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Metal chelating properties of pyridine-2,6-bis(thiocarboxylic acid) produced by *Pseudomonas* spp. and the biological activities of the formed complexes

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Abstract

We evaluated the ability of pyridine-2,6-bis(thiocarboxylic acid) (pdtc) to form complexes with 19 metals and 3 metalloids. Pdtc formed complexes with 14 of the metals. Two of these metal:pdtc complexes, Co:$(pdtc)_2$ and Cu:pdtc, showed the ability to cycle between redox states, bringing to 4 the number of known redox-active pdtc complexes. A precipitant formed when pdtc was added to solutions of As, Cd, Hg, Mn, Pb, and Se. Additionally, 14 of 16 microbial strains tested were protected from Hg toxicity when pdtc was present. Pdtc also mediated protection from the toxic effects of Cd and Te, but for fewer strains. Pdtc by itself does not facilitate iron uptake, but increases the overall level of iron uptake of *Pseudomonas stutzeri* strain KC and *P. putida* DSM301. Both these pseudomonads could reduce amorphous Fe(III) oxyhydroxide in culture. *In vitro* reactions showed that copper and pdtc were required for this activity. This reaction may derive its reducing power from the hydrolysis of the thiocarboxyl groups of pdtc.

Introduction

Pseudomonads are known for their ability to synthesize and excrete a variety of secondary metabolites, each of which may serve one or more recognized functions outside the cell. Antibiotics, such as 2,4-diacetylphloroglucinol, pyoluteorin, and pyrrolnitrin are antagonistic towards other microbial and fungal species (Duffy & Defago 1999). The virulence factors exotoxin A, rhamnolipids, and alginate exercise their antagonism towards host tissues (Maier & Soberon-Chavez 2000). Acyl-substituted homoserine lactones are used by several pseudomonads for quorum sensing (Fuqua *et al.* 1996). Pyocyanin, also an antibiotic and virulence factor, has the ability to reduce iron and may function to augment siderophore-based iron uptake systems (Cox 1986). Small organic siderophores such as pyochelin and cepabactin, from *P. aeruginosa* and *P. cepacia*, respectively, are produced for the purpose of iron acquisition (Cox *et al.* 1981; Meyer *et al.* 1990). The regulation of pyochelin by metals other than iron suggests that this secondary metabolite may be involved in acquisition of other essential metals as well (Visca *et al.* 1992).

The essential transition metals Co, Cu, Fe, Mn, Mo, Ni, V, and W are used by bacteria as cofactors for enzymes involved in redox or hydrolytic reactions. Zn, also essential, forms part of the recognition motif for some transcription factors and is present in some proteolytic enzymes. The toxic metals Ag, Al, Au, Bi, Cd, Hg, Pb, Sn, and Tl serve no known function in cells. Besides aluminum, which is a hard acid, all

these metals are toxic because they are soft acids and bind preferentially to sulfur ligands and displace the essential metals normally bound to biological molecules (Beveridge *et al.* 1997; Ho 1977). The metalloids As, Ga, Sb, and Se also serve no function in bacterial cells and are considered toxic to them (Beveridge *et al.* 1997).

Pyridine-2,6-bis(thiocarboxylic acid) (pdtc) (CAS number 69945-42-21) is a small secondary metabolite secreted by certain pseudomonads when grown aerobically or anaerobically in iron-limited laboratory media (Figure 1A) (Lee *et al.* 1999a,b; Ockels *et al.* 1978). Pdtc production has also been demonstrated under field conditions (Dybas *et al.* 1998). Pdtc was first purified from the medium of *Pseudomonas putida* DSM3601 and *Pseudomonas* sp. DSM3602 cultures (Ockels *et al.* 1978). The structures and characteristics of the Co, Fe, Ni, and Pd pdtc complexes were subsequently elucidated, including the redox cycling abilities of the Fe and Ni complexes (Krüger *et al.* 1990; Hildebrand *et al.* 1984, 1985, 1989; Espinet *et al.* 1994; Budzikiewicz 1993). While studying the carbon tetrachloride CCl_4 degradation activity of *Pseudomonas stutzeri* strain KC, we identified pdtc as the agent responsible for this organism's distinctive CCl_4 degradation activity (Lee *et al.* 1999a, b). Further study revealed that pdtc formed a 1:1 complex with Cu and that this complex, not pdtc alone, was the active agent of CCl_4 degradation (Lewis *et al.* 2001).

One of the unique properties of pdtc is its high affinity but low specificity for transition metal ions including Cu(II), Co(III), Fe(III), and Ni(II). The formation constants (log K) for Fe(III):$(pdtc)_2$ and Co(II):$(pdtc)_2$ were determined to be 33.36 and 33.96, respectively. A ligand competition study demonstrated that the formation constant for Cu(II):pdtc:Cl was the same order of magnitude (Stolworthy *et al.* 2001). *In vitro* CCl_4 degradation reactions (mediated by Cu:pdtc) are inhibited by the addition of Co but not Fe, so the relative affinity of pdtc for Cu(II) can be inferred to fall between Co(II) and Fe(III) (Lewis *et al.* 2001).

At 198 daltons, pdtc is among the smallest naturally-produced metal chelating compounds. Hydroxamate and catecholate functional groups, both of which are highly specific for ferric iron, are the two most common ligands used in siderophores to chelate ferric iron. The binding atom in both cases is oxygen, a hard base with high affinity for hard acids like Fe(II) (Ho 1977). Pdtc, in contrast, usually binds metals with two thiocarboxylate groups and a single pyridine nitrogen (Figure 2). These soft-base binding atoms differentiate pdtc from traditional siderophores containing oxygen ligands. Except for pdtc, examples of naturally-produced organic molecules with free thiocarboxylic groups are rare. The most studied molecule with a free thiocarboxylic group is the activated product of *thi*S in the thiamin biosynthetic pathway (Begley *et al.* 1999). A small secondary metabolite with unknown function produced by *Pseudomonas fluorescens*, 8-hydroxy-4-methoxy-monothiochinaldic acid, also has a single free thiocarboxylate group (Neuenhaus *et al.* 1980). Although sulfur-containing siderophores such as anguibactin (Jalal *et al.* 1989), pyochelin (Ankenbauer *et al.* 1988), yersiniophore (Chambers *et al.* 1996), and ferrithiocin (Naegeli & Zähner 1980) exist, none have been shown to use sulfur atoms as metal-binding ligands. Two pseudomonads with well-studied iron uptake systems are *P. fluorescens* and *P. aeruginosa*, both of which produce a large peptide-based siderophore as well as a smaller organic siderophore. The ligands of the smaller siderophores, pyochelin and quinolobactin from *P. aeruginosa* and *P. fluorescens*, respectively, are not the ferric iron-specific hydroxamate or catecholate functional groups used by larger peptide siderophores (Cox *et al.* 1981; Mossialos *et al.* 2000). While little is known about quinolobactin, studies of pyochelin suggest it may play a role in the acquisition of essential transition metals in addition to iron. In addition to ferric iron, pyochelin also binds Co, Cu, Ni, and Mo. This binding correlates to the repressive effect these metals have on pyochelin production, suggesting that this molecule may have a function in the uptake of essential transition metals (Visca *et al.* 1992). It remains to be shown whether any of these secondary metabolites produced by pseudomonads, including pdtc, play a role in the acquisition of essential metals other than iron.

We believe that the metal binding activity of pdtc plays a critical role in its biological function. In addition, pdtc's structural features could allow it to function in any of the secondary metabolite roles mentioned above. Here we report on the properties of the metal: pdtc complexes that we have examined as part of our investigation into the biological function of this unusual molecule.

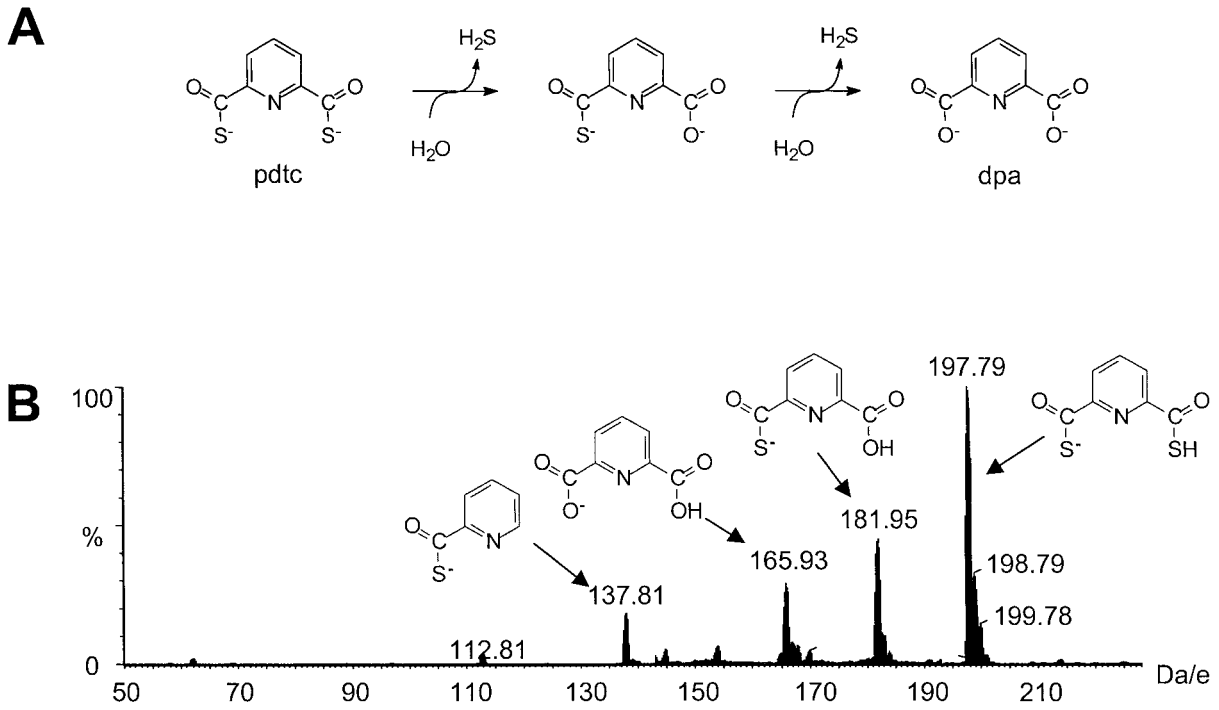


Fig. 1. Degradation of pdtc. A, Stepwise hydrolysis of pdtc to dipicolinic acid (dpa). B, Mass spectra of pdtc in water after 48 h incubation showing structures represented by ion peaks.

