BioMetals (2007) 20:717–726
DOI 10.1007/s10534-006-9035-x

The role of the siderophore pyridine-2,6-bis (thiocarboxylic acid) (PDTC) in zinc utilization by *Pseudomonas putida* DSM 3601

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Received 8 May 2006; accepted 16 August 2006

Key words: zinc, siderophore, transporter, outer membrane receptor, iron uptake, *Pseudomonas*, PDTC

Abstract

Previous work had suggested that in addition to serving the function of a siderophore, pyridine-2,6-bis(thiocarboxylic acid) (PDTC) may also provide producing organisms with the ability to assimilate other divalent transition metals. This was tested further by examining regulation of siderophore production, expression of *pdt* genes, and growth in response to added zinc. In media containing 10–50 μM $ZnCl_2$, the production of PDTC was found to be differentially repressed, as compared with the production of pyoverdine. The expression of PdtK, the outer membrane receptor involved in PDTC transport, was also reduced in response to added zinc whereas other iron-regulated outer membrane proteins were not. Expression of a chromosomal *pdt*I:: *xyl*E fusion was repressed to a similar extent in response to zinc or iron. Mutants that cannot produce PDTC did not show a growth enhancement with micromolar concentrations of zinc as seen in the wild type strain. The phenotype of the mutant strains was suppressed by the addition of PDTC. The outer membrane receptor and inner membrane permease components of PDTC utilization were necessary for relief of chelator (1,10-phenanthroline)-induced growth inhibition by Zn:PDTC. Iron uptake from ^{55}Fe:PDTC was not affected by a 32-fold molar excess of Zn:PDTC. The data indicate that zinc present as Zn:PDTC can be utilized by strains possessing PDTC utilization functions but that transport is much less efficient than for Fe:PDTC.

Introduction

The ability to acquire and appropriately maintain adequate concentrations of transition metals has undoubtedly been a driving evolutionary force shaping bacterial populations. Low dissolved concentrations of those elements characterize many environments, making high affinity transport capabilities essential for effective colonization. Conversely, toxic effects may ensue if intracellular concentrations of transition metals are too high. Iron toxicity, stemming largely from catalyzed reductions of reactive oxygen species (Andrews *et al*. 2003), must also be avoided. This can be prevented by finely tuned homeostatic mechanisms involving regulated expression (induction/repression) of high affinity transport, sequestration in intracellular storage proteins, and export from the cytoplasm by energy-dependent pumping systems (Neilands & Nakamura 1985; Braun 1997; Choudhury & Srivastava 2001).

Iron homeostasis is a well-described example of adaptation to the dual nature of metal chemistry in biological systems. Biologically, iron is somewhat unique among the transition metals. Iron is required in relatively large amounts, and has a

propensity to form insoluble and amorphous forms in neutral, oxic aqueous environments, thus creating a biological deficit of iron (Braun 1997; Schalk *et al*. 2004). To combat the paucity of iron, bacteria have evolved high affinity iron assimilation systems that include the excretion of siderophores. The evolution of siderophore systems is a case in point for pressure to acquire and regulate acquisition of this metal; a significant portion of bacterial genomes may be dedicated to the biosynthesis, export, and transport of iron complexes of the ferrisiderophore (Canovas *et al*. 2003; Ferguson & Deisenhofer 2004; Wandersman & Delepelaire 2004).

Bacteria have less elaborate machinery for the acquisition of other transition metals. For example, in *Escherichia coli* zinc is known to be transported by the dedicated ZnuABC system, part of the ABC-type transporter family (Patzer & Hantke 1998). Regulation of the *znu* ABC genes is afforded by the Zur repressor which uses zinc as a cofactor and is analogous to the well-known Fur repressor of iron homeostasis (Patzer & Hantke 1998, 2000). This type of minimal system would require diffusion across the cell envelope or transport by a non-specific and genetically unlinked outer membrane component. The apparent sufficiency of a simple cytoplasmic membrane transport apparatus for supplying zinc suggests that ambient dissolved concentrations are generally adequate to meet nutritional requirements for zinc, as opposed to iron.

