (Lewis *et al.*, 2004) was used to culture pseudomonads under iron-limiting conditions. PDTC was chemically synthesized by the method of Hildebrand *et al.* (1983), and was used at a concentration of 10 or 25 µM. Pyoverdine₃₆₀₁ was provided by J.-M. Meyer, Université Louis Pasteur, Strasbourg, and was prepared as described previously (Lewis *et al.*, 2004). Antibiotics were used at the following concentrations: chloramphenicol (Cm, 50 µg ml⁻¹), kanamycin (Km, 50 µg ml⁻¹), tetracycline (Tc, 15 µg ml⁻¹), gentamicin (Gm, 15 µg ml⁻¹) and ampicillin (Amp, 100 µg ml⁻¹). 1,10-Phenanthroline (o-p) was used at 50 µM.

For growth curves, cultures were grown in 96 well microtitre plates in PM, and turbidity was measured using a BioTek plate reader at 630 nm. Media were inoculated (0.1 %, v/v) from 24 h PM cultures adjusted to OD₆₀₀ 0.35.

Strain/plasmid construction. Strains used in this study are listed in Table 1. DNA amplified by PCR was cloned into pGEM-T easy (Promega). *P. putida* strain LLA3 was created through allelic exchange, replacing wild-type *pdtK* of strain DSM 3601 with a deletion/disruption allele containing a Gm-resistance (Gm^R) cassette in the same transcriptional orientation. DNA flanking an internal portion of *pdtK* was amplified and cloned, followed by insertion of the Gm^R cassette (Fig. 1). This construct resulted in the replacement of DNA encoding aa 189–425 of the 662 aa PdtK protein with the Gm^R cassette. The disrupted allele was inserted into the suicide vector pVT1460 via *EcoRI* sites, forming pVTΔK. *E. coli* BW20767 was used as the donor for conjugal transfer of this and other constructs into *P. putida* DSM3601 via the filter mating technique (DeLorenzo *et al.*, 1990). Gm^R *P. putida* were selected on tryptone soy agar (TSA) containing Gm and Cm (*P. putida* is inherently Cm^R). Gm^R isolates were then further screened for Km sensitivity to identify allelic replacements. Allelic replacement was verified by Southern analysis. Probe synthesis utilized the AlkPhos Direct Labelling kit according to the manufacturer's instructions (Amersham Biosciences).

PdtK₃₆₀₁ was cloned after amplification of the coding region and ~500 bp of upstream flanking sequences. For expression of the *P. stutzeri* KC *pdtK* gene in *P. putida*, *pdtK*_{KC} was inserted into the expression vector pJB861T. The entire *pdtK*_{KC} ORF was amplified from *P. stutzeri* KC genomic DNA using primers that contained *BspHI* sites for insertion into the *AflIII* site of pJB861T, allowing in-frame fusion to the start codon, provided by the vector and creating pKKC9. A construct with the insert in opposite orientation (pKKC10) was also obtained and used as a control for background activity.

Allelic exchange was also used to replace the *P. putida* DSM 3601 *pdtE* gene with a deletion/disruption allele containing a Gm^R cassette in the reverse transcriptional orientation. The disruption allele consisted of

Table 1. Bacterial strains and plasmids

DSMZ, Deutsche Sammlung von Mikroorganism und Zellkulturen; EEZ, Estacion Experimental del Zaidin, Granada.