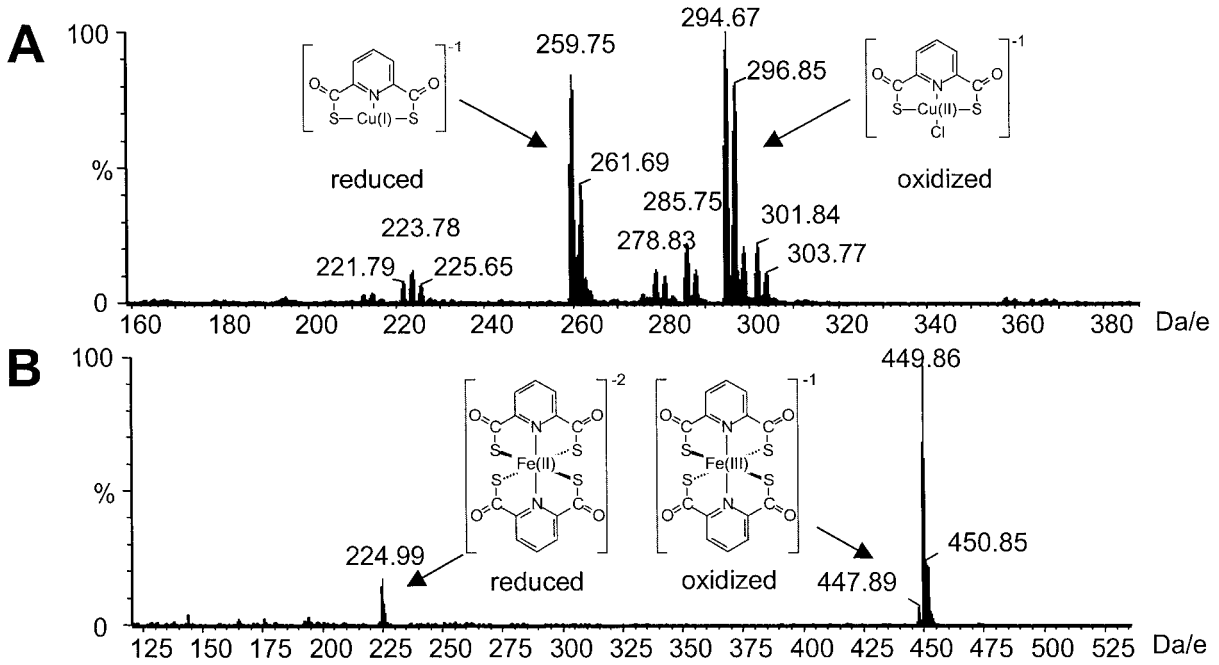


Fig. 2. Electrospray mass spectra of redox-active pdtc metal complexes showing both oxidized and reduced forms. Oxidized complex solutions were partially reduced with sodium dithionite and immediately analyzed. A, Cu:(pdtc); B, Fe:$(pdtc)_2$.

Materials and methods

Pdtc synthesis

Pdtc was synthesized using a previously described method (Hildebrand *et al.* 1983). Briefly, a complex containing a mixture of 1:1 pyridinium:pyridinium-2,6-bis-carbothioate was synthesized using pyridine (Fisher) saturated with H_2S (Matheson Gas Products). This was cooled on ice, and a solution of 2,6-pyridinedicarbonyldichloride (Aldrich) in acetone was slowly added, resulting in the formation of an orange-red pyridinium:pdtc complex. From an acidified water solution of this complex, a pure pdtc:H_2 was isolated using CH_2Cl_2 extraction. Using this method we obtained pdtc of more than 99% purity, which could be stored at 4 °C under nitrogen for several months without detectable degradation.

Complex synthesis and purification

The Co(III):$(pdtc)_2$, Fe(III):$(pdtc)_2$, and Ni(II):(pdtc) complexes were prepared in crystalline form as described earlier (Hildebrand *et al.* 1984; Hildebrand & Lex 1989). The Cu(II):pdtc:Cl complex was prepared by adapting a published method for the synthesis of Pd(II):pdtc:Br (Espinet *et al.* 1994). Pdtc complexes with other metals were formed by mixing equimolar solutions of pdtc:H_2 in dimethylformamide (DMF) with metal nitrate salts solubilized in 2% nitric acid in water. Resulting mixtures were analyzed within 2 h. If a precipitate formed, the pellet was washed twice with double distilled water and the washed precipitate was solubilized in DMF and analyzed. Pdtc in DMF was diluted to 1 mM with double distilled water and analyzed after 48 h for degradation products. Samples were analyzed for complex formation and structure by electrospray-ionization mass spectrometry (ES/MS) (Quattro II, Micromass). Solutions were delivered into the MS source at a flow rate of 5 μl min using a syringe pump (Harvard Apparatus). A potential of 2.5–3 kV was applied to the electrospray needle. The sample cone voltage was maintained at 10 V. The counterelectrode, skimmer, and RF lens potentials were tuned to maximize the ion beam for the given solvent. Detector resolution was set at 15 000, and source temperature was kept constant at 80 °C. The instrument was calibrated using a poly(ethylene glycol) solution. Structures of complexes were confirmed by MS/MS daughters analysis. The collision cell was operated at 20 to 40 eV with argon gas pressure adjusted to give approximately 90% parent ion fragmentation. All MS and MS/MS spectra were an average of 10–15 scans over a mass range of 30–800 Daltons.

Complex stability

The stability of Co(III):$(pdtc)_2$, Cu(II):pdtc:Cl, and Fe(III):$(pdtc)_2$ was investigated in buffers composed of either 50 mM sodium citrate-phosphate for pH 3 and 7, or 50 mM sodium bicarbonate for pH 10. Solutions of crystalline metal:pdtc complexes were prepared in DMF and added to give final concentrations of 0.2 mM. The final concentration of DMF in the preparations was approximately 25%. Solutions were incubated at room temperature with no provisions to exclude oxygen. Degradation of the complexes was monitored by periodically measuring the absorbance at 600 nm (for the Fe complex) or 400 nm (for the Co and Cu complexes) during the incubation period.

Cyclic voltammetry

Cyclic voltammetry of pdtc metal complexes was performed using a BAS-100B electrochemical analyzer (Bioanalytical Systems). All experiments were performed in a 20 ml volume. The working electrode was a platinum disk with an area of 2 mm^2. The counter electrode was a platinum wire. The reference electrode was saturated Ag/AgCl ($E^\circ = 0.199$ mV in water). All experiments were performed in a 0.2 M solution of tetrabutylammonium perchlorate in DMF. Potentials were measured at 25 °C versus SCE and were not corrected for junction potentials. The working electrode usually polarized at a rate of 10–50 mV per sec. A ferrocene/ferrocinium couple (200 mg/l ferrocene in DMF) was used to verify SCE potentials.

Absorbance spectra

Spectra were obtained using an Hewlett Packard 8453 Diode Array Spectrophotometer. To determine the spectrum of the oxidized iron complex, a solution of 1.67 mM purified crystalline Fe(III):$(pdtc)_2$:tetraethylammonium was constituted in DMF and diluted 20-fold to 83.5 μM with double distilled water. The instrument was blanked with a solution of 5% DMF in double distilled water. To determine the spectra of the oxidized copper complex, a 1.82 mM stock solution of purified crystalline Cu(II):pdtc:Cl:tetrabutylammonium was made up in DMF and diluted to 182 μM with 50% DMF in double distilled water. The instrument was blanked with a solution of 50% DMF in double distilled water. To

obtain the reduced species, 50 μl of 100 mM sodium dithionite was added to 1 ml of the above solutions. The absorbance of Cu(II):pdtc:Cl from 500 to 800 nm was also determined at a 10-fold higher concentration by using the undiluted stock solution and blanking with 100% DMF.