Transition metals other than iron can also be limited in bacterial habitats due to their propensity to form insoluble species or sequestration by other organisms (e.g. host cells) (Canovas *et al*. 2003; Finney & O'Halloran 2003; Ferguson & Deisenhofer 2004). The suggestion that siderophores may additionally function in acquisition of transition metals other than iron has come from characterization of the metal affinities for certain siderophores, and as a potential explanation of the apparent redundancy of siderophore systems in some bacteria (Visca *et al*. 1992). Pyochelin, a small salicylate-derived siderophore produced by *Pseudomonas aeruginosa*, is an example. Strains that produce pyochelin also produce another siderophore (pyoverdine, PVD) which has a higher affinity for ferric iron and a greater solubility, and is produced in larger amounts (Cox *et al*. 1981; Poole & McKay 2003). Those characteristics have led to the term secondary siderophore, to indicate that it is not likely the primary source of iron for the organism under conventional culture conditions (Mossialos et al. 2000; Cornelis & Matthijs 2002). This led to speculation that pyochelin may have an alternative function, rather than simply providing the bacterium with an additional iron scavenging source. In support of such an alternative function, pyochelin production was found to be uniquely downregulated in response to other transition metals (Ni, Co, Mo, Cu), and preformed complexes of pyochelin with those transition metals enhanced growth of the organism in a low transition metal-containing medium (Visca *et al*. 1992).

The novel thiocarboxylate siderophore pyridine-2,6-bis (thiocarboxylic acid) (PDTC) has also been the subject of such speculation. Three strains of bacteria belonging to the genus *Pseudomonas* are known to produce PDTC (Ockels *et al*. 1978; Lee *et al*. 1999). Those organisms also produce another siderophore, pyoverdines in the case of *P. putida* strains DSM 3601 and 3602, and defferioxamine E in the case of *P. stutzeri* KC (Lewis *et al*. 2004, J-M Meyer, unpublished). Pyoverdine and deferrioxamine are catecholate and hydroxamate, or hydroxamate ligands, respectively, with high stability constants for ferric iron (Meyer *et al*. 1987; Neu *et al*. 2000). PDTC offers N- and S-atom ligands for effective coordination of soft metals and is known to form complexes with 14 transition metal elements, some having high stability constants (Hildebrand *et al*. 1984; Hildebrand & Lex 1989; Kruger & Holm 1990; Espinet *et al*. 1994; Neu *et al*. 2001; Stolworthy *et al*. 2001; Cortese *et al*. 2002; Brandon *et al*. 2003). PDTC has been investigated for its ability to impart upon several bacteria enhanced resistance, or sensitivity, to a number of metals. Resistance effects were correlated with PDTC:metal precipitation (Cortese *et al*. 2002). The ability to produce PDTC has been associated with higher tolerance to the synthetic chelator 1,10-phenanthroline (*o*-phenanthroline, o-p) but not to ethylenediamine di(*o*-hydroxyphenyl)acetic acid (EDDHA) (Lewis *et al*. 2004). Since o-p has a higher affinity for divalent transition metals (Auld 1988) and EDDHA is a ligand with higher affinity for ferric iron (Cleton *et al*. 1963), a possible role for PDTC in supplying divalent metals was suggested (Lewis *et al*. 2004).

PDTC production is the basis of a bioremediation process requiring provision of a carbon and energy source (Dybas *et al*. 1998). Defined physiological advantages of PDTC producers might be exploited for more effective *in-situ* remediation e.g. minimizing wasteful feeding of ineffective competitors. We have examined physiological roles for PDTC, describing its function as a siderophore (Lewis *et al*. 2004) and genes required for the regulation of its production and utilization (Lewis *et al*. 2000; Morales & Lewis 2006; Leach & Lewis 2006). In this work, we have investigated a role for the PDTC system in zinc nutrition. We have used genetic and physiological approaches to resolve a nutritional benefit provided by the PDTC system from mitigation of zinc toxicity.