Strain or plasmid	Description	Source or reference
Strains		
<i>E. coli</i>		
BW20767	pir ⁺ , mob ⁺ host/donor	S. Minnoch, University of Idaho
DH5 α pir	pir ⁺ host	K. Mintz, University of Vermont
<i>P. putida</i>		
DSM 3601	Wild-type, PDTC producer	DSMZ; Ockels <i>et al.</i> (1978)
LLA3	DSM 3601 <i>pdtK</i> ::Gm ^R	This study
LLAE	DSM 3601 <i>pdtE</i> ::Gm ^R	This study
TLI1	DSM3601 <i>pdtI</i> ::mini-Tnxy/E Km ^R	This study
LLA3I ⁻	LLA3 with <i>pdtI</i> ::mini-Tnxy/E Km ^R	This study
LLAEI ⁻	LLAE with <i>pdtI</i> ::mini-Tnxy/E Km ^R	This study
KT2440	Wild-type	E. Duque, EEZ
<i>P. stutzeri</i>		
KC	Wild-type aquifer isolate; Pdt ⁺	Criddle <i>et al.</i> (1990)
CTN1	Spontaneous <i>pdt</i> ⁻ deletion mutant of KC	Lewis <i>et al.</i> (2000)
Plasmids		
pGEM-T easy	Ap ^R ; <i>ori</i> _{colE1} , cloning vector	Promega
pRK311	Tc ^R ; <i>ori</i> _{RRK2} , broad-host-range vector	Ditta <i>et al.</i> (1980)
pUCGM	Source of Gm ^R cassette	Schweizer (1993)
pACYC184	Source of Tc ^R cassette	V. Bandaru, University of Vermont
pT31	pRK311 with <i>pdt</i> _{KC} gene cluster	Lewis <i>et al.</i> (2000)
pJS52	pT31 with transposon insert in <i>pdtK</i>	Lewis <i>et al.</i> (2000)
pJS59	pT31 with transposon insert in <i>pdtF</i>	Lewis <i>et al.</i> (2000); this study
pJB3Tc20	Tc ^R ; broad-host-range vector	Blatny <i>et al.</i> (1997a)
pK3601	pJB3Tc20 with <i>pdtK</i> ₃₆₀₁ and flanking DNA inserted in multiple cloning site	This study
pJB861	Km ^R ; broad-host-range expression vector	Blatny <i>et al.</i> (1997b)
pQE30	<i>ori</i> _{colE1} expression vector	Qiagen
pJB861T	Km ^R ; pJB861 with 400 bp transcriptional terminator fragment from pQE30 between <i>Bam</i> HI and <i>Eco</i> RI sites	This study
pKKC9	pJB861T with <i>pdtK</i> _{KC} in forward orientation	This study
pKKC10	pJB861T with <i>pdtK</i> _{KC} in reverse orientation	This study
pE3601	pJB861T with <i>pdtE</i> ₃₆₀₁ in forward orientation	This study
pVT1460	<i>ori</i> _{R6K} , Ap ^R , mob _{RP4} suicide vector, Km ^R derivative of pGP704	K. Mintz; Miller & Mekalanos (1988)
pVTΔK	pVT1460 with <i>pdtK</i> ₃₆₀₁ ::Gm ^R allele	This study
pVTΔE	pVT1460 with <i>pdtE</i> ₃₆₀₁ ::Gm ^R allele	This study
pUTKmxy/E	Ap ^R , Km ^R ; <i>xy</i> /E, Km ^R mini-transposon delivery vehicle	DeLorenzo <i>et al.</i> (1990)
pEPGm2	Cm ^R , Gm ^R ; <i>ori</i> _{R6K} <i>tra</i> <i>sacB</i> suicide vehicle for allelic replacement	Lewis <i>et al.</i> (2004)
pSac2::SEMKm ^R	Cm ^R , Gm ^R , Km ^R ; pEPGm2 with <i>pdtI</i> ::mini-Tnxy/E <i>Kpn</i> I fragment for allelic exchange	This study
pEPSTc	Cm ^R , Tc ^R , Km ^R ; pSac2::SEMKm ^R with Tc ^R from pACYC184 in <i>Xba</i> I site replacing Gm ^R	This study
pJB321	Ap ^R ; broad-host-range vector	Blatny <i>et al.</i> (1997a)
pJB321Gm	Ap ^R , Gm ^R ; derivative of pJB321 with Gm ^R cassette inserted at <i>Bam</i> HI	This study
pPA21KΔXho	pJB321Gm with a 6.9 kb <i>Bgl</i> II fragment derived from a 21 kb fragment of DSM 3601 genomic DNA by deletion of <i>Xho</i> I fragment	This study

Fig. 1. Gene clusters encoding PDTC biosynthesis and utilization functions in *P. stutzeri* KC and *P. putida* DSM 3601. Open arrows indicate the positions and orientations of Gm^R cassettes inserted in *pdtK* and *pdtE*, with dashed lines indicating the extent of deletions in the respective alleles. The flag symbol indicates the position and orientation of a mini-Tnxy/E insertion in *pdtI* used as a transcriptional reporter. Angled arrows indicate predicted transcription start sites. The line at the bottom shows locations of restriction sites in the *P. putida* DSM 3601 genome segment: Bg, *BglI*; X, *XhoI*; N, *NotI*.

the first 177 bp and terminal 12 bp of the 1281 bp *pdtE* coding region, interrupted by the Gm^R cassette. PCR and Southern analysis were used to verify allelic replacement at *pdtE*.

