Biodegradation (2008) 19:417–424
DOI 10.1007/s10532-007-9147-1

ORIGINAL PAPER

Isolation and characterization of an NAD^+-degrading bacterium PTX1 and its role in chromium biogeochemical cycle

Geoffrey J. Puzon · Yan Huang · Alice Dohnalkova · Luying Xun

Received: 2 March 2007 / Accepted: 30 July 2007 / Published online: 15 August 2007

Abstract Microorganisms can reduce toxic chromate to less toxic trivalent chromium [Cr(III)]. Besides $Cr(OH)_3$ precipitates, some soluble organo-Cr(III) complexes are readily formed upon microbial, enzymatic, and chemical reduction of chromate. However, the biotransformation of the organo-Cr(III) complexes has not been characterized. We have previously reported the formation of a nicotinamide adenine dinucleotide (NAD^+)-Cr(III) complex after enzymatic reduction of chromate. Although the NAD^+-Cr(III) complex was stable under sterile conditions, microbial cells were identified as precipitates in a non-sterile NAD^+-Cr(III) solution after extended incubation. The most dominant bacterium PTX1 was isolated and assigned to *Leifsonia* genus by phylogenetic analysis of 16S rRNA gene sequence. PTX1 grew slowly on NAD^+ with a doubling time of 17 h, and even more slowly on the NAD^+-Cr(III) complex with an estimated doubling time of 35 days. The slow growth suggests that PTX1 passively grew on trace NAD^+ dissociated from the NAD^+-Cr(III) complex, facilitating further dissociation of the complex and formation of Cr(III) precipitates. Thus, organo-Cr(III) complexes might be an intrinsic link of the chromium biogeochemical cycle; they can be produced during chromate reduction and then further mineralized by microorganisms.

Keywords NAD^+ · Chromium · Organo-Cr(III) complex · Biodegradation

G. J. Puzon · Y. Huang · L. Xun (✉)
School of Molecular Biosciences, Washington State University, Abelson Hall 301, Pullman, WA 99164-4234, USA
e-mail: xun@mail.wsu.edu

A. Dohnalkova
Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99352, USA

Introduction

Trivalent chromium [Cr(III)] is the predominant form in the environment, while hexavalent chromium [Cr(VI)] is mainly introduced as a by-product of industrial use (Langard 1990). The biogeochemical process primarily converts Cr(VI), such as chromate, to less toxic Cr(III) (Bartlett 1991). Bacteria were first characterized to reduce Cr(VI) in 1979 (Lebedeva and Lyalikova 1979). Numerous bacteria have since been demonstrated to transform Cr(VI) to Cr(III) under various conditions, e.g., (Suzuki et al. 1992; Shen and Wang 1993; Lovley and Philips 1994; Garbisu et al. 1998; Tebo and Obraztova 1998; Park et al. 2000; Megharaj et al. 2003), highlighting the role of bacteria in the biogeochemical cycle of chromium and providing possible methods for in situ bioremediation of Cr(VI) contamination.

Microbial reduction of Cr(VI) was assumed to result in the formation of only insoluble Cr(III) precipitates

(Barnhart 1997). However, recent evidence has elucidated a more complex role for microorganisms in the chromium biogeochemical cycle. First, Cr(VI) reduction by several bacterial species has resulted in the production of soluble Cr(III) in the supernatant (Shen and Wang 1993; Campos et al. 1995; McLean and Beveridge 2001; Megharaj et al. 2003; Bencheikh-Latmani et al. 2007). Second, enzymatic reductions of Cr(VI) have formed organo-Cr(III) complexes, where Cr(III) is bound to the oxidized form of the reductants, such as NAD^+ (Puzon et al. 2002; Kwak et al. 2003) and cytochrome c_7 (Assfalg et al. 2002). Third, reduction of Cr(VI) in the presence of organics and common intracellular components produces various soluble organo-Cr(III) complexes (Buerge and Hug 1998; Puzon et al. 2005).

The increasing evidence on the formation of the soluble organo-Cr(III) complexes during chromate reduction leads to the necessity of addressing the possible fate of the complexes in the environment. Since the NAD^+-Cr(III) complex is the only product of NADH-dependent enzymatic reduction of Cr(VI) (Puzon et al. 2002; Kwak et al. 2003), it can be used as a representative compound to address the biotransformation of organo-Cr(III) complexes. We report here microbial transformation of the soluble NAD^+-Cr(III) complex to Cr(III) precipitates.