Materials and methods

Bacterial strains, growth media and culture conditions

Strains used in this study are listed in Table 1. Tryptic soy broth was used to maintain cultures of pseudomonads. Cultures were incubated at 30 °C. Small cultures (5 ml) were incubated on a roller drum. Larger (50 ml) cultures were grown in 250 ml Erlenmeyer flasks on a rotary shaker. PM, a PIPES-buffered minimal medium (Lewis *et al*. 2004), was used under iron-limiting conditions (tests performed during medium development indicated that iron and zinc were below 0.5 μM). Growth curves and single duration dose-response curves were performed in 96-well microtiter plates. Inocula consisted of a 1/1000 dilution of cultures previously adjusted to an OD_{600} of 0.35. Halving concentrations of Zn and Zn:PDTC were provided ranging from 1.6 to 100 μM. The optical density in each individual well was determined using a BioTek plate reader at 630 nm. Metals used for supplementation were $FeCl_3$, 98% purity, Aldrich; $ZnCl_2$, 99.99% purity, Acros; $ZnSO_4 \cdot 7H_2O$, >99% purity; $Na_2MoO_4 \cdot 2H_2O$, 99.99% purity, Aldrich.

PDTC was chemically synthesized by the method of Hildebrand *et al* (Hildebrand *et al*. 1984). $Fe(PDTC)_2$ was prepared using the protocol of Hildebrand *et al*. (Hildebrand *et al*., 1984). Zn:PDTC was synthesized by a modification of that used to prepare Pd:PDTC and Cu:PDTC (Espinet *et al*. 1994; Lewis *et al*. 2001). Zinc acetylacetonate $(Zn(acac)_2\ H_2O$, 99.9995%, Aldrich) (0.3206 g) was dissolved in 50 ml of nitromethane and filtered. A filtered solution of PDTC (0.2569 g, 1.21 mmol) in 50 ml of nitromethane, was added drop-wise to the $Zn(acac)_2$ solution. The mixture was stirred for 2 hours and filtered on a fine glass frit. The resulting solid was not readily dissolved in acetone/tetrabutylammonium bromide as used to prepare the Pd:PDTC and Cu:PDTC preparations. The solid was instead dissolved with a minimum amount of dimethylformamide (DMF) (~8 ml) with heating. The

Table 1. Bacterial strains and plasmids.

Strain/Plasmid	Description	Source/Reference
P. putida		
DSM 3601	wild type, PDTC producer	DSMZ (Ockels *et al*. 1978)
LLA3	DSM 3601 *pdt*K::Gm^R	(Leach & Lewis 2006)
LLΔE	DSM 3601 *pdt*E::Gm^R	This study
SMTL1	DSM 3601 *pdt*I:: mini-Tn *xyl*E Km^R	(Lewis *et al*. 2004)
SEM1	DSM 3601 *pdt*I:: mini-Tn *xyl*E Km^R *pfr*I::Tc^R	(Lewis *et al*. 2004)
Plasmids		
pJB3Tc20	Tc^R broad host range vector	(Blatny *et al*. 1997)
pK3601	pJB3Tc20 with $pdtK_{3601}$ and flanking DNA inserted in multiple cloning site	(Leach & Lewis 2006)
pJB861TGm	Gm^R, Km^R; pJB861 with 400 bp transcriptional terminator fragment from pQE30 between *Bam*HI and *Eco*RI sites, Gm^Rcassette at *Sna*BI site	(Leach & Lewis 2006)
pE3601	pJB861T with $pdtE_{3601}$ in forward orientation	(Leach & Lewis 2006)

DSMZ, Deutsche Sammlung von Mikroorganism und Zellkulturen

solution was cleared by filtration on a fine glass frit, and the product precipitated by the addition of 30 ml diethyl ether. The resulting solid was collected on a fine glass frit and analyzed by IR and NMR. ^{1}H-NMR spectra allowed assignment as a DMF solvento system (CDCl3, 500 MHz): 8.60 ppm (2H, d, J = 7.7 Hz), 8.02 ppm (1H,t,7.6 Hz), 2.90 ppm (3 H,s), 2.82 ppm (3 H,s). Proton-decoupled ^{13}C NMR in DMF: 187, 153.61, 146.74, and 126.81 ppm. Spectra were obtained on a Bruker AXR operated at 500 MHz with an SGI data system.