For expression of the *P. putida* *pdtE* gene, the amplified gene was inserted into pJB861T utilizing the *AflIII* site, as described for *pdtK_{KC}*.

To obtain a chromosomal *pdtI::xylE* reporter fusion in wild-type, *pdtK* and Δ *pdtE* backgrounds, a previously described *pdtI::xylE* allele (Lewis *et al.*, 2004) was inserted as a *KpnI* fragment into pEPGm, creating pSac2::SEMKm^R. For use with *pdtK* and Δ *pdtE* recipients, the construct was further modified by replacing the plasmid-borne Gm^R with Tc^R (amplified from pACYC184), yielding pEPSTC. Conjugal transfer into *P. putida* was followed by selection on Km-containing medium. Km^R transconjugants were then screened for the resistance marker of the vector (Gm^R or Tc^R). Allelic replacement or co-integration was verified by Southern analysis.

Inverse PCR. Cosmid DNA (pJS52 or pJS59) was digested with *PstI* and ligated with T4 ligase. Nested PCR was performed using two sets of primers, oriented in an outward direction from the mini-transposon KmlacZ1 (DeLorenzo *et al.*, 1990): set 1 consisted of Trp-lacinv (TCGCTGCTTTACCTGATCC) and KmRinv (TCAGCAACACCTTCTTACAG); and set 2 consisted of KmlacZF (GCCGCACTTGTGTAAAG) and KmlacZR (GGCCAGATCTGATCAAGA). Sequencing was done at the Vermont Cancer Center DNA Analysis Facility on an AB3100 genetic analyser. Cycle sequence chemistry employed the Big Dye Terminator Chemistry version 3.1 and was analysed using Sequence Analysis 5. The sequence data were compiled using Sequencher 4.2 (GeneCodes).

Xyle assays. Catechol dioxygenase was assayed by measuring the production of α -hydroxymuconic semialdehyde from catechol (Klecka & Gibson, 1981). The reaction was monitored at 375 nm on a Cary 50 spectrophotometer (Varian Instruments) with constant stirring at 30 °C. Cells were harvested upon entry into the stationary phase. Protein quantification for all experiments used the BCA protein assay kit (Pierce).

Outer-membrane protein analysis. Sarkosyl fractionation was used to isolate outer-membrane proteins of both *P. putida* and *P. stutzeri* (Cornelis *et al.*, 1989). Proteins (15 μ g per lane) were run on an 8% SDS-PAGE gel, and stained with 0.2% Coomassie blue (Laemmli, 1970). The broad-range molecular mass standard was

purchased from New England Biolabs. A 65 kDa band derived from strain CTN1/pT31, and corresponding to PdtK, was transferred from an SDS-PAGE gel to PVDF, and submitted for analysis. The N-terminal sequence was determined at the Protein Chemistry Laboratory, University of Texas Medical Branch at Galveston, TX, USA.

⁵⁵Fe uptake. Uptake assays were performed using a modification of our previous protocol (Lewis *et al.*, 2004). ⁵⁵Fe:PDTC was made by first adding 100 mM Tris/HEPES-buffered nitrilotriacetic acid (NTA, pH 6) to ⁵⁵FeCl₃ [NEN Biolabs; 69.64 mCi (2.577 GBq) (mg Fe)⁻¹, 0.5 M HCl] to stabilize the iron in soluble form. The mixture was adjusted to pH 6 by addition of NaOH (0.8 μ l 1 M NaOH to 10 μ l ⁵⁵Fe:PDTC). Immediately prior to each experiment, PDTC was mixed with ⁵⁵Fe/NTA in the stoichiometric ratio of 2:1. The mixture was then incubated for 20 min at room temperature. UV-visible spectrophotometry showed that this protocol produced quantitative yields of Fe(III):PDTC. Fe(II):PDTC, although readily resolved by UV-visible spectrophotometry (Cortese *et al.*, 2002), was not detected in this synthesis. The determination of statistical significance among uptake assay data involved a two-tailed *t* test.

Cells for iron-uptake assays were grown to early stationary phase (18 h, 30 °C), washed twice and resuspended in PM to OD₆₀₀ 0.8. *P. putida* DSM 3601 strains were pre-grown without antibiotic selection, as this reduced variability and mean uptake values for vector controls. Plating efficiencies on antibiotic-containing and control media were determined for cells used in assays, and were not significantly different. The addition of inducer (3-methylbenzoate) in conjunction with expression clones was omitted, as its inclusion did not affect results obtained for one of the constructs (LLAE/pE3601) in a control experiment.