Iron uptake assays

Labeled ferric pdtc complex was made by combining 7 μl of 133 μM $FeCl_3$ in 0.5 M HNO_3, 7 μl of 133 μM $^{59}FeCl_3$ in 0.5 M HNO_3, and 14.66 μl of 50 μg/ml pdtc in DMF for each 10 ml reaction (final concentrations ml^{-1}: 73 ng pdtc, 186 pmol Fe, 219 nCi). The Fe(III):$(pdtc)_2$ complex was allowed to form by incubating the solution at room temperature for 2 h prior to use. For iron only reactions, 7 μl of 133 μM $FeCl_3$ and 7 μl of 133 μM $^{59}FeCl_3$ were diluted to the same total volume per reaction as the Fe(III):$(pdtc)_2$ reaction mixtures with DMF. Radioactive iron (1.33 mM $^{59}FeCl$ in 0.5 M HCl, 5 mCi ml^{-1} specific activity) was obtained from NEN Life Sciences Products. The method of Cornelis (Cornelis *et al.* 1989), with modifications, was used for determination of iron uptake into washed cells. Cells were grown in 0.5× succinate medium (Meyer & Abdallah 1978) (SM) pH 7.3 with or without 200 μM ferric citrate to the desired density as measured by the absorbance at 600 nm. Pdtc production of the cultures was determined spectrophotometrically as previously described (Sebat *et al.* 2001). The cells were washed twice by centrifuging for 6 min at 3000 rpm using a JA-10 rotor (Beckmann) and resuspending in 0.5× nitrogen-free SM. After washing, the cells were resuspended in 0.5× nitrogen-free SM at an OD_{600} of 0.3 ($\sim 7 \times 10^7$ CFU ml^{-1}). Ten ml of washed cells were preincubated with or without 0.2% sodium azide for 10 min with agitation (100 rpm) at room temperature (24 °C) in 50 ml disposable centrifuge tubes (Falcon) before addition of 28.66 μl of labeled complex. Cells from 1 ml of reaction mix were filtered onto 0.45 μm pore size polycarbonate membranes (Isopore, Millipore Corp.), washed twice with 1 ml of 0.5× nitrogen-free SM, added to 15 ml of EcoLite (ICN) scintillation cocktail, and counted using a 0–466 keV energy window with a Tri-Carb 2100TR liquid scintillation counter (Packard) for each time point. For the determination of iron uptake into unwashed cells, a 1 ml aliquot of growing culture was incubated with 2.9 μl of either labeled ^{59}Fe(III):$(pdtc)_2$ or $^{59}FeCl_3$ for 20 min with shaking prior to sampling and counting as described above for unwashed cells.

Iron reduction assays

To screen strains for iron reduction activity, triplicate cultures were grown at room temperature without shaking (heterogeneous culture) in SM (pH 7.0) with 10 mg amorphous Fe(III) oxyhydroxide and 1 g of ferrozine (FerroZine® , Aldrich) (Stookey 1970) per liter. Ferrozine was used as a ferrous iron trap because the slow reaction rate prevented the iron reducing activity of the supernatant from being measured directly. Ferrozine was able to out-compete pdtc for ferrous iron, but did not reduce iron (data not shown). Amorphous Fe(III) oxyhyroxide was prepared according to the method of Lovley (Lovley & Phillips 1986) and sieved to select for a particle size of 0.197 ± 0.05 mm. Iron reduction was measured by determining Fe(II):ferrozine concentration by measuring the absorbance at 562 nm (Stookey 1970).

Abiotic reactions were carried out in 0.5× SM (pH 7.6) with ferrozine added. Trace metals were removed by stirring overnight at 4 °C with 5 g Chelex® 100 Resin (BioRad) and then removing the resin by filtration. All glassware was soaked in aqua regia for 1 h, rinsed with deionized water, and autoclaved prior to use. Pdtc was added from a 5.94 mM DMF stock. Cu was added from a stock made up from ICP standard solution (Fisher) diluted to 10 mM with 2% HNO_3. Amorphous Fe(III) oxyhydroxide was added by weight.

Toxicity assays

Metal and metalloid solutions were made up from either ICP standard solutions (Aldrich or Fisher) diluted in the same solvent as the stock solution (2% HCl for Al and Te; H_2O for Mo; 5% HNO_3 for Zn; or 2% HNO_3 for As, Cd, Co, Cr, Cu, Fe, Ga, Hg, Mn, Ni, Pb, Se, and Sr) or at least 99.9% pure nitrate salts (Aldrich) in 2% HNO_3 for Cs, Nd, Pd, and Sc. Pdtc free acid was dissolved in DMF. Metal:pdtc complexes and mixtures (for those metals which do not form complexes with pdtc) were made by mixing amounts of 30 mM pdtc in DMF and 30 mM solution of the respective metal or metalloid to achieve either 1:1 stoichiometry for As, Cd, Cu, Ga, Hg, Pb, Pd, Se, and Sr or 2:1 stoichiometry for all others. Cultures of tester strains (Table 1) were grown overnight in either SD broth (Difco) for *Candida albicans* or Mueller Hinton (MH) broth (Difco) for all others and diluted to an

Table 1. Strains used in this study.

Strain	Genotype	Source/reference
Pseudomonas stutzeri strain KC	Wild-type, aquifer isolate	(Criddle *et al.* 1990), ATCC 55595*
Pseudomonas stutzeri strain CTN1	Spontaneous pdtc$^-$ derivative of strain KC	(Lewis *et al.* 2000)
Pseudomonas putida DSM3601	Wild-type, pdtc producer	(Ockels *et al.* 1978), DSMZ 3601**
KC1896	pdtc$^-$ transposon mutant of *P. stutzeri* strain KC	(Sepulveda-Torres *et al.* 1999)
KC2753	pdtc$^-$ transposon mutant of *P. stutzeri* strain KC	(Sepulveda-Torres *et al.* 1999)
KC3164	pdtc$^-$ transposon mutant of *P. stutzeri* strain KC	(Sepulveda-Torres *et al.* 1999)
Staphylococcus epidermidis	Wild-type, clinical isolate	ATCC 35547*
Staphylococcus aureus	Wild-type, clinical isolate	ATCC 25923*
Arthrobacter sp.	Wild-type, soil isolate	ATCC 33790*
Bacillus cereus		T. Steffens, University of Idaho
Bacillus subtilis		ATCC 6633*
Escherichia coli	Wild-type, clinical isolate	ATCC 25922*
Pseudomonas putida mt-2	Wild-type, soil isolate	ATCC 33015*
Pseudomonas aeruginosa PAO1	Wild-type	N. Schiller, University of California, Riverside
Pseudomonas fluorescens F113	Wild-type, soil isolate	F. O'Gara, University of Cork, Ireland
Pseudomonas stutzeri 11607	Wild-type, clinical isolate	ATCC 11607*
Pseudomonas stutzeri 14405 (ZoBell)	Wild-type, marine isolate	ATCC 14405*
Pseudomonas stutzeri 17588	Wild-type, clinical isolate	ATCC 17588*
Candida albicans	Wild-type, clinical isolate	ATCC 90028*

*ATCC = American Type Culture Collection, Rockville, Maryland, USA.
**DSMZ = Deutsche Sammlung fur Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany

OD_{600} of 0.3 ± 0.5 with either SD or MH broth and then spread on 150 mm diameter SD or MH agar plates with a sterile cotton swab. Sterile 7.5 mm diameter disks of chromatography paper (Whatman 3030680) were saturated with individual metal or metal:pdtc complex solutions, touched to filter paper to wick off the excess solution, and placed on the inoculated plates. Complexes or mixtures that precipitated from solution (As, Cd, Hg, Pb, Se, and Te) were vortexed briefly to suspend the precipitate prior to soaking the filter disks. After incubation for 24 h at 30 °C for *Pseudomonas*, *Bacillus*, and *Arthrobacter* species, or 37 °C for all others, the diameters of the inhibition zones were measured to the nearest 0.5 mm with digital calipers. These data were corrected by subtracting the diameter of the zone of inhibition around the respective control disks (15 mM pdtc in DMF for complexes, 2% HCl, 2 or 5% HNO_3, or water for metals). All assays were done in triplicate with mean ± SD calculated for each individual metal, metal:pdtc complex, or control. The net increase or decrease in inhibition was determined by subtracting the corrected metal inhibition zone diameter from the respective corrected complex inhibition zone diameter. Standard deviations were combined by calculating the square root of the sum of the squares of the two standard deviation values. Positive values indicate that pdtc increased the toxicity of the particular metal while negative values indicate that pdtc decreased the toxicity of the par-

ticular metal. Differences of less than 3 mm within standard deviation were not reported.

Results

Pdtc hydrolysis

The mass spectrum of 48 h-old aqueous 1 mM pdtc solutions confirmed that pdtc underwent hydrolysis with the stepwise release of H_2S (Figure 1). Loss of either COO^- or COS^- to form the ion product at 138 was also observed.

Complex synthesis and analysis

Complex formation with pdtc was evident for some metals by the immediate color change when aqueous solutions of certain metals were added to pdtc dissolved in DMF (final concentration: 0.66–1% HNO_3 or HCl in 33 or 50% DMF). Metal additions that resulted in a color change from the yellow-colored pdtc/DMF solution were Cd (light green), Co (red), Cu (green), Fe (brown), Ni (green), Mo (red), and Pd (orange). These visible color changes were interpreted as an indication of complex formation. Metal additions that did not result in a detectable color change were Al, Ga, Mn, Nd, and Sr. Mixtures of pdtc with As, Cd, Hg, Pb, Se, and Te resulted in the formation of white, light green, yellow, orange, white, and orange precipitants, respectively.

