

Pyridine-2,6-bis(thiocarboxylic acid) produced by *Pseudomonas stutzeri* KC reduces chromium(VI) and precipitates mercury, cadmium, lead and arsenic

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Abstract

Interactions of the *Pseudomonas stutzeri* KC siderophore pyridine-2,6-bis(thiocarboxylic acid) (pdtc) with chromium(VI), mercury(II), cadmium(II), lead(II), and arsenic(III) are described. Pdtc was found to reduce Cr(VI) to Cr(III) in both bacterial cultures and in abiotic reactions with chemically synthesized pdtc. Cr(III) subsequently formed complexes with pdtc and pdtc hydrolysis products, and their presence was confirmed using electrospray ionization-mass spectrometry (ESI-MS). Cr(III):pdtc complexes were found to slowly release Cr(III) as chromium sulfide and possibly Cr(III) oxides. Pdtc also formed poorly soluble complexes with Hg, Cd, Pb, and As(III). Hydrolysis of those complexes led to the formation of their respective metal sulfides as confirmed by energy dispersive X-ray spectroscopy (EDS) elemental analysis. The pdtc-producing strain *P. stutzeri* KC showed higher tolerance to most of these metals as compared to a pdtc-negative mutant. A novel role of pdtc is postulated as its involvement in providing an extracellular pool of thiols that are used for redox processes in detoxification of the bacterial extracellular environment. These redox processes can be mediated by transition metal:pdtc complexes.

Introduction

Microbial metabolism can substantially influence metal speciation and thus metal mobility and toxicity. Microbially mediated processes affecting metal mobility are of great interest as they are a crucial component of biogeochemical cycles and are of importance to the field of environmental biotechnology (Diels *et al.* 2002; Valls & de Lorenzo 2002; Gadd 2004). For example, in aerobic environments bacterial siderophores greatly increase metal mobility. Siderophores are small organic molecules with strong affinity for ferric iron and are primarily produced to mediate iron acquisition. Siderophores are able to bind other metals, (Visca *et al.* 1992; Hu & Boyer 1996) and in some cases protect against metal toxicity (Fekete & Barton 1992; Cortese *et al.* 2002). Pyridine-

2,6-bis(thiocarboxylic acid) (pdtc) was identified as a siderophore having such multiple properties and also being capable of mediating carbon tetrachloride degradation (Lee *et al.* 1999). Pdtc is produced by *Pseudomonas putida* DSM 3601 and 3602 (Ockels *et al.* 1978) and *Pseudomonas stutzeri* KC (Lee *et al.* 1999); it was recently shown to promote iron transport into the bacterial cell (Lewis *et al.* 2004). Pdtc forms soluble complexes with iron and many other transition metals as well as with some heavy metals, lanthanides, and actinides. Complexes with Fe(III), Co(III), and Cu(II) have very high stability constants ($\log K \sim 33\text{--}34$) (Stolworthy *et al.* 2001), and complexes with iron, nickel, copper, and cobalt have the ability to redox cycle between oxidation states of bound metals (Cortese *et al.* 2002). Many heavy metals and metalloids have been found to form insoluble precipitates

with pdtc. Additionally, pdtc was effective in protecting some bacteria against metal toxicity (Stolworthy *et al.* 2001; Cortese *et al.* 2002) and was also shown to have antimicrobial properties (Sebat *et al.* 2001). The mechanism of pdtc interactions with toxic selenite and tellurite was recently elucidated, and pdtc was found to protect *P. stutzeri* KC against metalloid toxicity (Zawadzka *et al.* 2006). The properties of pdtc make it a good candidate for heavy metal remediation by metal reduction and complexation, selective precipitation of heavy metals from solution, or solubilization in the case of micronutrient metals. Pdtc also offers potential for use in remediation of metal and mixed metal-carbon tetrachloride contaminated wastes.

Toxic heavy metal contamination in the environment is a result of natural, industrial, and agricultural processes, and microorganisms inhabiting these contaminated environments utilize various mechanisms to reduce or eliminate metal toxicity. Chromium, mercury, cadmium, lead, and arsenic are among the most common toxic metal contaminants. Chromium is a widespread pollutant released mainly during industrial processing. The most common forms of chromium are hexavalent Cr(VI) occurring as chromate (CrO_4^{2-}) and dichromate ($\text{Cr}_2\text{O}_7^{2-}$) and trivalent Cr(III) species. Cr(VI) is the dominant form in aerobic conditions and is considered more toxic and more mobile than Cr(III). Cr(VI) can be reduced to Cr(III) by soil organic matter, sulfides, or ferrous iron under anaerobic conditions. At low pH, Cr(III) is bound to organic matter, clays, or oxide minerals; above pH 5, it is of low solubility due to the formation of insoluble Cr(III) hydrous oxides (Evanko & Dzombak 1997, Cervantes *et al.* 2001). Bacterial tolerance to chromium is thought to be associated mainly with chromate efflux systems (CHR protein family) (Nies *et al.* 1998; Nies 2003; Aguilera *et al.* 2004) or chromate reduction mechanisms (Cervantes *et al.* 2001; Lloyd 2003). Chromate reduction by several bacteria has been reported, with sulfate-reducing bacteria (SRB) being one of the most studied groups (Smith & Gadd 2000; Lloyd *et al.* 2001). Reduction of Cr(VI) by SRB was shown to be catalyzed by cytochrome *c* (Lovley & Phillips 1994) and by H_2S produced by the bacteria (Chardin *et al.* 2003). Mercury and cadmium are more toxic than other heavy metals to microorganisms due to their

strong affinity for thiol groups (solubility product (K_{sp}) of HgS is 6.38×10^{-53} ; K_{sp} of CdS is 1.4×10^{-29}). Mercury genetic resistance determinants (*mer*) that are widespread among microorganisms and living cells are able to reduce Hg(II) to elemental mercury, which can leave the cell by passive diffusion and volatilization. Under anaerobic conditions, higher pH values favor precipitation of HgS, whereas acidic conditions favor reduction or alkylation of Hg(II). No clear Cd toxicity mechanism has been elucidated. Microbial resistance to Cd is based on efflux mechanisms and in the case of cyanobacteria, production of metallothioneins. Under sulfidogenic conditions, cadmium precipitates as CdS (Evanko & Dzombak 1997, Nies 1999). Lead, on the other hand, is not considered very toxic to microorganisms due to its low solubility and thus low bioavailability (K_{sp} of PbPO_4 is 10^{-54}). Most lead released into the environment is retained in the soil and forms insoluble precipitates with carbonate, phosphate, sulfate, chloride, and sulfide anions or precipitates as oxides or hydroxides (Evanko & Dzombak 1997, Nies 1999). Finally, arsenic, a heavy metalloid, exists in several oxidation states. The dominant form in aerobic environments is moderately toxic As(V); under reducing conditions, highly toxic As(III) is found in the form of arsenite (AsO_3^{3-}). Most arsenic compounds sorb strongly to soils or coprecipitate with hydrous iron oxides (Evanko & Dzombak 1997, Rosen 2002). As(III) enters cells via aquaglyceroporins (Mukhopadhyay *et al.* 2002), while As(V) is transported into cells through phosphate transporters and after being reduced to arsenite, is either extruded from cells or sequestered as free arsenite or as a conjugate with glutathione or other thiols (Nies 1999; Rosen 2002; Oremland & Stolz 2003). Some microorganisms are capable of supporting growth through dissimilatory arsenate reduction that contributes to increased arsenic mobility (Lloyd 2003). In the case of *Desulfotomaculum auripigmentum*, respiratory arsenate reduction was linked to sulfate reduction and precipitation of As_2S_3 (orpiment) resulting in biomineralization of arsenic (Newman *et al.* 1997).