Reactions were started by the addition of 11.1 nmol ⁵⁵Fe:PDTC to 4 ml cell suspensions, and incubation at 30 °C. Aliquots (1 ml) were removed and the cells were sedimented in a microcentrifuge, and washed twice with 1 ml ice-cold PM. The washed cell pellet was suspended in Biosafe II scintillation fluid (Research Products International), and radioactivity was measured in a Beckman LS6000IC scintillation counter. Linearity was verified by time-course studies with *P. stutzeri* KC and *P. putida* cells. Thereafter, our standard protocol utilized a 30 min incubation. A negative control was run to report non-specific sorption of ⁵⁵Fe in the cell pellet. In that experiment, aliquots of a cell suspension were incubated at either 30 °C (experimental) or 4 °C (control), after the addition of ⁵⁵Fe:PDTC.

RESULTS

Identification of PdtK in outer-membrane fractions of *P. stutzeri* KC and *P. putida* DSM 3601

PdtK, a putative outer-membrane transporter, is encoded within the *pdt* gene clusters of *P. stutzeri* KC (Lewis *et al.*, 2000) and *P. putida* DSM 3601 (Fig. 1; GenBank AY319946). The proteins are 44 % identical, and PdtK₃₆₀₁ is 29 % identical to FepA of *E. coli*. In both *Pseudomonas* species, the translated genes were predicted to encode proteins of ~71 kDa after removal of a predicted Sec-dependent signal sequence (Bendtsen *et al.*, 2004). When grown under iron limitation, the outer-membrane protein profiles of both *P. putida* DSM 3601 and *P. stutzeri* strain KC showed an iron-regulated protein band migrating at ~65 kDa (Lewis *et al.*, 2004) (Fig. 2). To determine whether the band was PdtK, the outer-membrane profiles from a series of strains derived from *P. stutzeri* KC were analysed. Strain CTN1 is a spontaneously derived mutant of strain KC that lacks a 170 kb chromosomal segment that includes the entire *pdt* gene cluster. The Pdt[−] phenotype (PDTC production defect) of CTN1 was complemented by a cosmid (pT31) containing the *pdt* gene cluster (Lewis *et al.*, 2000). Outer-membrane proteins derived from the complemented strain (CTN1/pT31) grown under iron-limited conditions showed a band at 65 kDa, whereas the vector-bearing control (CTN1/pRK311) did not (Fig. 2A), indicating that the 65 kDa protein was likely encoded within the 25.7 kb *pdt* gene cluster. The N-terminal amino acid sequence was determined for the 65 kDa band derived from a CTN1/pT31 outer-membrane preparation. The sequence of the first 10 aa (APGSAASPDs) corresponded exactly to those predicted for the mature protein, and confirmed that the 65 kDa protein was PdtK_{KC}.

Outer-membrane protein preparations from iron-replete *P. putida* DSM 3601 contained additional bands near 65 kDa, compared to those from strain KC. However SDS-PAGE revealed an iron-regulated band within that size range (Lewis *et al.*, 2004). The 65 kDa band was not seen in the mutant bearing a *pdtK* deletion allele (LLA3), but was present when plasmid-borne *pdtK*₃₆₀₁ was provided (Fig. 2B), allowing the 65 kDa band to be classified as PdtK₃₆₀₁.

Uptake of iron from ⁵⁵Fe:PDTC is PdtK dependent

Uptake of ⁵⁵Fe from ⁵⁵Fe:PDTC is iron regulated, as would be expected for a siderophore system (Lewis *et al.*, 2004) (Table 2). The measured uptake was also curtailed by incubation at 4 °C, indicating a metabolic rather than a sorptive effect (data not shown). To determine if PdtK was essential for iron assimilation from Fe:PDTC, uptake assays of *pdtK* and *pdtK*⁺ strains were performed. *P. stutzeri* KC assimilated significantly more ⁵⁵Fe from ⁵⁵Fe:PDTC than did the *pdt* mutant CTN1. The iron-uptake phenotype of CTN1 was complemented by the cosmid pT31 (Table 2),

Fig. 2. Assignment of a 65 kDa outer-membrane protein as PdtK. Outer-membrane proteins were run on 8% polyacrylamide gels. (A) Lane 1, molecular size marker; lane 2, *P. stutzeri* KC; lane 3, *P. stutzeri* KC grown with 50 μ M FeCl₃; lane 4, *P. stutzeri* CTN1; lane 5, *P. stutzeri* CTN1/pRK311; lane 6, *P. stutzeri* CTN1/pT31. (B) Lane 1, *P. putida* DSM 3601; lane 2, LLA3; lane 3, LLA3/pJB3Tc20; lane 4, LLA3/pK3601; lane 5, molecular size marker. The positions of the band identified as PdtK by N-terminal sequencing, and the 97 and 66 kDa standards, are indicated.

demonstrating that the *pdt* gene cluster was sufficient for PDTC-bound iron uptake.