Materials and methods

Analytical techniques

Potassium chromate, K_2CrO_4 (Sigma, St. Louis, MO), was used as the Cr(VI) source in all experiments. Cr(VI) was measured by a reported method (Schmieman et al. 1998) using chromate as the standard and ChromaVer powder pillows (HACH, Loveland, CO) containing diphenylcarbazide. Total soluble Cr(III) was estimated as the difference between total soluble chromium and Cr(VI). Samples were prepared for total soluble chromium by first centrifugation at 16,500 × *g* for 5 min at 23°C and then filtered through a 0.22 μm MILLIPORE MILLEX^{TM}GP syringe filter (Millipore Corp., Bedford, MA). Samples were analyzed by using an HP 4500 Plus Series inductively coupled plasma/mass spectrophotometer (ICP/MS) (Agilent Technologies Inc., Palo Alto, CA). $Cr(NO_3)_3$ (Aldrich, Milwaukee, WI) in distilled water was used to generate a standard curve for ICP/MS analysis. NAD^+ concentrations were measured at $OD_{260\ nm}$, using a molar extinction coefficient of 18,000 M^{-1} cm^{-1} and also by HPLC analysis with a reverse phase C-18 column, essentially using a previously reported method (Stocchi et al. 1985).

Media and growth conditions

Mineral medium (MM) for PTX1 growth was composed of the following components per liter of distilled water: 3 g of Na_2HPO_4, 1 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, and 1.5 ml of trace mineral solution (containing the following per liter, 10 g of $MgSO_4$, 2 g of $CaCO_3$, 4.5 g of $FeSO_4{\cdot}H_2O$, 1.4 g of $ZnSO_4{\cdot}7H_2O$, 1.1 g of $MnSO_4{\cdot}4H_2O$, 0.2 g of $CuSO_4{\cdot}5H_2O$, 0.2 g of $CoSO_4{\cdot}7H_2O$, 0.06 g of H_3BO_3, and 10 ml of concentrated HCl). NAD^+ (Sigma) or the NAD^+-Cr(III) complex was added to 2 mM or as specified as the sole carbon and energy source. The NAD^+-Cr(III) complex was the product of enzymatic reduction of Cr(VI), prepared as previously described (Puzon et al. 2002). Briefly, 40 mM chromate in 40 mM potassium phosphate buffer (pH 7) was reduced by 10 μg of NADH:flavin oxidoreductase with 80 mM NADH (Sigma) under anaerobic conditions. Chromate was completely reduced to the soluble NAD^+-Cr(III) complex within 1 h. The sample was then dialyzed in dialysis tubing with molecular weight cutoff of 3,500. When dialyzed against 40 mM potassium phosphate buffer (pH 7.0), the NAD^+ to Cr(III) ratio was 2:1 (Puzon et al. 2002). A ratio of 1:1 of the NAD^+-Cr(III) complex was produced by dialysis against distilled water overnight to further remove loosely bound NAD^+. The NAD^+-Cr(III) complex dialyzed against distilled water was sterilized by filtration and use for bacterial growth.

The seed culture of PTX1 was pre-grown in either Luria–Bertani (LB) or MM medium with NAD^+ as the sole carbon and energy source. The cells were transferred into fresh MM medium with NAD^+ or the NAD^+-Cr(III) complex and into controls without any carbon and energy source. Cultures were normally incubated aerobically at 30°C with 200 rpm shaking. Bacterial growth was monitored by increased turbidity at an optical density of 600 nm. The PTX1 cultures grown with the NAD^+-Cr(III) complex were centrifuged to remove soluble NAD^+-Cr(III), which

also absorbs at 600 nm, and re-suspended in 10 mM potassium phosphate buffer (pH 7) before turbidity measuring.

Electron microscopy

Preparation of samples for transmission electron microscopy (TEM) was as follows: bacterial suspensions were diluted 1:10 in distilled H_2O, 3 µl was then immediately placed on a 200 mesh nickel grid with a formvar support coated with carbon and air dried. Samples were examined without staining using a JEOL 2010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operated at 200 kV coupled with an Oxford electron dispersive spectroscopy system. Images were collected digitally and analyzed using Gatan's Digital micrograph software and ISIS Links system (Oxford, England).

Phylogenetic analysis

Genomic DNA was purified using the Puregene™ DNA isolation kit (Gentra Systems, Minneapolis, MN). The 16S rRNA gene was amplified by PCR with the primers GB-13 and GB-14 (Table 1) and purified using the Qiagen QIAquick PCR purification Kit (Qiagen Inc, Valencia, CA). The purified products were sequenced in both directions with the corresponding PCR primers (Table 1) to obtain the entire PCR product sequence. The PTX1 sequence was compared to sequences in the National Center for Biotechnological Information (NCBI) database (http://www.ncbi.nlm.nih.gov) by using the BLAST program (Altschul et al. 1997). The 10 closest matching 16S rRNA gene sequences were aligned using CLUSTAL W (Thompson et al. 1994) at the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw). Phylogenetic trees were constructed with the 16S rRNA gene sequences by using the PHYLIP phylogeny inference program package version 3.65c, and evolutionary distance matrices were estimated using DNADIST (Felsenstein 1993). Phylogenetic trees were constructed using the distance matrices generated as inputs for the NEIGHBOR (neighbor-joining method) program and viewed using TreeView 1.6.6 (Page 1996). Bootstrap analysis of tree topologies was evaluated using 1000 replicate alignments with the SEQBOOT program. The bacteria and their 16S rRNA genes accession number were as follows: *Leifsonia shinshuensis*, DQ232614; *L. xyli* strain s29n, AY509236; Naphthalene-utilizing bacterium IS1, AF531474; *L. naganoensis*, DQ232612; *L. xyli* CV86, AJ717351; *L. xyli* subsp. *cynodontis*, M60935; *L. aquatica*, DQ232618; *L. xyli* subsp. *xyli* str. CTCB07, NC006087; *Microbacteriaceae* KVD-1921-08, DQ490452; *Microbacteriaceae* KVD-unk-64, DQ490453; and PTX1, DQ901014.