Work described here was focused on pdtc interactions with chromium(VI), mercury(II), cadmium(II), lead(II), and arsenic(III) and on the nature of the precipitates formed with these metals. We describe the ability of chemically synthesized pdtc and pdtc produced by *P. stutzeri* KC to

reduce toxicity of dichromate by reducing Cr(VI) to Cr(III) and subsequently chelating the Cr(III). We also describe the compositions of heavy metal and metalloid insoluble precipitates formed by pdtc. Additionally, we included in our studies desferrioxamine siderophores, also referred to as proferrioxamines (Feistner 1995) that are produced by *P. stutzeri* KC in addition to pdtc (Zawadzka, unpublished data). Since desferrioxamines are known to chelate and solubilize several metals (Winkelmann 1991), we tested their influence on pdtc activities. To our knowledge, the reported work constitutes the first documentation of bacterial use of a siderophore (pdtc) to carry thiol groups for use as extracellular reducing agents. The most unique observation resulting from the research was that of bacteria using pdtc to form complexes with heavy metals, which were then hydrolyzed leading to heavy metal precipitation in insoluble sulfide forms. The data reported here help make our understanding of the biological role of pdtc and its mechanisms of interactions with metals more complete.

Materials and methods

Bacterial strains and culture conditions

The strains used in this study included *P. stutzeri* strain KC (wild type, aquifer isolate; Criddle *et al.* 1990) and strain CTN1 (spontaneous mutant of strain KC; Lee *et al.* 1999). All strains were maintained on tryptic soy agar plates (Difco). Media were prepared with deionized water purified to $< 18 \text{ m}\Omega$ resistivity using a WATER PROTM PS filtration unit (Labconco). Bacteria were grown in iron-depleted acetate medium (DRM) (Lee *et al.* 1999) for 48–96 h at 30 °C with constant shaking at 150 rpm.

Growth in the presence of Cr(VI)

The influence of dichromate on growth of strain KC and CTN1 cultures was tested in iron-depleted DRM medium. Fresh media (10 ml) were inoculated (0.1% v/v) from overnight iron-depleted cultures, and 200 μl per well were transferred to 96-well microtiter plates. Dichromate was added from the filter-sterilized stock solution (1 mM) to final concentrations of 5, 10, 25, and 50 μM in triplicate.

The plates were covered with breathable membranes (Sigma) and incubated for 48 h at 30 °C with constant shaking at 150 rpm. The optical density at 600 nm (OD_{600}) was measured automatically using a microtiter plate reader (PowerWave EL800, Bio-Tek) every 15 min.

Cr(VI) reduction during bacterial growth

Triplicate cultures (50 ml) were inoculated from aerobically grown overnight cultures (0.1% v/v), and dichromate was added from a filter-sterilized stock solution (100 mM) to final concentrations of 10 and 25 μM . Bacteria were grown for 96 h at 30 °C with constant shaking at 150 rpm. Samples (2 ml) were taken periodically to determine optical density, remaining Cr(VI) concentration, and siderophore concentration. The samples were centrifuged ($13,000 \times g$ for 10 min) to remove the cells, and Cr(VI) and siderophore concentration were determined in culture supernatants. Sterile controls and controls without added dichromate were also prepared and monitored for Cr(VI) concentration.

Cr(VI) determination

The method used for Cr(VI) measurement was adopted and modified from standard methodology for hexavalent chromium determination in water, wastewater, and solid waste extracts (Dionex Corporation 1996). The postcolumn reagent consisted of 2.0 mM diphenylcarbazide, 10% methanol, and 1.0 N sulfuric acid (Dionex Corporation 2000). The reagent forms a pink color complex with Cr(VI), which was detected by measuring absorbance at 530 nm. The reagent (50 μl) was added to samples (150 μl) in a 96-well microtiter plate and incubated for 10 min at room temperature to allow for color development prior to absorbance measurement using a microtiter plate reader (PowerWave EL800, Bio-Tek). A standard solution of potassium dichromate was prepared by appropriate dilutions from a 0.1 M stock solution of $\text{K}_2\text{Cr}_2\text{O}_7$ in water.

Determination of siderophore concentration during bacterial growth

Siderophore concentration during bacterial growth was determined spectrophotometrically for

ferrioxamine and pdtc-ferrous iron complexes. To measure ferrioxamine concentration, freshly prepared FeCl_3 solution in water was added to 950 μl of culture supernatant to a final iron concentration of 5×10^{-4} M. The mixture was incubated for 1 h and the absorbance measured at 430 nm. Ferrioxamine concentration was calculated using the molar absorption coefficient $\varepsilon_{430} = 2720 \text{ cm}^{-1} \text{ M}^{-1}$, typical of a trihydroxamate ferric complex (Winkelman 1991; Hou *et al.* 1998). Pdtc concentration was assayed spectrophotometrically as described previously (Lewis *et al.* 2000). The concentration of Fe(II):(pdtc)_2 complex was calculated using the molar extinction coefficient $\varepsilon_{687} = 8435 \text{ cm}^{-1} \text{ M}^{-1}$.

Cr(VI) reduction by two-day cultures and culture media

Two-day iron-depleted cultures of strains KC and CTN1 and culture filtrates were tested for dichromate-reducing activity. Bacteria were removed from culture samples by centrifugation at $6000 \times g$ for 30 min at 4 °C followed by filtration through 0.22- μm syringe filters. Aliquots (1 ml) of both the cultures containing bacterial cells and cell-free culture filtrates were incubated for 24 h at 25 °C with 10 or 25 μM dichromate added. Cells from the cultures were removed by centrifugation at $13,000 \times g$ for 10 min, and the remaining Cr(VI) concentration was assayed. Additionally, culture filtrates were separated into high- and low-molecular-weight fractions by size-exclusion chromatography using a Sephadex G-25 pre-packed PD-10 column (Amersham Biosciences). The low-molecular-weight fraction was assayed for dichromate reducing activity as described above.