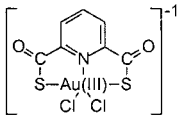
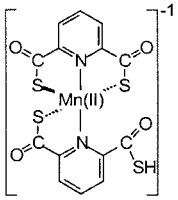
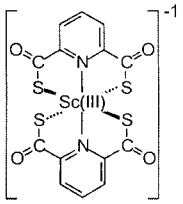
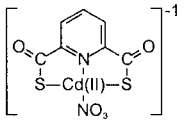
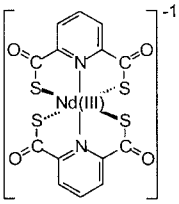
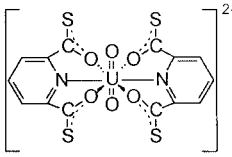
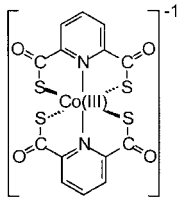
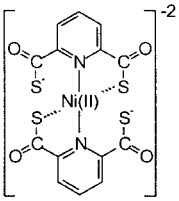
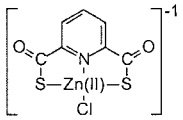
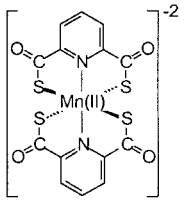
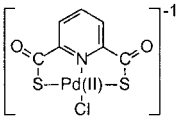
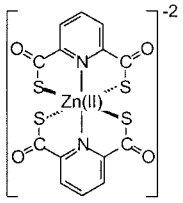
By analyzing the ion peaks from electrospray tandem mass specra of formed complexes and visible absorbance spectra of metal:pdtc titrations (data not shown), we found that either one pdtc molecule could bind one metal ion, forming a tetracoordinate planar complex such as Au(III):pdtc:Cl, Cd(II):pdtc, Cu(I):pdtc, Cu(II):pdtc:Cl, Pb(II):pdtc, Pd(II):pdtc, and Zn(II):pdtc (Table 2, Figure 2A, Figure 3C), or two pdtc molecules could bind a metal ion forming hexacoordinate octahedral complexes such as Bi(III):$(pdtc)_2$, Co(III):$(pdtc)_2$, Cr(III) $(pdtc)_2$, Fe(II):$(pdtc)_2$, Fe(III):$(pdtc)_2$, Mn(II):$(pdtc)_2$, Nd(III):$(pdtc)_2$, Ni(II):$(pdtc)_2$, Sc(II):$(pdtc)_2$, and Zn(II):$(pdtc)_2$ (Table 2, Figure 2B, Figure 3A and B,). Complexes with a 1:1 stoichiometry could gain one or two anions from solution (Table 2, Figure 2A,). These could be halogens such as Cl^-, Br^-, or acid anions like NO_3^-, depending on the composition of the solution. Ion peaks and complex structures determined during ES/MS for Au, Cd, Co, Mn, Nd, Ni, Pd, Sc, and Zn pdtc complexes are listed in Table 2. Although a color change was noted when pdtc was added to Hg, Mo, Se, and Te solutions, indicating complex formation, the structures for these complexes were not determined. No complex formation was detected by ES/MS when Al, Ga, and Sr were combined with pdtc.

Pdtc hydrolysis was observed when the mixtures of pdtc with Bi(III), Cr(III), and Pb(II) were analyzed by ES/MS (Figure 3). Complexes were formed between the metals and pdtc as well as the products of pdtc hydrolysis. ES/MS spectra of the Bi(III), Cr(III), and Pb(II) pdtc complexes showed 5, 3, and 2 masses, respectively, related to the structures shown in Figure 3. The relative peak heights of the various complexes seen in the spectra of Bi(III):$(pdtc)_2$ and Cr(III):$(pdtc)_2$ suggest that complexes other than those formed between the metal and pdtc are the most predominant (Figure 3A, B). The higher abundance of non-pdtc complexes in these reactions may be due to the higher stability constants of these complexes (as compared to metal:pdtc) combined with an uncharacterized reaction that could promote pdtc hydrolysis.

Data obtained from analysis of electrospray mass spectra showed that the Cu and Fe complexes could exist in oxidized or reduced forms (Figure 2). Solutions of these two complexes could undergo repeated redox cycling as evidenced by the visual color changes of the solution when reduced by addition of sodium dithionite or after oxidization by exposure to air (data not shown). The oxidized states were green and brown colored while the reduced states were orange and blue colored for Cu and Fe complexes, respectively. We then determined the absorbance spectra of the reduced and oxidized forms of the copper and iron pdtc complexes. The absorbance spectrum of Fe(III):$(pdtc)_2$ showed peaks at 345, 468, 604, and 740 nm with extinction coefficients of 11177, 3450, 1831 and 707 (cm^{-1} M^{-1}), respectively, while that of Fe(II):$(pdtc)_2$ showed peaks at 314 and 687 nm with extinction coefficients of 26094 and 6876 (cm^{-1} M^{-1}), respectively (Figure 4A). The absorbance spectrum of Cu(II):pdtc:Cl showed peaks at 392 and 614 nm with extinction coefficients of 6124 and 489 (cm^{-1} M^{-1}), respectively, while that of Cu(I):pdtc showed peaks at 358 and 510 nm with extinction coefficients of 7324 and 425 (cm^{-1} M^{-1}), respectively (Figure 4B).

These observations were supported by cyclic voltammetry data. As mentioned above, the redox cycling abilities of the complexes Fe:$(pdtc)_2$ and Ni:$(pdtc)_2$ were previously known. We found that the Co:$(pdtc)_2$ and Cu:pdtc complexes were also ca-

Table 2. Molecular ion peaks from ES/MS spectra and associated structures for the complexes.

Metal	Mol. Ion (*m/z*)	Structure	Metal	Mol. Ion (*m/z*)	Structure	Metal	Mol. Ion (*m/z*)	Structure
Au	464		Mn	450		Sc	439	
Cd	373		Nd	538		UO_2[c]	N.D.	
Co[a]	453		Ni[a]	226		Zn	298	
Mn	224.5		Pd[b]	N.D.		Zn	229	

[a](Hildebrand & Lex 1989; Krüger & Holm 1990)
[b](Espinet *et al.* 1994)
[c](Neu *et al.* 2001)
N.D.: Not determined in this study.

pable of redox cycling. Cyclic voltammetry of both iron and cobalt complexes shows a separation of cathodic and anodic waves in a relatively narrow range (73 and 112 mV, respectively), suggesting that quasi-reversible electrochemical reactions with single electron exchange occurred (Figure 5). However, we were unable to find a reductant that could satisfactorily reduce Co:$(pdtc)_2$. In contrast to the Fe and Co complexes, the wide separation in cathodic and anodic waves seen for Cu:pdtc suggests a two electron reduction that could result in the formation of Cu^0, leading to disassociation of the complex or the reduction to Cu(I) coupled with the dissociation of the ligand (i.e., Cl^- in Figure 2A) in the 4th position, which would result in an unstable, perhaps more reactive, complex.

The copper complex also proved to be less stable than the cobalt and iron complexes at acidic and basic pH values when tested for stability at different pHs. Co(III):$(pdtc)_2$ and Fe(III):$(pdtc)_2$ were relatively stable at pH 3, 7, and 10 over a 1 week period (Figure 6).