Analytical procedures

PDTC was quantitated by measuring the absorbance of the Fe^{2+}:PDTC complex at 687 nm (Budzikiewicz *et al.* 1983). Pyoverdine was assayed by titration with ferric citrate, measuring absorbance at 450 nm (Visca *et al.* 1992). Protein quantitation used the BCA protein assay kit from Pierce. Catechol dioxygenase was assayed by measuring the conversion of catechol to α-hydroxymuconic semialdehyde (Klecka & Gibson 1981). Reactions were monitored at 375 nm on a Cary 50 spectrophotometer (Varian Instruments, Walnut Creek, CA) with constant stirring at 30 °C. Cells were harvested for cell-free extract preparation upon entry into stationary phase (OD_{600} ca. 1.0–1.2). Sarkosyl fractionation was used to isolate outer membrane proteins (Cornelis *et al.* 1989). Protein samples (15 μg/lane) were separated by SDS-PAGE with 8% polyacrylamide, and stained with 0.2% Coomassie blue (Laemmli 1970). The broad range molecular weight standard from New England Biolabs was used as a standard.

Strain/plasmid construction

Details of strain construction are described in Leach & Lewis (2006). Briefly, strain LLA3 carries a deletion/disruption allele of the *pdt*K outer membrane transporter gene interrupted by a gentamycin resistance (Gm^R) cassette. Strain LLΔE carries a deletion/disruption allele of the inner membrane permease gene *pdt*E interrupted by a Gm^R cassette. Wild type alleles, for complementation of each respective gene, were obtained by PCR amplification and cloning in broad host range vectors.

^{55}Fe Uptake

Iron uptake assays were performed by a protocol modified from one described previously (Lewis et al. 2004). ^{55}Fe:PDTC was made by first adding 100 mM Tris-HEPES buffered nitrilotriacetic acid (NTA, pH 6) to $^{55}FeCl_3$(NEN Biolabs; 69.64mCi/mg Fe, 0.5 M HCl) to stabilize the iron in soluble form. The mixture was adjusted to pH 6 by addition of NaOH (0.8 μl of 1 M NaOH to 10 μl ^{55}Fe:PDTC). Immediately prior to each experiment PDTC was mixed with ^{55}Fe:NTA in the stochiometric ratio of 2:1. The mixture was incubated for 20 min at room temperature before use. Spectrophotometic titrations had shown this to give 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.

Cells used for iron uptake assays were grown to early stationary phase (18 h, 30 °C), washed twice and re-suspended in PM to an OD_{600} of 0.8. Reactions were started by the addition of 11.1 nmol ^{55}Fe:PDTC to 4 ml cell suspensions. 1 ml samples were removed and cells were sedimented in a microcentrifuge and washed twice with 1 ml of ice-cold PM. The washed cell pellet was suspended in Biosafe II scintillation fluid (Research Products International) before measuring radioactivity in a Beckman LS6000IC scintillation counter. Time course studies were performed to verify linearity. Thereafter, a standard protocol with a 30-min incubation was adopted.

Results and discussion

Effect of zinc on siderophore production

If PDTC production was part of a system for acquiring transition metals, that function should be reflected in its regulation; bacteria would be expected to limit expression of a high affinity uptake system when adequate amounts of the target substrate can be obtained by other means. We tested several transition metals for their effects on growth and siderophore production in *P. putida* DSM 3601. Pyoverdine production was also measured, as it would reflect any metal-dependent regulation due to cross-specificity of the Fur

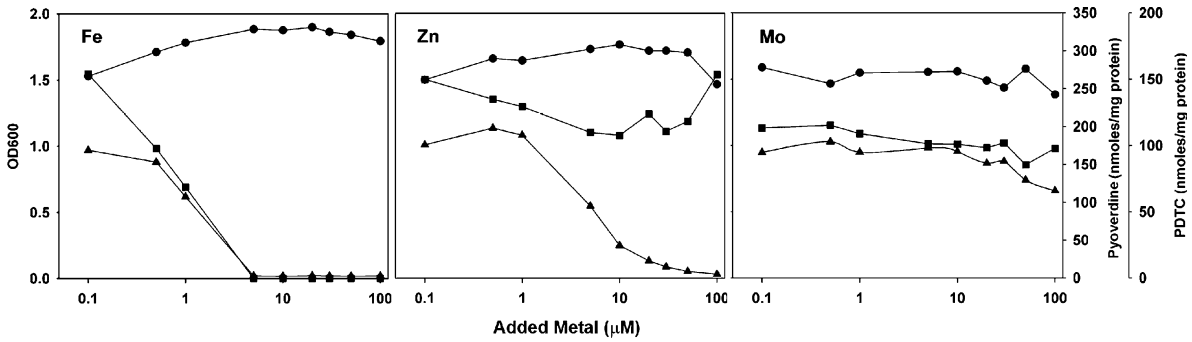

Figure 1. Differential regulation of PDTC production by zinc. Optical density (•), pyoverdine production (■), and PDTC production (▲), by cultures of *P. putida* DSM 3601 grown in PM medium with different concentrations of $FeCl_3$, $ZnCl_2$ and Na_2MoO_4.