Iron-uptake assays were also performed on *pdtK* and *pdtK*⁺ *P. putida* DSM 3601 strains. The *pdtK*₃₆₀₁ mutant LLA3 showed a severe defect in ⁵⁵Fe uptake from ⁵⁵Fe:PDTC, similar to the results observed with iron-replete DSM 3601 cells. The uptake deficiency of strain LLA3 was partially restored upon complementation with plasmid-encoded *pdtK*₃₆₀₁ (Table 2), confirming a role for PdtK in the transport of Fe:PDTC. To determine if the two PdtK proteins were functionally equivalent, a plasmid-borne copy of *pdtK*_{KC} was introduced into strain LLA3. Significant ⁵⁵Fe uptake in that strain (LLA3/pKKC9) confirmed that the two genes are functional orthologues (Table 2).

The *pdtE* gene product is necessary for iron assimilation from Fe:PDTC

The predicted eleven-transmembrane-domain protein PdtE shares sequence similarity with the single-polypeptide-type

Table 2. Uptake of ⁵⁵Fe from ⁵⁵Fe:PDTC by *Pseudomonas* strains

Strain (genotype)	⁵⁵ Fe uptake* [pmol (mg protein) ⁻¹]
<i>P. stutzeri</i>	
KC (wild-type)	111 ± 32
KC + Fe†	10 ± 7.6
CTN1 (<i>pdt</i> ⁰)	7.8 ± 4.3
CTN1/pRK311‡	41 ± 21
CTN1/pT31‡	230 ± 58
<i>P. putida</i>	
DSM 3601 (wild-type)	46 ± 10
DSM 3601 + Fe†	7.0 ± 2.5
LLA3 (<i>pdtK</i>)	13 ± 5.2
LLA3/pJB3Tc20	12 ± 3.6
LLA3/pK3601	23.6 ± 3.4
LLA3/pKKC9	63.6 ± 6.9
LLA3/pKKC10	11 ± 5.4
LLAE (<i>pdtE</i>)	1.8 ± 1.1
LLAE/pJB861T	2.8 ± 3.7
LLAE/pE3601	15 ± 2.1
KT2440 (wild-type, <i>pdt</i> ⁰)/pJB321Gm‡	1.3 ± 0.5
KT2440/pPA21KΔXho‡	80 ± 4.4

*Cell-pellet-associated ⁵⁵Fe from 30 min incubations with ⁵⁵Fe:PDTC; means ± SD from at least three independent experiments.

†+Fe signifies cells grown with 50 μM FeCl₃ prior to assay.

‡Cells were grown with antibiotic selection prior to assay; all other cultures were grown without antibiotics and plasmid retention was verified (see Methods).

permeases of the rhizobactin (*rhtX*) and pyochelin (*fptX*) utilization systems (Cuiv *et al.*, 2004). These characteristics suggest that PdtE constitutes the cytoplasmic-membrane component of the Fe:PDTC-uptake machinery (Paszczynski *et al.*, 2004). A *P. putida* DSM 3601 mutant with a deletion/disruption allele of *pdtE* (LLAE) was found to be devoid of detectable iron uptake from ⁵⁵Fe:PDTC, a phenotype that could be partially restored by the presence of *pdtE* on a multicopy plasmid (Table 2).

Comparing the membrane transport mutants, the *pdtE* mutant was more severely compromised for ⁵⁵Fe assimilation than was the *pdtK* mutant (Table 2, *P*=0.004). A possible explanation is that there are other modes of transport of Fe:PDTC into the periplasm that are much less efficient than PdtK, but that cytoplasmic transport depends solely upon PdtE.