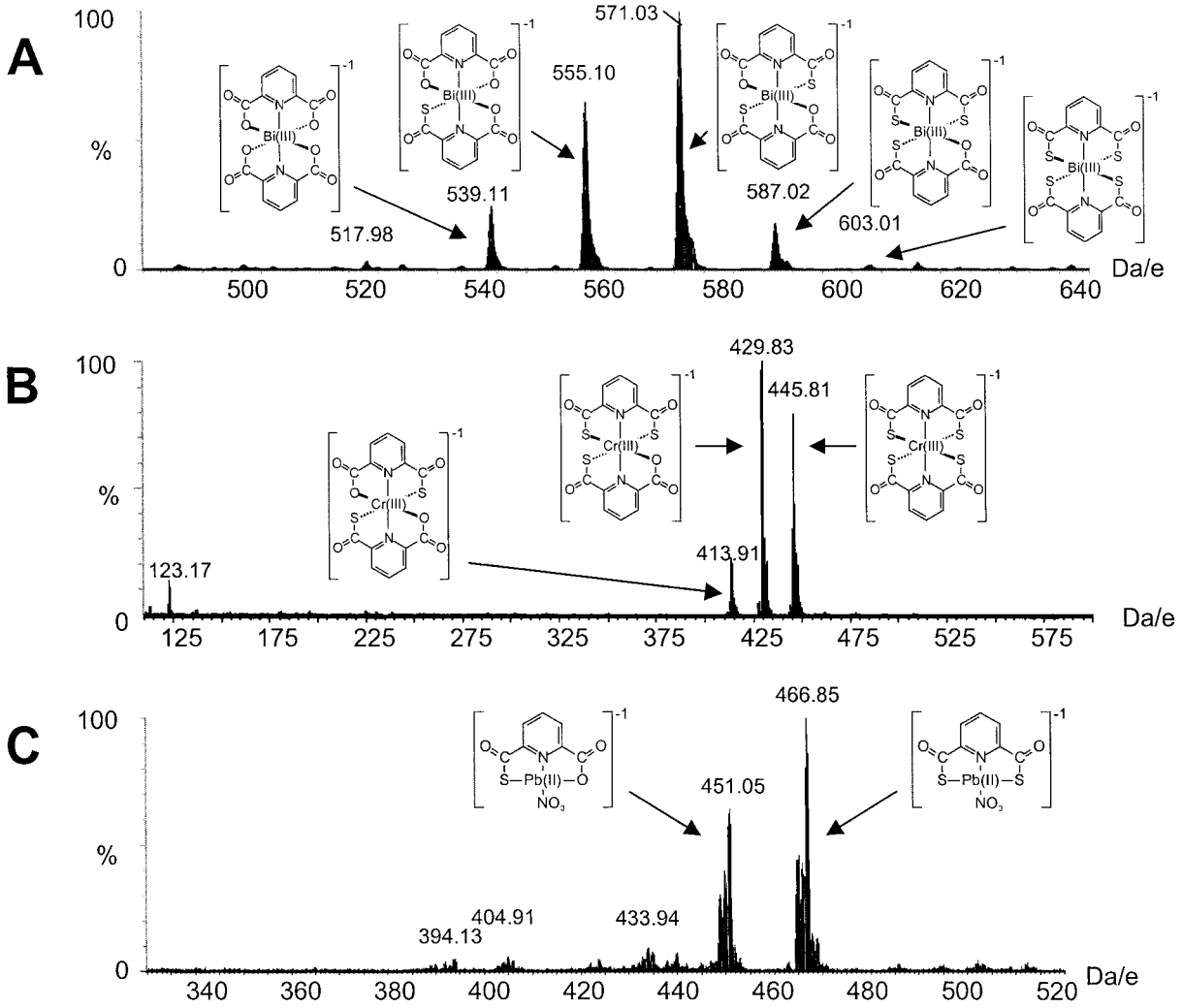


Fig. 3. Electrospray MS spectra of Bi(III):$(pdtc)_2$, Cr(III):$(pdtc)_2$, and Pb(II):pdtc complexes showing additional complexes formed between the metal ions and pdtc hydrolysis products.

Cu(II):pdtc:Cl was also stable at pH 7, but showed marked degradation at pH 3 and 10.

Iron uptake

Based on its regulation by iron, its secretion into the growth media, and the high binding constant for Fe(III):$(pdtc)_2$, it seemed likely that pdtc could function as a siderophore. To test this hypothesis, we first used the established technique of incubating washed bacterial cells with the postulated siderophore complexed with ^{59}Fe. Results of washed-cell iron uptake experiments for strain KC are shown in Figure 7. Labeled Fe(III):$(pdtc)_2$ was taken up by induced (pdtc-producing) washed cells at the same rate as uninduced and killed cells, while Fe(III) alone was taken up at a much greater rate. Parallel experiments with *P. putida* DSM3601 yielded similar results (data not shown). This experiment was repeated three times with each strain, with the same outcome each time. Approximately 66% of the supplied Fe(III) was taken up into the cells after 55 min of incubation with iron only. The absence of pdtc-mediated iron uptake in washed cells lead us to investigate the uptake of Fe(III):$(pdtc)_2$ into unwashed cells. In these experiments, the addition of pdtc did facilitate increased iron uptake over ^{59}Fe alone (Figure 8). Without added pdtc, the unwashed cells removed 78.4 $\pm$ 3.6% of the supplied iron from the medium in 20 min. With added pdtc, iron uptake into unwashed cells became very efficient, with cells

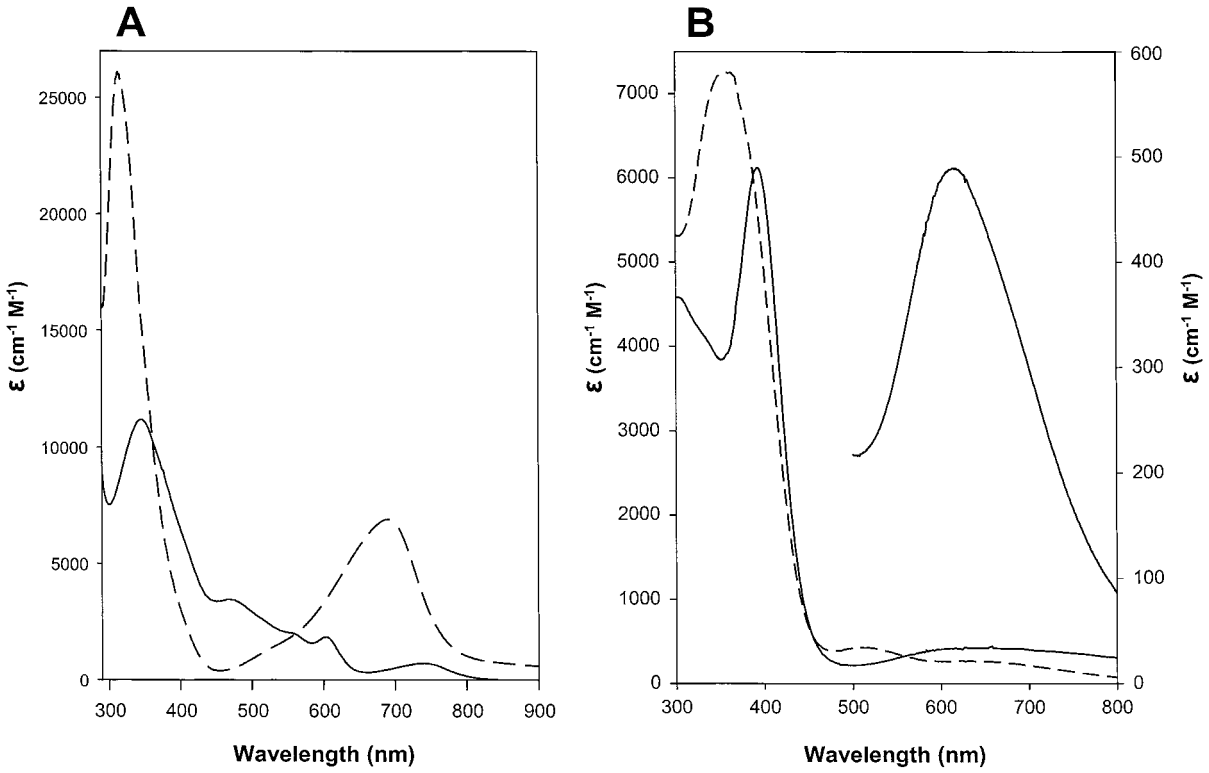


Fig. 4. A, Absorbance spectra of redox active complexes of Fe:$(pdtc)_2$. B: Absorbance spectra of Cu:pdtc. Right Y-axis is for high concentration absorbance spectra from 500 to 800 nm. Solid lines = oxidized states, dashed lines = reduced states.

Table 3. Iron reduction activity of strains measured after 6 days of heterogeneous growth. Values are the mean ± SD of triplicate cultures based on the absorbance of Fe(II):ferrozine at 556 nm.

Strain	[Fe(II)] (μM)
P. stutzeri strain KC	36.0 ± 5.93
P. putida DSM3601	20.8 ± 0.536
E. coli	29.9 ± 15.0
CTN1	10.2 ± 2.61
KC1896	6.16 ± 3.30
KC2753	5.87 ± 3.19
KC3164	6.41 ± 1.52

taking up 99.5 ± 3.1% of the supplied iron over a 20-min incubation period. Mean and standard errors for iron uptake by unwashed cells were calculated by combining all time points for each condition since the uptake efficiencies appeared constant throughout the experiment.

Iron reduction

Since the iron uptake results suggested that pdtc did not function as a stand-alone siderophore but might increase the efficiency of iron acquisition through some other process, we decided to test pdtc for iron reducing activity. We began by testing a heterogeneous collection of aerobically-grown bacteria strains for the ability to reduce iron using ferrozine as a trap for Fe(II). *E. coli*, which produces an iron reductase, was used as a positive control (Vartivarian & Cowart 1999). Both *P. putida* DSM3601 and *P. stutzeri* strain KC exhibited levels of iron reducing activity comparable to *E. coli*, while the $pdtc^-$ mutants CTN1, KC1896, KC2753, and KC3164 show significantly less activity (Table 3). Three other isolates of *P. stutzeri*, ATCC 11607, 14405, and 17588 showed no appreciable activity (data not shown). Since these results suggested that pdtc was the agent responsible for iron reduction, abiotic experiments were carried out using synthetic pdtc. Pdtc was shown to reduce carbon tetrachloride only when complexed with copper, but this was only clearly shown when conditions