repressor. Iron was used as a standard for siderophore repression. Molybdate was also compared as a negative control since the oxyanion would not be transported via siderophores, but once inside cells may have non-specific effects on regulation. Some of the metals tested (Ni^{2+}, Co^{2+}) interfered with the PDTC quantitation measures used and their effects upon siderophore production were not examined further. Zinc did not interfere with the PDTC assay, but did result in differential decreases in PDTC as compared with pyoverdine (Figure 1). Though iron was the more potent repressor, zinc effects were seen at concentrations that appeared to be physiologically relevant. A maximum in growth yield (OD_{600nm}), indicating benefit by provided zinc, corresponded to the minimum in PDTC production.

Zinc has been shown to increase pyoverdine production by *P. aeruginosa* 7NSK2 when added at high concentrations (>0.1 mM) (Höfte *et al.* 1993). We saw an increase in pyoverdine production by *P. putida* DSM 3601 between 50 and 100 μM zinc that also corresponded to a decrease in growth yield, indicating that this organism may respond similarly in terms of pyoverdine production, but is more sensitive to zinc.

Effect of zinc on regulation of pdt genes

Since zinc supplementation led to decreased PDTC production with little effect on pyoverdine, a selective repression of *pdt* genes was suggested. One means of testing preferential regulation was by observing outer membrane protein profiles as the most highly expressed siderophore receptors should be in evidence in those preparations. The electophoretic migration of PdtK, the outer membrane receptor critical for the transport of Fe from Fe:PDTC, corresponds to a size of approximately 65 kDa (Leach and Lewis 2006), and can be seen as an iron-repressed band in PAGE analysis (Figure 2). It was found that PdtK decreased in abundance in the zinc-supplemented culture whereas two higher molecular weight iron-regulated proteins (likely to be pyoverdine receptors; Lewis *et al.* 2004) did not show such marked decrease (Figure 2). Interestingly, another band corresponding to a protein larger than 65 kDa was observed to be decreased in abundance in the zinc-supplemented culture, however we have no data regarding the identity of that protein.

Another means of measuring expression of *pdt* genes was afforded by a chromosomal transcriptional reporter fusion (*pdt*I:: *xyl*E) (Lewis *et al.* 2004). The gene disruption created by that fusion leads to abolishment of PDTC production and a

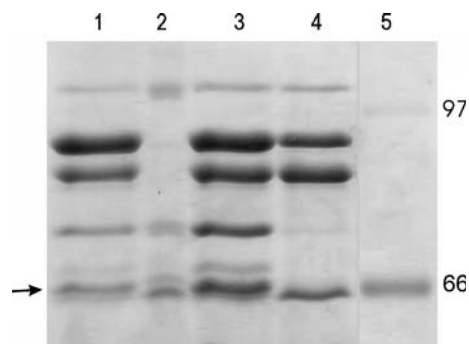

Figure 2. Expression of outer membrane receptor proteins by *P. putida* DSM 3601 in response to zinc. Outer membrane proteins were run on an 8% polyacrylamide gel. Lanes: 1, *P. putida* DSM 3601 grown on PM medium; 2, DSM 3601 PM + $FeCl_3$; 3, DSM 3601 PM + $NaMoO_4$; 4, DSM 3601 PM + $ZnCl_2$; 5, molecular weight marker. Metals were added at 50 μM. The arrow indicates the position of PdtK.