Influence of pH, metals, and desferrioxamine B on Cr(VI) reduction by pdtc

Pdtc was chemically synthesized using 2,6-pyridinedicarbonyl dichloride (Sigma) as a starting compound (Hildebrand *et al.* 1983). Pdtc stock solution in dimethylformamide (DMF) was prepared fresh (10 mM). Desferrioxamine B (dFO B) as a mesylate salt (Sigma) was dissolved in water (10 mM). The 50 mM phosphate-citrate buffers containing 5% DMF at pH 7.0 and 8.0 were

prepared and treated with Chelex-100 to remove trace metals. Stock metal solutions of Fe(III) , Cu(II) , Zn(II) , Ni(II) , Co(III) , and Cr(III) were prepared in water (10 mM). Influence of metals was tested by adding metal solutions to the reaction mixtures (25 μM final concentration of each metal). The reaction mixtures in each buffer (1 ml) all contained 25 μM $\text{K}_2\text{Cr}_2\text{O}_7$; control reactions without and with metals were prepared along with controls containing dichromate and dFO B but no pdtc. Pdtc was added alone to a final concentration of 50 or 125 μM as well as with dFO B (50 or 125 μM final concentration) to a series of reaction mixtures with and without metals. Reaction mixtures were incubated at 25 °C for 30 days and periodically assayed for Cr(VI) concentration. Metal:pdtc complex speciation in the reaction mixtures was monitored using electrospray ionization-mass spectrometry (ESI-MS). A 25- μM dichromate concentration (corresponding to approximately 2.6 mg l^{-1} Cr(VI)) was chosen for the use in the *in vitro* experiments for study of Cr(VI) reduction by chemically synthesized siderophores. Dichromate concentrations of 25 μM and higher were shown to inhibit growth of *P. stutzeri* KC, and the concentration falls within the range encountered in contaminated soil and groundwater (0.1 to several thousand mg l^{-1} ; EPA 2000). Pdtc concentrations added to the reaction mixtures (50 and 125 μM) were chosen to mimic the constant siderophore supply produced in bacterial cultures and reflected the concentration range detected in *P. stutzeri* KC cultures that depended on culture media (28 μM in this study to over 90 μM (Zawadzka *et al.* 2006)).

Electrospray ionization-mass spectrometry (ESI-MS)

Samples were analyzed using negative and positive ESI-MS (Quattro II, Micromass Ltd.). Samples were delivered into the source at a flow rate of $5 \mu\text{l min}^{-1}$ using a syringe pump (Harvard Apparatus). A potential of 3.4 kV was applied to the electrospray needle, and the sample cone voltage was maintained at 15–20 V. Detector resolution was set at 15,000 with source temperature kept constant at 80 °C. All MS spectra were an average of 18 scans over a mass range of 20–1000 Da.

Minimal inhibitory concentration (MIC)

The MICs of mercury, lead, cadmium, and arsenic were determined on agar plates with DRM medium solidified with noble agar (Difco) prepared with the addition of increasing concentrations of metal salts (0.01–0.2 mM of $\text{Hg}(\text{NO}_3)_2$, 0.5–3.0 mM of $\text{Pb}(\text{NO}_3)_2$, 0.01–0.1 mM of $\text{Cd}(\text{NO}_3)_2$, and 0.5–3.0 mM of As_2O_3). Plates were inoculated with bacteria from overnight cultures with cell densities of about 10^7 ml^{-1} colony forming units. The MIC was defined as the metal concentration higher than the highest concentration that did not inhibit bacterial growth.

Metal precipitation by pdtc

A 100 mM stock solution of chemically synthesized pdtc in DMF was mixed with freshly prepared metal solutions in equimolar concentration (10 mM final concentration each) in 2 M Tris-HCl buffer at pH 7.8 treated with Chelex-100. The following metal salts dissolved in water were used: $\text{Hg}(\text{NO}_3)_2$, $\text{Pb}(\text{NO}_3)_2$, $\text{Cd}(\text{NO}_3)_2$, and As_2O_3 . Additionally, an aqueous solution of $\text{K}_2\text{Cr}_2\text{O}_7$ was mixed with pdtc (10 mM final concentration each). The precipitates were allowed to stand at room temperature for 1 wk and then washed several times with water, dried at 50 °C overnight, and analyzed using scanning electron microscopy-energy dispersive X-ray spectrometry (SEM-EDS). The combinations of 10 mM dFO B together with pdtc or dFO B alone were also tested as controls but only for the formation of the precipitates. The relatively high pdtc and metal concentrations used (10 mM) followed from the fact that milligram amounts of precipitates were needed for SEM-EDS elemental analysis. Additionally, for ESI-MS analysis of the initially formed metal:pdtc complexes, which are extremely insoluble, reactants were required in excess in order to have enough complex solubilized in DMF so that the complexes were detectable. The same experiments were completed for 1 mM concentrations of metals and pdtc (reported for chromium only; other data not shown). Metal concentrations used here have biological relevance only in the case of high natural background levels of Pb in soil (2–200 mg kg^{-1}) (NRC 1972) and also in case of areas highly contaminated by mining or smelting activities (Gustin *et al.* 1994; EPA 2000; IPCS 2001; Kim *et al.* 2003).

Scanning electron microscopy-energy dispersive X-ray spectrometry (SEM-EDS)

SEM-EDS was used for elemental analysis of metal precipitates formed by pdtc. Dry precipitates formed by pdtc were mounted on carbon tape. Samples were examined with a scanning electron microscope LEO Supra 35 VP FESEM (Zeiss) equipped with a Thermo Electron System Six EDS. Elemental ratios were calculated as the average of atom % of two to three sample measurements.

Results

Cr(VI) reduction monitored by ESI-MS

The reaction mixture containing pdtc and dichromate was analyzed by ESI-MS after a 1 h incubation period and compared to a solution containing pdtc and chromium (III) (Figure 1). In both samples, $[\text{Cr}(\text{III}):(\text{pdtc})_2]^{1-}$ complex peaks (m/z 445.8) were visible together with complexes of Cr(III) with pdtc hydrolysis products where sulfur atoms were substituted with oxygen. The presence of these complexes indicates dichromate-induced pdtc hydrolysis, pdtc-driven Cr(VI) reduction to Cr(III), and subsequent formation of Cr(III) complexes with pdtc and pdtc hydrolysis products. The products of pdtc hydrolysis, pyridine-2-carboxylic-6-thiocarboxylic acid (pctc) and dipicolinic acid (pyridine-2,6-bis(carboxylic acid), dpa), were also observed in ESI-MS spectra (Figure 1). When the same samples containing pdtc and dichromate were analyzed after 90 days, the peaks corresponding to Cr(III) complexes with pdtc, pctc, and dpa were still detectable; however, their abundance decreased significantly to 1%, 4.7%, 8.5%, and 3.5% of the initial peak intensity of m/z 445.8, m/z 429.8, m/z 413.7, and m/z 397.8, respectively.