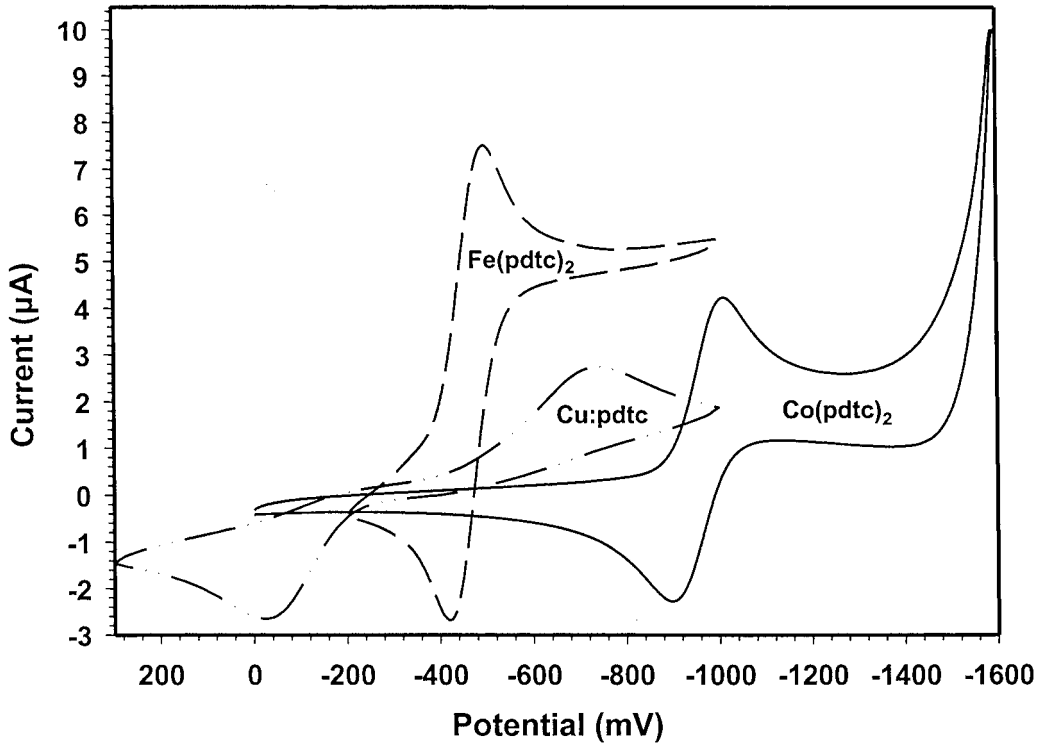


Fig. 5. Cyclic voltammograms for Co:(pdtc)$_2$ (solid line), Cu:pdtc (dash-dot line), and Fe:(pdtc)$_2$ (dashed line) complexes. Synthetic crystalline preparations of complexes were prepared as described in the text.

were carefully controlled to exclude extraneous metals (Lewis *et al.* 2001). We duplicated these conditions in our experiments to determine if copper was a specific requirement for pdtc-mediated iron reduction. Both sodium dithionite and Cu:pdtc reduced Fe(III) oxyhydroxide to a similar extent, but no iron reduction was seen in the pdtc only, Cu only, or media only controls (Table 4). The iron reduction by sodium dithionite was immediate, but the Cu:pdtc reaction was extremely slow (15 nM Fe(II) h^{-1}), taking 8 days to reduce a similar amount of iron.

Table 4. Abiotic Fe(III) oxyhydroxide reduction in 0.5× SM after 8 days of incubation at room temperature. All reactions contained 1 g ferrozine and 10 mg amorphous Fe(III) oxyhyroxide per l. Values are the mean ± SD of triplicate reactions based on the absorbance of Fe(II):ferrozine at 556 nm.

Reaction Formulation			[Fe(II)]
[pdtc] (μM)	[Cu] (μM)	[dithionite] (μM)	produced (μM)
0	0	0	0
0	0	500	3.96 ± 1.13
0	250	0	0
250	0	0	0
250	250	0	2.89 ± 0.72

Toxicy testing

Nineteen metals and 2 metalloids were tested alone as well as combined with pdtc to determine the affect of pdtc on the toxicity of the metals towards 15 different bacterial strains and one yeast strain (Table 1). No growth inhibition was seen around the DMF control disks for any strain. The following strains were inhibited by 15 mM pdtc in DMF (inhibition zone diameter – disk diameter): *S. epidermididis* (8.67 ± 1.26 mm), *S. aureus* (7.00 ± 0.75 mm), *Arthrobacter* (6.83 ± 0.95 mm), *B. subtillus* (7.83 ± 1.04 mm), and *E. coli* (5.83 ± 1.04 mm). The combination of pdtc with a particular metal could result in three different outcomes: an increase, a decrease, or no change in toxicity when compared to the metal alone. Addition of pdtc to Al, As, Cu, Ga, Mo, Nd, Pb, Sc, Se, Sr, or Te resulted in increased toxicity towards some strains (Table 5). Most strains showed a dramatic decrease in sensitiv-

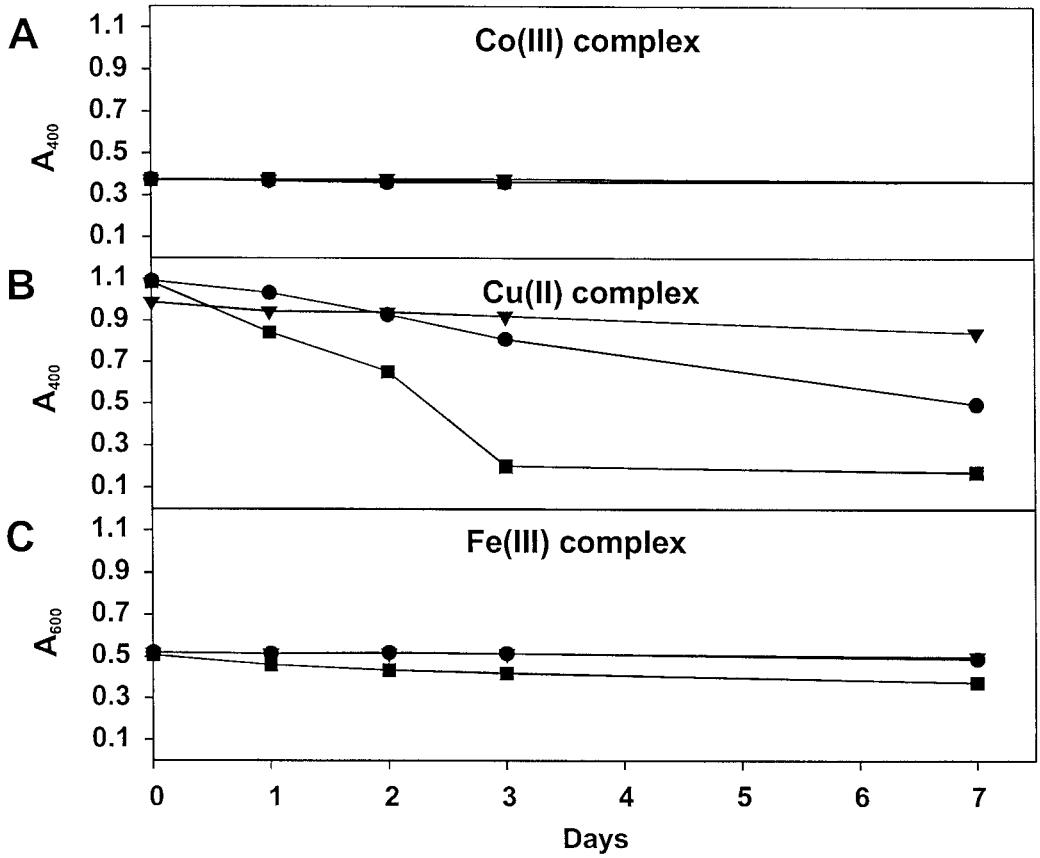


Fig. 6. Stability of redox-active pdtc metal complexes over time at different pH-values. Symbols: (●), pH 3; (▼), pH 7; (■), pH 10. A) Co(III):$(pdtc)_2$ complex, B) Fe(III):$(pdtc)_2$ complex, C) Cu(II):pdtc:Cl complex. The day 14 time point for the Co complex is not shown.

ity to Cd and Hg when pdtc was present compared to the metals alone. Three strains also showed decreased sensitivity to Te in the presence of pdtc. There was no significant difference in the toxicity of Co, Cr, Cs, Fe, Mn, Ni, Pd, or Zn for any strain in the presence or absence of pdtc (data not shown).

Discussion

Pdtc is a promiscuous metal chelator. Of the 22 metals and metalloids tested, 14 formed complexes with pdtc that could be detected by electrospray MS. Addition of pdtc to solutions of As, Hg, and Se resulted in the formation of precipitants, indicating that a reaction had taken place, but the structures for pdtc complexes with these elements were not detected. Precipitation of metals by addition of pdtc may be due to the formation of an insoluble uncharged complex between the metal ion and pdtc or the formation of an insoluble metal sulfide. The nature of these precipitants is currently under investigation. Pdtc formed complexes with both hard and soft metals. While larger molecules can have a greater number of binding atoms that can serve to increase the specificity of chelation, small molecules such as pdtc are intrinsically restricted by their size to the number of options available to increase the specificity of ligand binding. Pdtc's unique structure contributes to its relative nonspecificity for ligands. Sulfur and nitrogen, both soft bases, are more specific for metals with soft character. So, by virtue of its binding atoms, pdtc has a natural affinity for soft, easily polarizable metals like, Au(I), Cd(II), Cu(I), Hg(I), Hg(II), and Pd(II). However, the proximity and orientation of pdtc's two sulfur and single nitrogen binding atoms, which essentially form a tridentate 'binding pocket', evidently increases the binding affinity of this molecule for hard metals like Bi(III), Co(II), Co(III), Cr(III), Cu(II), Fe(II), Fe(II), Ni(II), Pb(II), and Zn(II) over that which would be expected for independent S and N binding atoms. The abilities of pdtc

Table 5. Increase (+ values) or decrease (− values) in sensitivity of various cells to 15 mM metal:pdtc complexes when compared to 15 mM metal alone. Values are the average inhibition zone diameter of three replicates minus the larger of the disk diameter or the inhibition zone of the solvent blank. Values in parentheses are SD. Differences less than 3 mm are not reported.