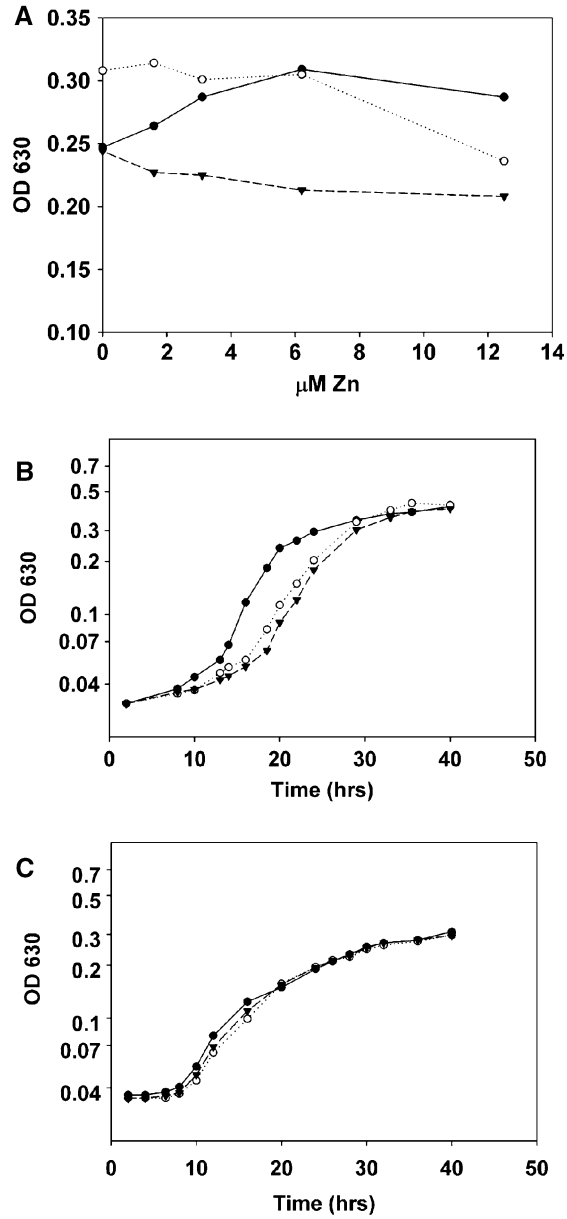

Figure 3. Effect of zinc on Pdt$^-$ mutants. Symbols: (•), DSM 3601 (WT); (○), SEM1 (Pvd$^-$ Pdt$^-$); (▼), SMTL1 (Pdt$^-$). A. Optical density after 24 h incubation in PM medium with the indicated amounts of $ZnCl_2$. B. Growth curves in PM medium supplemented with 3 μM $ZnSO_4$. C. Growth curves in PM medium (no metal supplementation).

low baseline level of transcription through the respective genes. That basal level of expression was reduced further by iron or zinc supplementation (Table 2). This again differed from a response described for pyoverdine biosynthesis genes from another pseudomonad (*P. fluorescens*) which showed marked induction in response to high levels (1 mM) of zinc (Rossbach *et al*. 2000).

Growth enhancement by zinc supplementation: requirement for PDTC and PDTC-specific transporters

As seen in Figure 1, zinc at concentrations near 10 μM gave higher growth yields of *P. putida* DSM 3601. We tested whether a growth benefit in response to zinc required PDTC and/or PDTC

Table 2. Transcriptional regulation of *pdt* genes in the presence of exogenous PDTC, iron, and zinc.

Treatment (50 μM)	Catechol dioxygenase activity[a] (μmoles/min/mg protein)
SMTL1	80.8 ± 29.0
SMTL1 + PDTC	1297.9 ± 135.0
SMTL1 + Fe	22.8 ± 3.0
SMTL1 + Zn	12.9 ± 7.7

[a] mean ± standard deviation of triplicate determinations.

transport machinery. In comparison to wild type, mutants that are unable to produce PDTC (Pdt^-) showed no growth benefit in response to zinc (Figure 3A). The apparent cell yields (OD_{630nm} values at 24 hrs) varied by as much as 20–25% between experiments. However, the dose response was entirely consistent; an increase in the yield at 24 hours for the wild type and no increase or a decrease for Pdt^- mutants. Furthermore the dose response of Pdt^- mutants was independent of their ability to make pyoverdine.

Twenty-four hour measurements of growth were not maximal readings but instead corresponded to a point late in the exponenential growth phase or early stationary phase in our cultures. The effect of added zinc in the low transition metal minimal medium used was illustrated by comparing growth curves of Pdt^+ and Pdt^- strains (Figure 3B and C). Those data showed that zinc addition led to a lag in growth for Pdt^- strains that was not seen for the wild type, whereas all strains grew with similar kinetics in the control medium. Variability in the single point measurements was likely to have been due to variations in inocula, despite standardized procedure.