Cr(VI) toxicity, reduction, and siderophore production during bacterial growth

Pseudomonas stutzeri KC and CTN1 were grown in iron-limited media supplemented with potassium dichromate (Figure 2A and B). Strain KC was able to grow in the presence of up to 25 μM Cr(VI). At this concentration, however, its growth was negatively affected as manifested by elonga-

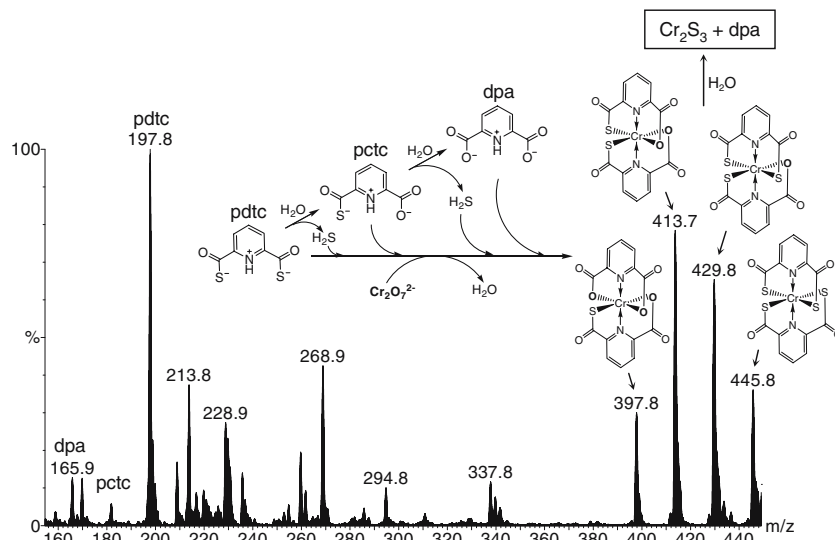


Figure 1. ESI-MS spectrum and structures of the Cr(III) complexes with pdtc and pdtc hydrolysis products pyridine-2-carboxylic-6-thiocarboxylic acid (pctc) and pyridine-2,6-bis(carboxylic acid) (dipicolinic acid, dpa) formed upon interaction of pdtc with Cr(VI). A schematic representation of pdtc-mediated Cr(IV) (as dichromate) reduction is also presented. The Cr(III) complexes with pyridine thiocarboxylic and carboxylic acids form a series of peaks differing by m/z 16 indicating stepwise substitution of sulfur with oxygen.

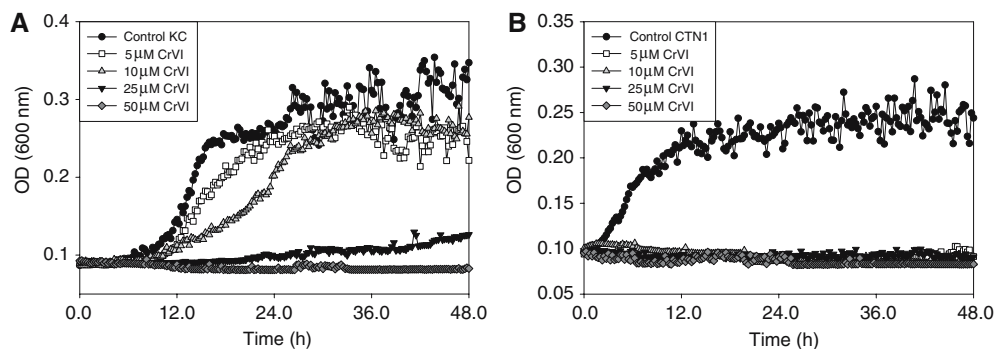


Figure 2. Growth curves of *P. stutzeri* (A) KC and (B) CTN1 cultured in iron-limited media with Cr(VI) added as potassium dichromate in microtiter plate format.

tion of the logarithmic growth phase and a lower final optical density than in control and 5 and 10 μM Cr(VI)-containing cultures. On the other hand, strain CTN1 that lacks the ability to produce pdtc was not able to grow at any dichromate concentration tested.

Cr(VI) reduction was monitored during bacterial growth of both strains in iron-restricted media in the presence of 10 and 25 μM Cr(VI) (Figure 3A and B). Only strain KC was able to reduce 10 and 25 μM Cr(VI) in the actively growing cultures, and its Cr(VI) reduction activity intensified after 24 h of growth. The lag time for the beginning of growth was longer when 25 μM Cr(VI)

was present. Pdtc (20 μM) could be detected in the culture media of dichromate-free controls after 24 h. The growth of strain KC coincided with the decrease in dichromate concentration that was reduced after 96 h of growth by 90% and 86% in 10 and 25 μM Cr(VI)-supplemented cultures, respectively. Strain CTN1 was not able to grow in 25 μM Cr(VI), and only modest growth occurred after 72 h in 10 μM Cr(VI), which was accompanied by a slight decrease in Cr(VI) concentration. Both strains produced desferrioxamine siderophores whose concentrations reached over 140 μM in both cultures after 24 h of growth; this concentration only slightly increased over 96 h. The

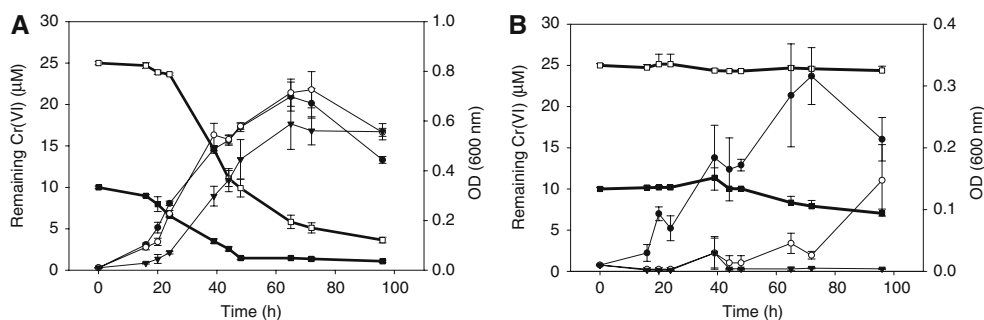


Figure 3. Growth of *P. stutzeri* A) KC and B) CTN1 in the absence and presence of dichromate (10 and 25 μM ; right axis) and Cr(VI) concentration remaining in the cultures. ● – growth control; ○ – growth with 10 μM Cr(VI); and ▼ – growth with 25 μM Cr(VI). ■ – 10 μM Cr(VI) initial concentration and □ – 25 μM Cr(VI) initial concentration.

reduction of Cr(VI) in sterile controls was negligible.