	Differences between metal and complex inhibition zone diameters (mm)												
Metal:	Al	As	Cd	Cu	Ga	Hg	Mo	Nd	Pb	Sc	Se	Sr	Te
Stoichiometry:	2:1	1:1	1:1	1:1	1:1	1:1	2:1	2:1	1:1	2:1	1:1	1:1	2:1
Pdtc producers													
P. stutzeri strain KC			−17.0			−27.7							
			(0.6)			(1.7)							
CTN1			−15.0			−19.5		3.3		4.7			
			(1.3)			(0.8)		(0.1)		(0.9)			
P. putida DSM3601						−22.2							
						(1.0)							
Gram positives													
S. epidermidis						−9.8							
						(0.6)							
S. aureus		4.5	−28.2			−20.0			17.5			7.0	−14.2
		(0.1)	(0.6)			(1.4)			(2.2)			(2.3)	(1.7)
Arthrobacter			−19.5			−13.3							−8.8
			(0.1)			(0.1)							(2.9)
Bacillus cereus				7.3		−10.0							
				(0.4)		(1.6)							
Bacillus subtillus	9.7	10.5	−22.3		7.9	−10.8	8.8		7.9			12.7	−9.2
	(1.9)	(1.3)	(2.0)		(1.5)	(1.7)	(3.5)		(2.1)			(2.3)	(2.3)
Gram negatives													
E. coli						−11.8		4.5					−12.2
						(0.3)		(1.2)					(1.7)
P. putida mt-2						−18.3		5.6		5.4			
						(0.3)		(1.4)		(0.5)			
P. aeruginosa PAO1						−22.1		6.8					
						(1.8)		(1.8)					
P. fluorescens F113						−22.6							
						(0.5)							
P. stutzeri 11607			−14.9		5.8	−18.4					4.0		
			(0.1)		(0.6)	(1.7)					(0.1)		
P. stutzeri 14405						−23.7		52				7.5	
						(0.8)		(0.3)				(1.9)	
P. stutzeri 17588			−6.4							7.1			
			(1.5)							(0.4)			
Eucaryotic													
Candida albicans	7.8		−14.2		12.8			6.3	5.7		3.7	10.8	
	(0.3)		(0.3)		(1.6)			(1.9)	(1.0)		(0.3)	(0.8)	

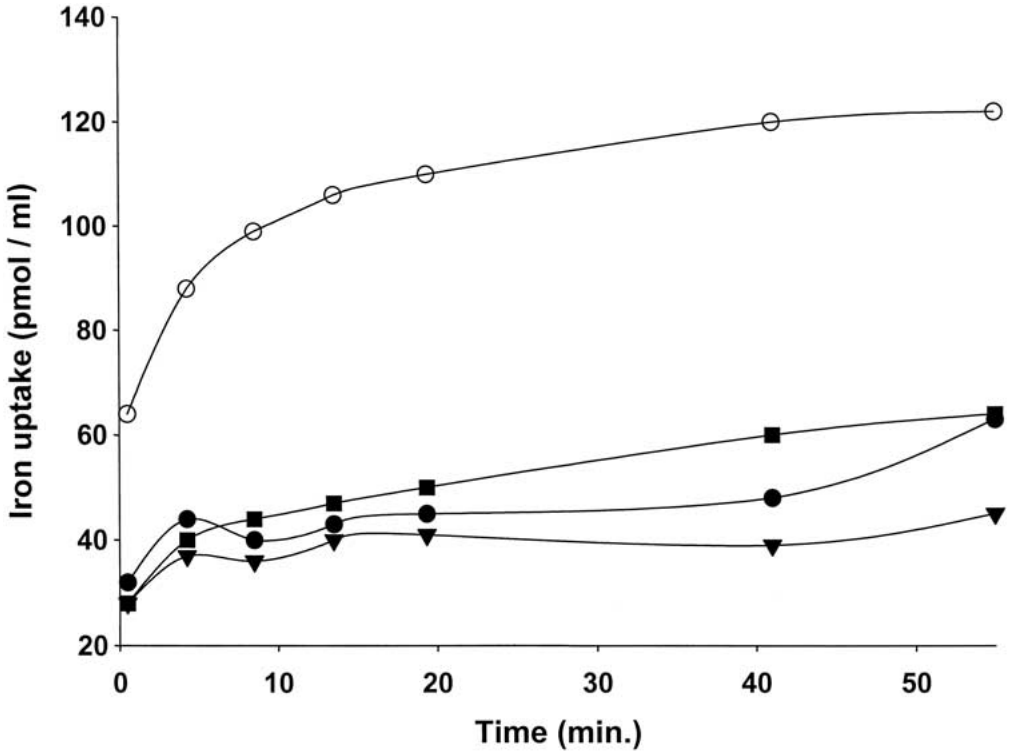


Fig. 7. Uptake of ^{59}Fe(III):(pdtc)$_2$ into washed *P. stutzeri* strain KC cells. Symbols: (○), pdtc-producing cells incubated with $^{59}FeCl_3$; (●), pdtc-producing cells incubated with ^{59}Fe(III):(pdtc)$_2$; (■), cells repressed for pdtc production by growth in medium supplemented with 200 μM ferric citrate incubated with ^{59}Fe(III):(pdtc)$_2$; (▼), killed pdtc-producing cells incubated with ^{59}Fe(III):(pdtc)$_2$. Reactions contained 186 pmol of Fe(III) per ml.

to form complexes in 1:1 or 2:1 stoichiometric ratios and to use either S or O as a binding atom (Neu *et al.* 2001) further increase the flexibility of pdtc's binding and expands the range of metals with which pdtc can form complexes. These structural features combine to form a small molecule with broad specificity but high affinity for metals.

Comparison of the list of metals and metalloids that form soluble complexes with pdtc (Au, Bi, Co, Cr, Cu, Fe, Mn, Nd, Ni, Pb, Pd, and Zn) and those that pdtc causes to precipitate from solution (As, Cd, Hg, Pb, Se, Sn, Te, Tl) reveals an interesting relationship. In general, pdtc forms soluble complexes with essential metals, but causes toxic metals and metalloids to be precipitated from solution. This suggests that complexation with pdtc could facilitate the uptake of essential metals into the cells of pdtc producers, as has been suggested for pyochelin (Visca *et al.* 1992), but this has not been investigated for any metal except iron. The precipitation of As, Cd, Hg, Pb, Se, and Te from solution with the addition of pdtc results in decreased availability of these metals and could therefore be expected to protect living cells from their toxic effects. This hypothesis is supported by our toxicity results for Cd, Hg, and Te where 8, 14, and 4 out of 16 strains respectively were protected from the toxic effects of these metals by pdtc. The fact that pdtc causes the precipitation of toxic elements may prove beneficial to pdtc-producing bacteria by detoxifying the local environment. Regardless of whether such 'environmental conditioning' is a true physiological function of pdtc or simply an artifact, the consequence is a benefit and would therefore serve as a selection criteria for pdtc production in metal contaminated environments. The fact that pdtc forms insoluble complexes with many toxic elements could be applied to bioremediation of aqueous solutions or to immobilize these elements *in situ* when it is desired to stop their migration in aqueous environments (Gadd 2000).

S. epidermididis, *S. aureus*, *Arthrobacter* sp., *B. subtillus*, and *E. coli* were found to be sensitive to pdtc alone. The mechanism of this toxicity was not investigated, but given the affinity of pdtc towards a wide range of metals, one or more metal:pdtc

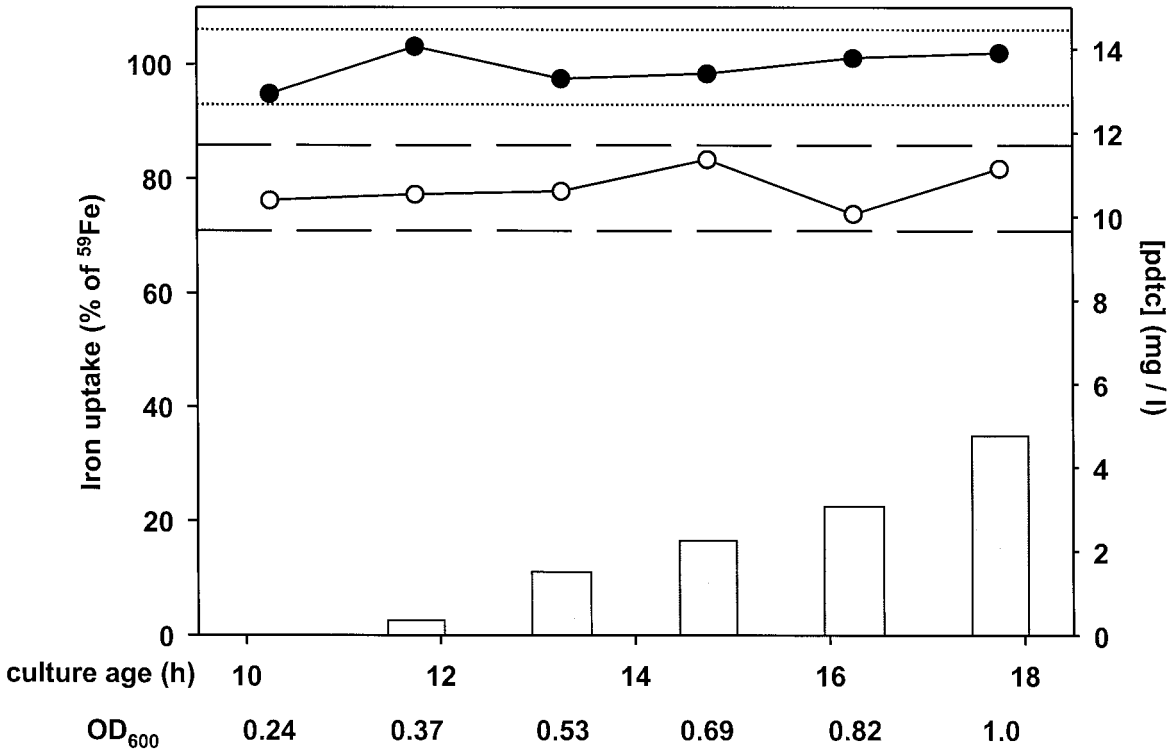


Fig. 8. Uptake of $^{59}Fe(III):(pdtc)_2$ into unwashed *P. stutzeri* strain KC cells. One ml aliquots of an aerobic shaken culture were spiked with either $^{59}Fe(III):(pdtc)_2$ or $^{59}FeCl_3$ and assayed for iron uptake after 20 min of incubation. Optical density (2^{nd} X axis) and pdtc production (right Y-axis) were determined before addition of radioactive iron. Dotted and dashed lines represent 2 sigma values for averaged $^{59}Fe(III):(pdtc)_2$ or $^{59}FeCl_3$ values, respectively. Reactions contained 186 pmol of Fe(III) per ml. Symbols: (○), incubated with $^{59}FeCl_3$; (●) incubated with $^{59}Fe(III):(pdtc)_2$