To determine whether the phenotype of altered zinc response was due solely to a lack of PDTC or whether the mutation had pleiotrophic effects unrelated to PDTC, the ability to suppress that phenotype with exogenously added PDTC was tested. Addition of PDTC was sufficient to restore a growth benefit from added zinc upon the Pdt^- mutant SEM1, enabling us to assign the defect as being a result of loss of PDTC production (Figure 4).

If PDTC facilitated zinc transport, it seemed likely that this would require transport machinery specific for PDTC:metal complexes. Two gene products are known to be necessary for wild type levels of iron uptake from Fe:PDTC, the outer membrane receptor PdtK and the transmembrane

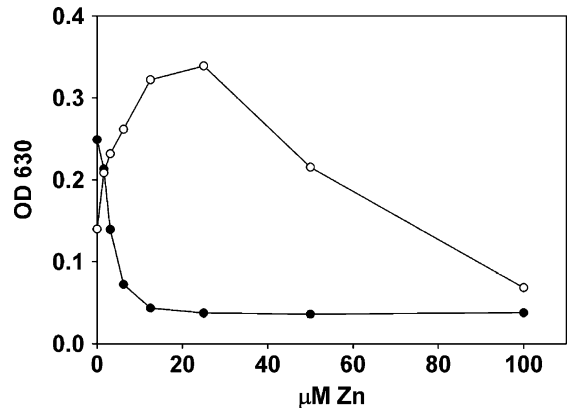

Figure 4. Suppression of the zinc-dependent growth phenotype of a Pdt^- mutant by PDTC. Optical density of strain SEM1 (Pdt^- Pvd^-) after 24 h incubations in PM medium with the indicated concentrations of $ZnSO_4$. Symbols: (•), control; (○), 25 μM PDTC.

permease PdtE (Leach and Lewis 2006). We tested whether those gene products were also necessary for the growth benefit provided by zinc, examining the dose-response of mutants defective in those components and their *trans* complements (Figure 5). Instead of using free zinc ions in standard low transition metal minimal medium, we used synthetic Zn:PDTC and a medium further decreased in metal availability by addition of 1,10-phenanthroline (o-p). The transporter mutants showed a severe inhibition due to o-p addition, despite their ability to produce PDTC (Leach and Lewis 2006). That growth inhibition

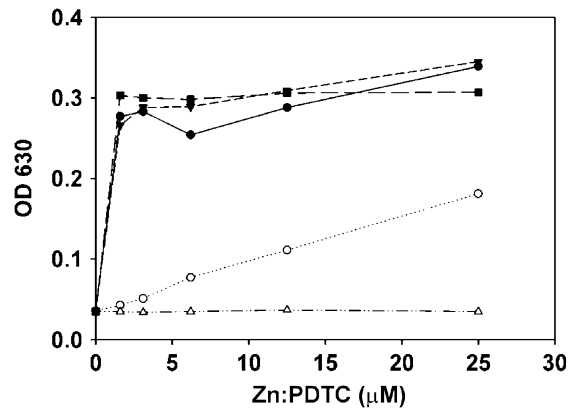

Figure 5. Dependence on PDTC transport proteins to overcome 1,10-phenanthroline-induced growth inhibition with Zn:PDTC. Optical density measured after 48 h in PM medium with 50 μM 1,10-phenanthroline and the indicated concentrations of Zn:PDTC. Symbols: (•), DSM 3601; (○) LLA3/pJB3Tc20 (*pdt*K); (▼), LLA3/pK3601 (*pdt*K/*pdt*K$^+$); Δ, LLΔE/pJB861T (*pdt*E); (■), LLΔE/pE3601 (*pdt*E/*pdt*E$^+$).

was not alleviated by Zn:PDTC unless a wild type copy of *pdt*E was present. The *pdt*K mutant was capable of a growth response to Zn:PDTC, but required much higher concentrations of Zn:PDTC relative to the strain carrying a wild type copy of *pdt*K. The data were consistent with iron uptake data obtained for these mutants. In ^{55}Fe uptake assays, the PdtK mutant retained some ability to transport iron provided as Fe:PDTC, whereas no detectable uptake was observed for the PdtE mutant (Leach and Lewis 2006). This has been interpreted as evidence for an alternative, but inefficient, means of PDTC:metal complexes traversing the outer membrane. No such alternative route across the cytoplasmic membrane appears to exist in this system.