Cr(VI) reduction by two-day bacterial cultures

Cr(VI) reduction capabilities of 48 h strain KC and CTN1 cultures were compared. The low-molecular-weight fraction of the strain KC culture filtrate was responsible for the reductive activity (Table 1). The 48 h cultures had an optical density of about 1.0 at 600 nm and desferrioxamine contents of 170 μM (KC) or 145 μM (CTN1). Only strain KC produced pdtc (28 μM). The Cr(VI) reducing activity of the KC cultures was comparable to that of the culture filtrates, and about

70% of the dichromate was reduced in both 10 and 25 μM Cr(VI)-containing treatments. The low-molecular-weight fraction of the KC culture filtrate was able to reduce 30% of the dichromate, comparable to the culture filtrate diluted to the same fold. In the case of strain CTN1, some Cr(VI) reduction was observed: less than 10% in cultures with 10 μM Cr(VI) added and 20–30% in cultures and culture filtrates supplemented with 25 μM Cr(VI). The low-molecular-weight fraction showed no Cr(VI) reducing activity.

Influence of pH, metals, and desferrioxamine B on Cr(VI) reduction by pdtc

The reduction of 25 μM dichromate by synthetic pdtc was assayed at two physiologically relevant pH values (7.0 and 8.0). Additionally, the influence of a metal mixture in the solution and the presence of desferrioxamine B were examined (Table 2). At pH 7, spontaneous Cr(VI) reduction was observed in the control samples, but over 70% Cr(VI) remained after the 30 d incubation. In controls with dFO B added to dichromate, over 60% of the Cr(VI) remained; when the metal mixture was also present along with dFO B, 35% Cr(VI) remained. In the control reaction with metals added to dichromate, Cr(VI) was completely removed. At pH 7, pdtc alone was not able to completely reduce Cr(VI), but in the presence of pdtc and metals, only up to 4% Cr(VI) remained. The presence of dFO B particularly together with metals allowed for complete reduction of Cr(VI) at this pH. At pH 8, no spontaneous Cr(VI) reduction was observed in the control samples. Some Cr(VI) was reduced in controls when metals were present. In controls with dFO B added to dichro-

Table 1. Concentration remaining of initially added 10 and 25 μM Cr(VI) (dichromate) after 24 h incubation periods with different fractions of 48 h cultures of *P. stutzeri* strains KC and CTN1. Culture fractions included whole cultures, cell-free culture filtrates, six-fold diluted culture filtrates to match the dilution of low-molecular-weight fractions of culture filtrates, and low-molecular-weight fractions of culture filtrates obtained using Sephadex G-25 chromatography.

Culture Fraction	Cr(VI) remaining concentration	
	10 μM	25 μM
KC culture	3.3 \pm 0.3	6.8 \pm 0.4
KC filtrate	3.4 \pm 0.4	7.5 \pm 0.7
CTN1 culture	8.8 \pm 0.3	17.7 \pm 1.4
CTN1 filtrate	9.0 \pm 0.8	19.3 \pm 2.0
KC diluted filtrate	6.3 \pm 0.2	155 \pm 0.2
KC LMW fraction of filtrate	7.2 \pm 0.1	17.8 \pm 0.1
CTN1 diluted filtrate	10.9 \pm 1.2	24.6 \pm 0.2
CTN1 LMW fraction of filtrate	11.2 \pm 0.1	25.2 \pm 0.2

Abbreviations used: LMW, low-molecular-weight.

Table 2. Remaining percentage of initially added 25 μM Cr(VI) (dichromate) after incubation with different concentrations of chemically synthesized siderophores of *P. stutzeri* KC: desferrioxamine B (dFO) and pyridine-2,6-bis(thiocarboxylic acid) (pdtc) with and without addition of 25 μM of each of the following metals: Fe(III), Cu(II), Zn(II), Ni(II), Co(III), and Cr(III) in 50 mM phosphate-citrate buffer at pH 7 and 8.

Metals present	Incubation time (d)	Control	50 μM pdtc	50 μM pdtc + 50 μM dFO	125 μM pdtc	125 μM pdtc + 125 μM dFO
pH		7				
–	1	98.9 \pm 1.1	66.8 \pm 0.9	59.3 \pm 2.9	39.2 \pm 0.2	32.3 \pm 1.9
–	14	85.3 \pm 3.3	51.0 \pm 23.2	34.4 \pm 4.7	27.9 \pm 11.9	0.4 \pm 0.9
–	30	71.7 \pm 4.7	41.9 \pm 22.3	8.2 \pm 2.2	21.2 \pm 18.0	nd
+	1	63.4 \pm 5.3	46.3 \pm 1.2	39.1 \pm 2.0	26.3 \pm 0.9	2.9 \pm 0.3
+	14	0.2 \pm 0.2	8.2 \pm 0.6	4.5 \pm 1.3	0.0 \pm 0.1	0.7 \pm 0.1
+	30	nd	4.2 \pm 0.6	nd	0.3 \pm 0.3	nd
pH		8				
–	1	103.7 \pm 4.2	70.0 \pm 2.7	63.2 \pm 1.2	43.4 \pm 1.2	39.4 \pm 0.4
–	14	99.7 \pm 0.5	78.7 \pm 3.9	55.7 \pm 15.2	48.5 \pm 2.3	25.0 \pm 4.8
–	30	102.8 \pm 3.2	85.9 \pm 6.1	46.4 \pm 11.5	62.8 \pm 5.0	20.8 \pm 2.9
+	1	87.9 \pm 2.1	81.8 \pm 1.9	69.5 \pm 3.6	77.5 \pm 2.4	46.7 \pm 3.8
+	14	82.7 \pm 3.2	75.7 \pm 0.8	56.5 \pm 1.6	57.0 \pm 0.7	1.5 \pm 0.1
+	30	82.8 \pm 2.8	73.8 \pm 1.1	53.4 \pm 0.9	53.2 \pm 0.4	1.5 \pm 0.4

Abbreviations used: nd, not detected; +, metals added; –, metals not added.

mate, over 70% of the Cr(VI) remained; when the metal mixture was also present along with dFO B, 50% Cr(VI) remained at pH 8. Analogous trends to the observations at pH 7 were noted at pH 8 where the most effective Cr(VI) reduction was observed in the presence of pdtc, dFO B, and metals together. Almost complete dichromate reduction at pH 8 was possible only when both siderophores were added and the metal mixture was present. The reaction mixtures containing dichromate, pdtc, metals, and dFO B were also monitored using ESI-MS. In the presence of all metals added to the reaction (Fe, Cu, Zn, Ni, Co, Cr(III)) and where dichromate was found by the spectrophotometric assay to be reduced, the Co(III):(pdtc)₂, Cu(II):pdtc, and Cu(I):pdtc complexes were mainly detected with traces of Zn(II):(pdtc)₂, Fe(III):(pdtc)₂, Cr(III):(pdtc)₂, and pdtc hydrolysis product complexes (spectra reported by Cortese *et al.* 2002).