PdtK and PdtE are necessary for full tolerance to transition metal deprivation

The iron-uptake data predicted that *pdtK* and *pdtE* mutants would have growth defects under conditions in which PDTC is normally required. Mutants defective in PDTC production are more sensitive to the transition metal chelator o-p

than their isogenic counterparts, and tolerance to o-p can be restored to these mutants by addition of PDTC to the culture medium (Lewis *et al.*, 2004). Although DSM 3601 can also produce pyoverdine, that capacity contributes less to o-p resistance than does PDTC production (Lewis *et al.*, 2004). Therefore, we used o-p tolerance to assess the roles of PdtK and PdtE, as a means of physiological corroboration of the iron-uptake data (Fig. 3).

Although both strains retained the capacity to produce PDTC (data not shown), they showed an increased sensitivity to the effects of o-p (Fig. 3). The *pdtK* mutant was more adversely affected by 50 μM o-p than the wild-type, but was still capable of growth. The *pdtE* mutant was even more sensitive to 50 μM o-p, and was unable to overcome o-p-induced growth inhibition. Both mutants could be complemented for o-p sensitivity phenotypes by the respective genes introduced singly *in trans* (Fig. 3). An unexpected result was that the phenotype of the *pdtK* mutant was more severe when harbouring a plasmid vector. The data confirmed that PdtK and PdtE were essential for the o-p-resistance phenotype of DSM 3601, and corroborated the iron-uptake data, indicating that the *pdtK* mutant LLA3 possessed residual capacity to transport Fe:PDTC complexes into the cytoplasm.

***pdtKCPE* is sufficient for ⁵⁵Fe:PDTC uptake and PDTC bioutilization**

A two-protein system consisting of individual outer-membrane and cytoplasmic-membrane transport proteins is sufficient for utilization of rhizobactin and pyochelin siderophores by heterologous hosts (Cuiv *et al.*, 2004). Therefore, to test whether PdtK and PdtE were sufficient for PDTC utilization, a segment of the DSM 3601 *pdt* gene cluster containing complete ORFs for PdtK and PdtE, as well as putative regulatory components (PdtC and PdtP), was transferred to the *P. putida* reference strain KT2440 on a multicopy plasmid. The strain KT2440 genome has been sequenced (Nelson *et al.*, 2002), and no homologues of *pdt* genes were found by BLAST searches (Altschul *et al.*, 1997). *pdtKCPE in trans* conferred upon strain KT2440 the ability to take up ⁵⁵Fe from ⁵⁵Fe:PDTC (Table 2), and also relieved a PDTC-sensitive phenotype seen for KT2440 (Fig. 4). The fact that this small subset of genes could confer utilization upon a heterologous host supported the two-protein uptake system model, and indicated that the PDTC antimicrobial effect was due solely to a lack of these components.

Transcriptional activation by PDTC in transport mutants

To assess the roles of the described transporters in expression of *pdt* genes, we measured *pdt* gene expression in *pdtK* and *pdtE* backgrounds. Transcriptional regulation of *pdt* genes in response to exogenously added PDTC has been demonstrated previously by measuring expression of a chromosomal *pdtI* :: *xylE* transcriptional fusion (Lewis *et al.*, 2004). The same *pdtI* :: *xylE* reporter fusion was recombined

Fig. 3. Roles for PdtK and PdtE in tolerance to o-p. Growth curves for (A) DSM 3601, (B) LLA3 (*pdtK*), (C) LLA3/pJB3Tc20, (D) LLA3/pK3601, (E) LLΔE (*pdtE*), (F) LLΔE/pJB861T, and (G) LLΔE/pE3601. ●, PM minimal medium; ○, PM + 50 μM o-p. Values are the averages of two parallel experiments. The experiments were repeated with similar results.

into the *pdtK* and *pdtE* backgrounds in order to assess the roles of the respective genes in that activation. Since the allele containing the reporter abolishes endogenous PDTC production, any response seen would have been due solely to

exogenously added PDTC. Assays of reporter expression showed that the *pdtK* and *pdtE* reporter strains responded to 25 μM PDTC in a manner indistinguishable from that of the isogenic (*pdtK*⁺ *pdtE*⁺) strain TL11 (Fig. 5). This demonstrated that transcriptional activation by exogenously added PDTC still occurred, even in strains carrying mutations that severely reduced or abrogated PDTC-dependent iron uptake.