In order to more directly test whether Zn:PDTC was transported across cellular membranes, we performed competition experiments with ^{55}Fe:PDTC. Initially we used synthetic Fe:PDTC to compete with ^{55}Fe:PDTC; the decreased cellular accumulation of radiolabeled iron corresponded to decreased calculated specific activity from dilution with unlabeled Fe:PDTC. However, no inhibition of iron uptake from ^{55}Fe:PDTC was detected at a 32-fold molar excess of Zn:PDTC relative to iron and using a concentration of ^{55}Fe:PDTC that was below saturation (data not shown). Therefore, Zn:PDTC is not an effective competitor for Fe:PDTC transport proteins, but it cannot be ruled out that it is transported by the same proteins with a substantially higher K_m.

The Zn:PDTC preparation used in our experiments was shown by ^{1}H-NMR to be predominantly a symmetric complex. A 1:1 stoichiometry of Zn:PDTC is expected since a molecule of dimethylformamide is present, likely as a ligand occupying the fourth coordination position of the zinc atom. Electrospray ionization MS has shown that a 1:2 Zn:PDTC complex can be detected from aqueous solution (Cortese *et al*. 2002) but no information regarding the ligand environment is obtained from that type of analysis. X-ray diffraction data for the structure of a 1:2 Zn:PDTC crystal showed an asymmetric arrangement of S, N, and O atoms, with one carbonyl oxygen atom and one thiocarboxyl sulfur atom of each PDTC contributing to coordination (Morris, J.C. and Clark, G.R., unpublished data). Since Fe:PDTC is a 1:2 complex (Hildebrand *et al*. 1984), it would seem likely that a 1:2 Zn:PDTC complex could be recognized and transported by the same receptor/transporter system that recognizes Fe:PDTC due to their structural similarity, whereas the 1:1 complex would present a very different structure. If the 1:2 Zn:PDTC complex is relatively rare (i.e. low steady-state concentration due to greater stability of the 1:1 complex), it would not be surprising that we did not see competition in our experiments since Fe:$PDTC_2$ could still be in much higher concentration. In addition, the asymmetric complex may indeed allow discrimination by the receptor, yielding a decreased K_m for transport.

The response of *P. putida* DSM 3601 to zinc and its dependence upon PDTC differed from responses described previously for other pseudomonads with respect to siderophore production and gene expression. In those studies it was shown that pyoverdine biosynthesis, the probable pyoverdine outer membrane receptor, and the putative receptor for pyochelin, were all up-regulated in response to zinc (Höfte *et al*. 1993; Rossbach *et al*. 2000). The given explanation implied that zinc interfered with iron uptake since the addition of high concentrations of iron did not overcome the zinc-induced phenotype (Höfte *et al*. 1993). Therefore, pyoverdine and possibly pyochelin may represent means of overcoming zinc toxicity; in fact, pyoverdine mutants were shown to be more sensitive to zinc.

Conversely, our data indicated that at low concentrations of zinc (10–20 μM) the growth of *P. putida* DSM 3601 is enhanced, specifically due to the ability to produce and transport PDTC. It seems likely that PDTC has an effect due to a contribution to zinc nutrition rather than simply avoidance of zinc toxicity. The fact that growth was enhanced relative to un-supplemented media is difficult to explain on the basis of enhanced iron uptake, as PDTC is produced in higher amounts in the un-supplemented media.

The lack of interference with Fe:PDTC formation in our detection assay, and the apparent use of a transport system which also recognizes Fe:PDTC when the most stable complex has different stoichiometry and ligation suggest that Zn:PDTC may be a rather inefficient means of acquiring zinc. The nutritional benefit may appear to be a fortuitous consequence of a structural similarity; however, the regulatory phenomena indicate that it is an evolved trait. The data describe another function for PDTC, and novel regulation for a siderophore.

Acknowledgements

The authors would like to thank George Clark for sharing X-ray diffraction data prior to publication. Funding for this work was supplied by the Vermont Agricultural Experiment Station.

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