Composition of chromium precipitate

When pdtc (10 mM) and dichromate (10 mM) were mixed, a yellowish-brown precipitate immediately formed. Within hours, the precipitate darkened and turned black. The black precipitate was analyzed using SEM-EDS (Figure 4). Ele-

mental analysis showed chromium and sulfur present in a ratio of 1:0.7. When pdtc was mixed with dichromate in lower concentrations (1 mM each), no visible precipitate formed initially, but within 12 wks of incubation time, a black precipitate did appear.

Minimal inhibitory concentration (MIC)

Metal MICs for *P. stutzeri* KC and CTN1 were determined in iron-limited media. The growth of strain KC was inhibited in the presence of more than 0.05 mM Hg(II), 0.01 mM Cd(II), 2.0 mM Pb(II), or 2.0 mM As(III). Strain CTN1 was incapable of growth in Hg(II) concentrations above 0.025 mM, Cd(II) concentrations above 0.01 mM, Pb(II) above 1.0 mM, and As(III) above 1.0 mM.

Composition of heavy metal precipitates

Mixing of mercury, cadmium, lead, and arsenic(III) solutions with pdtc resulted in formation of poorly water-soluble precipitates that were different shades of white in the case of Hg, Cd, and As and orange in the case of Pb. The precipitates were partially soluble in DMF, and using ESI-MS we confirmed that pdtc formed complexes with Cd and Pb. The

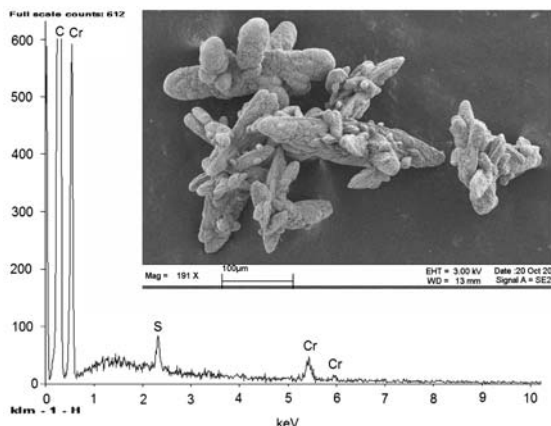


Figure 4 EDS spectrum and SEM image of black precipitate formed in the dichromate and pdtc mixture. The black precipitate is possibly Cr_2S_3 .

structures of $\text{Cd}:\text{pdtc}$ and $\text{Pb}:\text{pdtc}$ complexes were reported previously (Cortese *et al.* 2002). Only after addition of excess of pdtc to the mercury solution could we observe the $\text{Hg(II)}:(\text{pdtc})_2\text{H}$ complex using ESI-MS and for the first time determine its composition (Figure 5). An $\text{As(III)}\text{-pdtc}$ complex structure was not detected. After 7 days of incubation, Hg precipitates turned black,

Cd precipitates yellow, Pb precipitates black, and As(III) precipitates yellow. EDS analysis of metal precipitates formed by pdtc showed the presence of the respective metals accompanied by sulfur (Figure 6). As calculated from the atom percentage, the $\text{Hg}:\text{S} = 1:2$, $\text{Cd}:\text{S} = 1:1.4$, $\text{Pb}:\text{S} = 1:1.8$, and $\text{As(III)}:\text{S} = 1:1.7$ suggesting the presence of metal sulfides possibly accompanied by the remains of these metal:pdtc complex precipitates.

Discussion

The evaluation of chromium(VI) toxicity on *P. stutzeri* KC showed that Cr(VI) inhibited growth of the strain at concentrations higher than $25\ \mu\text{M}$. Strain CTN1, lacking the ability to synthesize pdtc, was incapable of growth at all dichromate concentrations tested (Figures 2 and 3). Both strains produced desferrioxamine siderophores, but only strain KC produced pdtc. These observations suggest a correlation between pdtc production and chromium resistance. An observed toxic effect of chromium on *P. stutzeri* KC was the lengthening of the logarithmic growth phase and

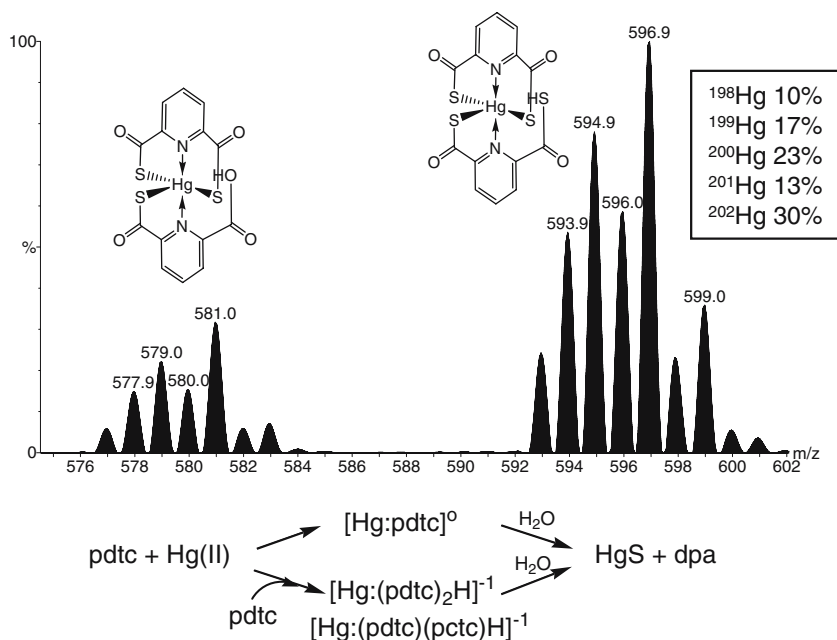


Figure 5. ESI-MS spectrum (negative ion mode) and structures of the Hg(II) complexes with pdtc and pdtc hydrolysis products (pyridine-2-carboxylic-6-thiocarboxylic acid, pctc) in DMF. Peak m/z distribution reflects the natural abundance of mercury isotopes that is shown in the box. A schematic representation of pdtc interaction with Hg(II) is shown below the spectrum. At low pdtc concentrations, pdtc molecules initially form insoluble zero valent complexes of $\text{Hg(II)}:\text{pdtc}$ that precipitate as a white amorphous sediment. The sediment is hydrolyzed to HgS and dipicolinic acid (dpa) resulting in a color change to black. At higher concentrations, pdtc forms the charged, partially soluble complexes $\text{Hg}:(\text{pdtc})_2\text{H}$ and $\text{Hg}:(\text{pdtc})(\text{pctc})\text{H}$ that were detected using ESI-MS.