Characterization of cosmid clones assigned as *pdtK_{KC}* insertions

Previous data suggest a role for *pdtK* in the regulation of PDTC production by *P. stutzeri* KC (Lewis *et al.*, 2000); however, these data are equivocal. Two transposon insertions were mapped within a cosmid-borne copy of *pdtK_{KC}*, and led to either abolition of (pJS59), or no effect on, PDTC production (pJS52). We determined the precise positions of these transposon insertions by inverse PCR and sequencing. The pJS59 transposon integration site was determined to lie at nt 872 of *pdtF*, and thus the resulting phenotype did not reflect an effect of the disruption of *pdtK*. The pJS52 insertion site was determined at nt 1066 of the 2063 bp *pdtK_{KC}* ORF, likely generating a *pdtK* null phenotype. Therefore, since pJS52 conferred levels of PDTC production

Fig. 4. Resistance to the antimicrobial effect of PDTC is conferred by *pdtK_{CPE}*. *P. putida* KT2440 bearing either plasmid vector (pJB321Gm, ●) or cloned *pdtK_{CPE}* genes from *P. putida* DSM 3601 (pPA21KΔXho, ○) were inoculated into PM with 10 μM PDTC. Values are the averages of two parallel experiments. The experiments were repeated with similar results.

Fig. 5. Transcriptional activation of a chromosomal *pdtI::xy/E* reporter fusion in response to PDTC. Cell-free extracts were prepared from the following cultures, grown on PM (black bars) or PM with 25 μM PDTC (grey bars). TL1, *pdtI::xy/E*; LLA31[−], *pdtI::xy/E pdtK*; LLΔEI[−], *pdtI::xy/E pdtE*.

indistinguishable from those of the uninterrupted cosmid (Lewis *et al.*, 2000), no role was indicated for PdtK_{KC} in regulating PDTC genes present in multicopy.

DISCUSSION

The data presented clarify the physiological role for which PDTC production has evolved, distinguishing it from other activities that can be also be attributed to PDTC. A role for PdtK was demonstrated as an outer-membrane component in the receptor-mediated uptake of iron from the Fe:PDTC complex. Orthology between *pdtK* genes from two PDTC-producing strains was also established. Previous attempts to demonstrate receptor-mediated uptake of iron from Fe:PDTC have not been successful (Cortese *et al.*, 2002), most likely due to the filtration-based protocol used to assay iron uptake in these experiments. That filtration-based technique led to very high background counts, not observed with the centrifugation protocol used in both this and our previous study (Lewis *et al.*, 2004). It seems likely that the high background obscures the physiological accumulation of iron. Another important finding derived from the ⁵⁵Fe-uptake assays, and the dependence of the observed activity upon an outer-membrane transporter, was that the bulk of the uptake of iron occurred without reduction to ferrous ion by added PDTC; the Fe:PDTC preparations used were confirmed as Fe(III):PDTC spectrophotometrically. In addition, the ferric-reducing activity, shown previously for PDTC (at ~50-fold higher concentrations than those used in our uptake assays) incubated with insoluble ferric

hydroxides, was ~10-fold slower than the uptake rates measured in our work (Cortese *et al.*, 2002; data for DSM 3601, Table 2, normalized to h^{−1}).

⁵⁵Fe uptake and bioutilization assays also showed that PdtE was necessary for PDTC-dependent iron uptake. By homology to the rhizobactin and pyochelin systems (Cuiv *et al.*, 2004), PdtK and PdtE were predicted as the analogous Fe:PDTC utilization system, providing outer-membrane/TonB-dependent, and cytoplasmic-membrane permease functions, respectively. This supposition was supported by data demonstrating that *pdtKCPE* was sufficient for relieving a growth-inhibitory effect of PDTC seen for a strain of *P. putida* devoid of *pdt* gene orthologues (Fig. 4). Therefore, the observed antimicrobial effect described for PDTC (Sebat *et al.*, 2001) is likely to be due to the avid iron-chelating property of PDTC (Stolworthy *et al.*, 2001) in the absence of a cognate uptake system. In light of the avid iron affinity, it is interesting that the small set of genes was sufficient to relieve growth inhibition caused by PDTC, since it also implies that iron removal from Fe:PDTC can occur once the complex is translocated into the cell. This may arise through non-specific reduction, since the affinity of PDTC for Fe²⁺ is drastically lower than that for Fe³⁺ (Brandon *et al.*, 2003).