complexes may be a factor. Alternatively, the high formation constants for metal:pdtc complexes could result in reduced availability of essential metals to those organisms that do not possess metal acquisition systems that can compete with pdtc. Antimicrobial activity exhibited by pseudomonads is thought to be due, in part, to iron chelation by siderophores (Vachee *et al.* 1997). Antimicrobial activity was also observed in tests of metal:pdtc complexes. Al, As, Cu, Ga, Mo, Nd, Pb, Sc, Se, and Sr were found to be more toxic to some cells in the presence of pdtc than the equivalent concentration of metal. However, no clear trend was evident, and this activity was not investigated further.

When pdtc undergoes hydrolysis, first one and then the second sulfur atoms are replaced by oxygen (Figure 1). This degradation reaction releases H_2S and ultimately leads to the formation of dipicolinic acid (dpa). H_2S can be detected when vials of crystalline or highly concentrated solutions of pdtc that have not been purged with nitrogen are opened (Hübner *et al.* 1990). We found that Bi, Cr, and Pb promote this sulfur-to-oxygen substitution, as observed by ES/MS. We did not investigate the fate of the sulfur atoms lost in the reactions with these metals, but it is possible that the sulfur atom may be protonated and remain as H_2S as mentioned above, or it may form a metal sulfide with free metal ions. This degradation of pdtc by certain metals and subsequent formation of H_2S may be linked to the precipitation of As, Cd, Hg, Pb, Se, Sn, and Tl. Although we did not characterize the precipitated product of these reactions, it is possible that the H_2S released by the hydrolysis of pdtc could combine with metals in solution and result in the formation of insoluble sulfides. Thermal decomposition of Cd:thiocarboxylate complexes at temperatures as low as 25 °C has been shown to result in the formation of CdS (Nyman *et al.* 1997). Our stability studies (Figure 5B) showed that the copper complex was unstable at high and low pH values. The nature of this degradation was undetermined but could be the result of hydrolysis, loss of carbonyl sulfide, or a combination of both (Figure 1B).

When metal-metal competition studies were performed with pdtc free acid, the complexes with the highest formation constants were found to be the Co, Cu, Fe, and Ni complexes (Stolworthy *et al.* 2001). Our stability studies, which showed that solutions of Co(II):$(pdtc)_2$, Cu(II):pdtc:Cl, and Fe(III):$(pdtc)_2$ were stable at neutral pH, are consistent with this finding. Interestingly, the four metals for which pdtc has the highest affinity also form redox-active complexes with pdtc. The redox cycling capabilities of Fe:$(pdtc)_2$ and Ni:$(pdtc)_2$ have been previously reported (Ockels *et al.* 1978; Hildebrand *et al.* 1984; Krüger & Holm 1990). It is doubtful that Co:$(pdtc)_2$, with its high redox potential (Figure 5), serves any biological function, but our addition of Cu:pdtc to the list of redox-active pdtc complexes is significant in the fact that it is a 1:1 complex, unlike the other three redox-capable complexes that form 2:1 complexes (Figure 2, Table 2). Because of this open configuration, which is accentuated by the loss of a ligand in the reduced form, the Cu electron orbitals are exposed and are available to participate in reactions and may contribute to the reactivity of this complex. The metal atoms are more shielded in 2:1 complexes of pdtc. Cu:pdtc was previously shown to be essential for pdtc-mediated CCl_4 degradation (Lewis *et al.* 2001), and we have shown that this complex is capable of reducing iron as well.

Pdtc shares many traits with siderophores: its production is regulated by iron, it has a high affinity for ferric iron, its ferric complex has a reduction potential of about −400 mV, it promotes the growth of pdtc-producing pseudomonads (Sebat *et al.* 2001), and it is excreted from cells that produce it. *P. stutzeri* strain KC produces siderophores other than pdtc, as evidenced by positive results obtained from chromeazurol assays of $pdtc^-$ mutants that are also able to grow in iron limited media (data not shown). Additionally, hydroxamate- and catecholate-containing siderophores have been detected in supernatants of iron-limited cultures of *P. stutzeri* strain KC (Dybas *et al.* 1995). Only three organisms to date have been shown to produce pdtc, suggesting that pdtc production is relatively rare. While our results show that pdtc is not a stand-alone siderophore, it may enhance iron uptake in conjunction with a specific siderophore (Dybas *et al.* 1995) or membrane-based iron uptake system. The results of our iron uptake studies support the hypothesis that pdtc interacts with some other secreted factor to increase iron uptake efficiency. Uptake efficiency for both $^{59}Fe(III)$:$(pdtc)_2$ and $^{59}FeCl_3$ by unwashed cells remained constant at apparent saturation levels over a wide range of cell densities and pdtc production levels (Figure 8). This pdtc-mediated increase in iron uptake efficiency may work in conjunction with another system, perhaps a ferrous iron uptake system. The main difference between the three recognized modes of iron uptake from siderophores, the shuttle, the direct shuttle, and the indirect shuttle (Crowley *et al.* 1991), is where the reduction (and therefore release) of iron takes place. However, in each of the three models, this location is cell-associated. Since pdtc enhanced iron uptake only in unwashed cells when other secreted species such as siderophores and reductases could be present, we suggest that pdtc works in conjunction with one or more of these molecules or could represent a new model. However, iron uptake with a pdtc-mediated reductive step would require three bi-molecular reactions (as opposed to the two bi-molecular reactions required for iron acquisition from a stand-alone siderophore) and would appear to be an inefficient means of obtaining iron. The existence of this mechanistically inefficient iron uptake system seems inconsistent with the efficiency expected of natural biological systems. It may be that pdtc is utilized only in confined spaces such as soil or mineral pores where it cannot defuse away faster than it can be recycled. On the other hand, perhaps pdtc's contribution to iron acquisition is fortuitous and is not its primary function.

Given that Cu:pdtc is capable of redox cycling and has been shown to reduce carbon tetrachloride, we predicted that Cu:pdtc would be able to reduce iron and that this activity may be its natural physiological function. Our findings show that pdtc can mediate the reduction of iron, but only in the presence of copper. *P. stutzeri* strain KC and *P. putida* DSM3601 reduced amorphous Fe(III) oxyhydroxide, but $pdtc^-$ derivatives of strain KC did not. *In vitro* experiments showed that copper and pdtc were both required for Fe reduction. Given the high formation constant of Cu(II):pdtc:Cl, the active species was most likely this redox-active complex. Fe(III):$(pdtc)_2$ was probably present in these reactions as well, again because of its high formation constant, and may play a role in pdtc-mediated iron reduction. However, the source of electrons required for reductive activity could not be accounted for except from H_2S that could be generated from the hydrolysis of pdtc. Knowing that pdtc hydrolyzed with the production of H_2S when exposed to air (Figure 1) (Hübner *et al.* 1990), we hypothesize that oxidation of this H_2S provided the electrons for the Cu:pdtc-mediated iron reduction reaction. Since

H_2S can reduce Fe(III) directly, the rate of reductive activity should depend upon the rate of pdtc hydrolysis. However, the precise role that copper or the redox cycling of the copper and/or iron complex(es) play in this reaction is unknown. Paradoxically, iron reduction was not detected in any pdtc-only reaction although H_2S is generated as pdtc degrades. Copper may accelerate the hydrolysis of free pdtc, or Cu:pdtc itself may be more prone to hydrolysis under certain conditions.

Iron reducing secondary metabolites are rare. Literature references exist for pyocyanin and an iron reducing, protease-resistant, low molecular weight factor identified from *Histoplasma capsulatum* culture supernatant (Timmerman & Woods 1999). This organism was also found to produce and secrete a ferric reductase. Many species of bacteria also produce ferric reductases. These reductases are usually membrane or periplasmic in location and function to release iron bound to siderophores in order to facilitate uptake into the cell (Fontecave 1994). The inability of our $pdtc^-$ mutants to reduce amorphous Fe(III) oxyhydroxide above background levels suggests that strain KC produces only one ferric reductive activity, that mediated by pdtc. It is unknown whether this reductive activity works in conjunction with siderophores. Whether iron reduction, siderophore dependent or not, is the primary physiological role for pdtc and pyocyanin remains a question for future investigations. These extracellular redox systems could have activity towards additional substrates, including a target or targets that constitute the natural function of pdtc for the pseudomonads that produce it.

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