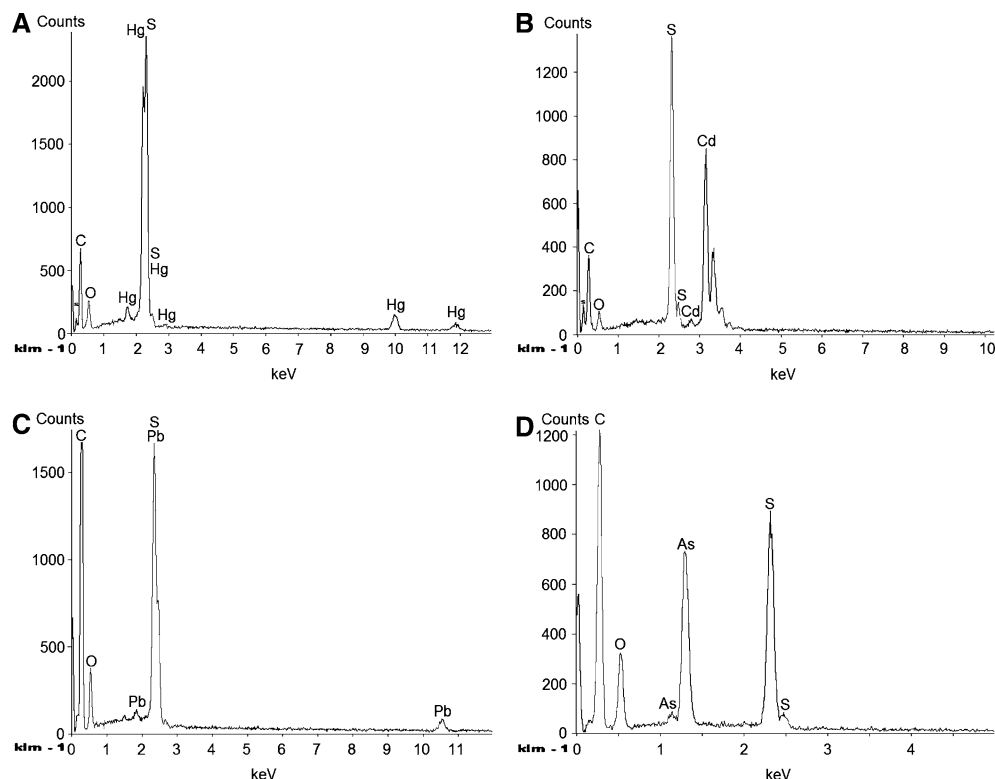


Figure 6. EDS spectra of precipitates formed by pdtc with: (A) mercury, (B) cadmium, (C) lead, and (D) arsenic (III).

decreased growth rates, an effect similar to that of chromium on algae (Cervantes *et al.* 2001). When strain CTN1 was grown in the presence of 10 μ M of Cr(VI), some growth was recorded after 72 h accompanied by a slight decrease in dichromate concentration. This observation may indicate the existence of an alternative mechanism for chromium resistance based on, for example, an inducible efflux mechanism (Aguilera *et al.* 2004) or enzymatic reaction (Park *et al.* 2000), but these being less effective than pdtc activity at reducing Cr(VI) toxicity.

Two-day culture filtrates of *P. stutzeri* KC containing biologically produced pdtc were effective at dichromate removal to the same extent as cell-containing cultures (Table 1), an observation that confirms the involvement of pdtc in Cr(VI) removal by reduction. The low-molecular-weight fraction of the culture filtrate was responsible for the reducing activity, suggesting that neither the bacterial cells nor a possible extracellular enzyme system played a major role in dichromate reduction. At the same time, strain CTN1 removed significantly lower amounts of

dichromate. CTN1 cultures were slightly more efficient than CTN1 culture filtrates at Cr(VI) removal, likely due to adsorption to the cell surface (Cervantes *et al.* 2001; Gadd 2004) or other non-specific processes.

The results of ESI-MS analysis of reaction mixtures containing chemically synthesized pdtc and dichromate revealed that pdtc reduced Cr(VI) to Cr(III) and complexes of Cr(III) with pdtc and pdtc hydrolysis products were formed (Figure 1). Cr(III) was previously recognized as one of the metals inducing pdtc hydrolysis, and the structures of the complexes have been determined (Cortese *et al.* 2002). Cortese *et al.* (2002) did not recognize reduction of dichromate anions by pdtc. We postulate that Cr(VI) is reduced to Cr(III) possibly by H_2S released during pdtc hydrolysis and/or by electrons derived from the pdtc hydrolysis and oligomerization reaction (Zawadzka *et al.* 2006). The H_2S -induced Cr(VI) reduction mechanism may be similar in nature to Cr(VI) reduction caused by sulfide produced by sulfate-reducing bacteria (Smillie *et al.* 1981; Chardin *et al.* 2003). The reduced chromium is then complexed by

remaining pdtc and its hydrolysis products, pyridine-2-thiocarboxylic-6-carboxylic acid (pctc) and dipicolinic acid (dpa). The presence of the Cr(III) complexes and free pctc and dpa in solution provide evidence for this reaction scenario. The complexes were fairly stable for up to 90 days; however, their concentrations decreased greatly indicating slow hydrolysis of these complexes. Elemental analysis of the black precipitate formed upon reaction of pdtc with dichromate revealed the presence of chromium and sulfur that may correspond to water-insoluble black Cr_2S_3 . Although the ratio of Cr to S may suggest different stoichiometry, the elevated content of chromium may come from the coprecipitation of chromium oxides ($\text{Cr}_2\text{O}_3(\text{H}_2\text{O})_n$) (Evanko & Dzombak 1997, Cervantes *et al.* 2001) both released from Cr(III) complexes with pctc and dpa and from unchelated chromium after its reduction.