In light of the ability to confer resistance to PDTC-induced growth inhibition on a heterologous host, it is unclear why plasmid-borne *pdtK* or *pdtE* only partially complemented the iron uptake and o-p-resistance phenotypes of strains LLA3 and LLΔE, respectively. Possible explanations include poor expression, complicating effects of plasmids or antibiotic selection, or additional defects caused by the constructed alleles. We did not quantitatively assess expression of the respective plasmid-borne genes, but we did observe a corresponding band in SDS-PAGE (pK3601, Fig. 2), and verified promoter integrity by sequencing (pE3601). At least two effects related to plasmids or antibiotic selection were observed: growth of control strains (containing empty vectors) in the presence of antibiotics led to high counts in uptake assays, and significant reduction in siderophore (pyoverdine) production was seen in strains harbouring the RK2-based plasmids used in this work (data not shown). The high counts seen in iron-uptake assays were not physiologically relevant, since the bioutilization experiments showed that no advantage was gained by harbouring control plasmids in a chelated medium with antibiotic selection. In fact, strain LLA3 showed impaired ability to grow in the chelated medium while harbouring a control plasmid (Fig. 3). These observations indicate that plasmid functions and/or antibiotic selection result in changes in the physical properties of the cell envelope, which confound uptake assays and impede transition metal uptake. Although we cannot resolve the potential factors contributing to the poor complementation, we have revealed complexity in the genetic requirements of the *P. putida* DSM 3601 transition metal-acquisition systems.

In addition to clarifying the physiological role of PDTC, further information regarding regulation of PDTC

biosynthesis was obtained in the present work. Current models to explain siderophore signalling include membrane transporters acting as direct signal transducers, and transport activities potentially bringing necessary effectors into the cytoplasm. Evaluation of the *pdt* gene cluster has identified similarity between the PDTC system and the pyochelin system of *P. aeruginosa*, in that both have *araC*-like regulatory genes, *pdtC* and *pchR*, respectively. The PchR/ferrisiderophore co-effector model, in which transcriptional activation requires a protein activator and a ferrisiderophore, has received experimental support. It has recently been demonstrated that PchR binds to a consensus 'PchR box' element found in two locations within the *pch* gene cluster, and that binding requires the presence of pyochelin (Michel *et al.*, 2005). We have shown that a *pdtK* mutation, which does not support transport of iron from Fe:PDTC above iron-repressed levels, did not eliminate transcriptional activation by exogenous PDTC. The predicted cytoplasmic-membrane permease PdtE was found to have an even more important role in transport, and was likewise shown not to be essential for transcriptional activation. In other work, we have found that *pdtC* alone is sufficient to allow PDTC-dependent transcriptional activation of a *pdt* promoter by *P. putida* KT2440 (S. Morales and T. Lewis, unpublished data). Transporter-independent signalling has also been indicated for other siderophore-responsive regulatory systems, including that of yersiniabactin in *Yersinia pestis* (Fetherston *et al.*, 1996) and enterochelin in *Bordetella* spp. (Anderson & Armstrong, 2004). Models to reconcile our data with a PdtC/co-effector model would require cellular import/diffusion of the effector without *pdtK* or *pdtE*, and that the necessary amount of effector import does not alleviate iron deprivation. It is possible that Fe:PDTC is transported across the outer membrane by an alternative transporter, or it may diffuse through porins at a low rate, as has been seen for other siderophores (Ghysels *et al.*, 2004, 2005; Meyer, 1992). There could also be cytoplasmic transporters with a limited cross-specificity for Fe:PDTC. The first suggestion might explain the residual ability of the *pdtK* mutant to take up iron from Fe:PDTC. However, transport of Fe:PDTC in the absence of PdtE was below our detection limit, and the residual ability to respond would have required a very sensitive sensing mechanism to explain the observed transcriptional activation. Alternatively, the zwitterionic form of PDTC may be cell-permeable and, once inside the cell, it may bind iron from cytosolic sources. However, since the pK_a for conversion to a species with no net charge is 2.58 (Stolworthy *et al.*, 2001), the steady-state concentration of that species would be very low at physiological pH. In summary, the ferrisiderophore effector model of transcriptional activation could explain activation in the absence of specific transporters, but the uncoupling between physiological levels of transport and regulation remains to be explained mechanistically.

An appealing element of the ferrisiderophore effector model is that it could also explain the phenomenon of coordination

between two separate siderophore systems of the same organism (Poole & McKay, 2003). It was not an explicit goal of this study to test that model, but it would be interesting to determine how, or whether, transport components affect the interplay between siderophore systems. Unravelling the regulatory circuits involved may not only yield information vital to an optimal bioremediation strategy, but also inform us about regulatory coordination between important competitive and virulence determinants of bacteria.

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