Abiotic reactions using chemically synthesized pdtc showed that pdtc was effective in dichromate reduction, but this process was more efficient in the presence of desferrioxamine and transition metals (Table 2), suggesting a synergistic effect of these two components in natural environments. Pdtc was most effective in Cr(VI) reduction at pH 7; however, at this pH, the metals themselves catalyzed dichromate reduction, a process that is well recognized and used in Cr(VI) remediation via zero valent iron-containing permeable barrier technology (Fendorf & Li 1996; Evanko & Dzombak 1997). At pH 8, pdtc mixed with metals was more efficient at Cr(VI) reduction than pdtc alone, especially when present together with dFO B. This process cannot be attributed to metals alone because dichromate levels remained unchanged in controls containing metals without siderophores. Because we have confirmed metal:pdtc complex formation under experimental conditions using ESI-MS, it appears that metal-siderophore complexes were even more effective at Cr(VI) reduction than pdtc-derived hydrogen sulfide. This is not surprising since Fe, Cu, Co, and Ni within pdtc complexes are redox active. Redox potentials of these complexes range from -400 mV for Fe(III):(pdtc)_2 to -900 mV for Co(III):(pdtc)_2 (Ockels *et al.* 1978; Cortese *et al.* 2002), sufficient for mediating the reduction of Cr(VI) to Cr(III) (Nies 1999). Additionally, metal complexes of ferrioxamines with -470 mV redox potential of ferric complex (Boukhalfa & Crum-

bliss 2002) can exhibit synergistic effects in transfer of electrons during pdtc hydrolysis.

Pdtc was found to form poorly soluble precipitates with mercury, cadmium, lead, and arsenic. The precipitates were identified as complexes of metals with pdtc. The structure of cadmium and lead complexes are known (Cd(II):(pdtc) and Pb(II):(pdtc)) (Cortese *et al.* 2002). For the first time, we were able to detect a complex with mercury and elucidate its structure as $\text{Hg(II):(pdtc)}_2\text{H}$ with one carbonyl sulfide ligand protonated (Figure 5) when pdtc was present in excess of Hg. Complexes of Hg with pdtc hydrolysis products were also observed (Figure 5). It is possible that the zero valent Hg(II):(pdtc) 1:1 complex also forms as in the case of other divalent metals but is insoluble and therefore not detectable using ESI-MS. An arsenic-pdtc complex was not detected, probably due to its low solubility. Precipitate colors changed gradually to colors corresponding to respective metal sulfides, and their elemental compositions were confirmed by SEM-EDS analysis (Figure 6). Because all metals were accompanied by sulfur, we can conclude that the following sulfides were formed as a result of hydrolysis of these metal:pdtc complexes: HgS , CdS , PbS , and As_2S_3 . The atom percentage of As and S was 2:3, suggesting presence of the As_2S_3 precipitate (orpiment). Microbiological formation of arsenic trisulfide, although by a different mechanism, has been previously reported (Newman *et al.* 1997). In other metal precipitates, the sulfur content was nearly double the metal content, indicating the possibility of elemental sulfur co-precipitation during complex hydrolysis reactions where one metal cation is complexed by two sulfur ligands. We can conclude that pdtc forms complexes with Hg, Cd, Pb, and As as with other metals, but these complexes are chargeless, poorly soluble in water, and readily form precipitates. Heavy metal:pdtc complexes are also less stable and hydrolyze, releasing metal sulfides. For example, $\text{Cd:thiocarboxylate}$ complexes have been shown to release CdS as a result of thermal decomposition at temperatures as low as 25°C (Nyman *et al.* 1997).

We observed pdtc binding of heavy metals into insoluble complexes that are hydrolyzed to heavy metal sulfides of further increased insolubility, indicating that bacteria may utilize pdtc excretion as an environmental detoxification mechanism. The MIC of Hg, Pb, and As(III) was twice as high

for strain KC as compared to strain CTN1, confirming that KC cultures benefit from a pdtc-mediated heavy metal detoxification mechanism. Only cadmium exhibited similar toxicity in both strains. Since little is known about the fate of pdtc complexes with metals other than iron (Lewis *et al.* 2004), we can speculate that some complexes are transported into cells as a means of micronutrient acquisition. Some solubilized cadmium complexes may also be transported in, causing toxicity rather than preventing it. Cortese *et al.* (2002) showed that supplementation with pdtc protected several bacterial strains including *P. stutzeri* KC and CTN1 from both Hg and Cd toxicity. Our experimental conditions were different, however, and a toxic Cd effect may have occurred before the bacteria from the inoculum had a chance to produce enough pdtc for detoxification in the iron-limited media containing Cd.

Our results suggest that pdtc serves not only as a siderophore but also as a multi-functional metabolite that plays a role in bacterial environment conditioning (Budzikiewicz 2003). Here we report novel abilities of pdtc: to reduce the more mobile and toxic chromium(VI) to less toxic chromium(III) under aerobic conditions; and to chelate Cr(III) and subsequently release it in the form of chromium sulfides and possibly chromium oxides. We also confirmed that precipitates formed by pdtc with heavy metals are metal:pdtc complexes that decompose releasing metal sulfides. MIC data indicate that pdtc-producing *P. stutzeri* KC was more resistant to metal toxicity than the pdtc-negative mutant CTN1 suggesting involvement of pdtc in metal detoxification of biological systems.

Presented results clearly suggest a function for pdtc in heavy metal and metalloid detoxification. Similarly to cellular thiols like glutathione, pdtc's properties originate from reactivity of its thiol groups. Glutathione was shown to be involved in bacterial resistance to cadmium (Figueira *et al.* 2005), selenium (Kessi & Hanselmann 2004), and tellurium (Turner *et al.* 2001). Pdtc, however, is unique due to its electron-rich center, which is composed of two carbonyl sulfide groups and pyridine nitrogen. This unique composition gives pdtc metal chelation abilities that are not exhibited by other thiols (Stolworthy *et al.* 2001; Cortese *et al.* 2002; Budzikiewicz 2003). Pdtc can act as a biochelator-reducer supplying hydrogen sulfide

from its hydrolysis. The redox active metal:pdtc complexes can transfer electrons to a substrate like Cr(VI) reducing it to Cr(III). The mechanism of pdtc-mediated heavy metal detoxification involves the high affinity of the metals for sulfur. Pdtc mediates precipitation of metals as their insoluble sulfides, yielding metals present outside the cells bio-unavailable (Nies 1999; Raab & Feldmann 2003). In aerobic conditions, pdtc provides an extracellular pool of thiols conferring the advantage of mediating reactions outside the cells and thus preventing intracellular metal toxicity. The energetic expense of pdtc synthesis may be in the end far less costly than the metabolic expense needed to reduce, sequester, or extrude metals or to repair metal-induced damage inside the cell (Nies 1999). The chemistry of pdtc's interactions with Cr(VI), Hg, Pb, Cd, and As(III) elucidated here and with Se(IV) and Te(IV) elucidated in our earlier studies (Zawadzka *et al.* 2006) add new information to our understanding of the biological role of pdtc in metal detoxification of the bacterial environment. The great diversity of pdtc interactions with environmental contaminants including metals, metalloids, and carbon tetrachloride make *P. stutzeri* KC an excellent candidate for use in bioremediation (Dybas *et al.* 1998).

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