Table 1 Primers for 16S rRNA gene amplification and sequencing[a]

Primer name	Primer sequence
GB-13 (Forward)	GAGAGTTTGATC(C or A)TGGCTCAG
GB-14 (Reverse)	ACGG(C or T)TACCTTGTTACGACTT
SC2F	GAGCGTTGTCCGGAATTA
SC2R	GTTCTTCGCGTTGCATCG
SC3F	CGTGTCGTGAGATGTTGG
SC3R	CGTTGCTGCATCAGGCTT

[a] GB-13 and GB-14 were used for amplifying the 16S rRNA gene sequence and for the first round of DNA sequencing. F and R refer to forward and reverse primers, respectively. The numbers 2 and 3 refer to primers used in determining the complete sequence of the PCR product

Results

Degradation of the NAD^+-Cr(III) complex

A sterile solution of the NAD^+-Cr(III) complex in 40 mM potassium phosphate buffer (pH 7.0) was stable with no precipitation or any change in concentrations of the complex. However, pale green Cr(III) precipitates were clearly visible at the bottom of the tube of a non-sterile solution of the NAD^+-Cr(III) complex in 40 mM potassium phosphate buffer (pH 7.0) after 18 months incubation at room temperature. Microscopic examination showed dense bacterial cells in the precipitates, indicating bacterial growth. The initial NAD^+-Cr(III) complex was prepared by dialysis against 40 mM potassium phosphate buffer, resulting in a 2:1 ratio of NAD^+ and Cr(III); the initial NAD^+-Cr(III) complex solution consisted of 16.4 mM NAD^+ and 8.8 mM Cr(III). After 18 months, NAD^+ and Cr(III) concentrations

decreased to 6.22 and 5.73 mM, respectively, with the remaining complex at approximately 1:1 ratio. Microscopic observations identified two bacterial morphologies present, rod and coccus, with the rods being the dominant morphology. The solution was diluted and plated on LB agar plates. Individual bacterial colonies were formed after 3–4 days incubation at 23°C, giving two different colony types, designated PTX1 and PTX2. PTX1 colonies were small, pale yellow, round with smooth edges, and glossy. In comparison, PTX2 colonies were large, orange, round with rough edges, and matte. PTX1 colonies were present in greater numbers, estimated at a 5:1 ratio to PTX2. A single colony of each morphology was selected from the highest dilutions, pre-grown on LB, washed and inoculated (with a final $OD_{600\ nm}$ about 0.014) into MM medium containing the NAD^+-Cr(III) complex as the carbon and energy source. After incubation for 4 months no change in NAD^+ and soluble Cr(III) was detected with PTX2 cultures. PTX1 cultures showed removal of 668 μM NAD^+ and 675 μM soluble Cr(III) (Fig. 1). Thus, PTX1 was shown to consume NAD^+ of the complex.

TEM/EDS analysis of PTX1 grown on NAD^+-Cr(III) complex

The decreased soluble Cr(III) should be present in some insoluble forms, which was investigated by using a TEM coupled with EDS. Images of unwashed PTX1 cells grown on the NAD^+-Cr(III) complex revealed several areas of electron dense materials. Phosphate granules, with trace amounts of Cr(III) inside the cells (Fig. 2A) and Cr(III) precipitates surrounding the cells (Fig. 2B) were identified. In cultures grown for over a year, even greater amounts of crystalline Cr(III) surrounding the cells were visible (Fig. 3). The presence of Cr(III) precipitates only in cultures containing PTX1 indicates that the bacterium was capable of transforming the soluble NAD^+-Cr(III) complex to insoluble Cr(III) precipitates.

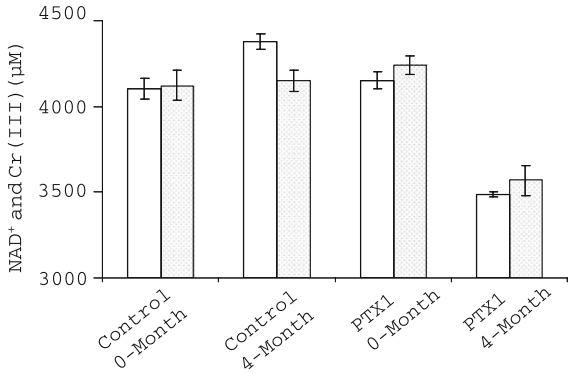

Fig. 1 PTX1 consumption of the NAD^+-Cr(III) complex. Cultures were grown for 4 months at 23°C. Open column represent NAD^+ and shaded columns represent soluble Cr(III) of the complex solution. Results are averages of triplicate experiments with standard deviations

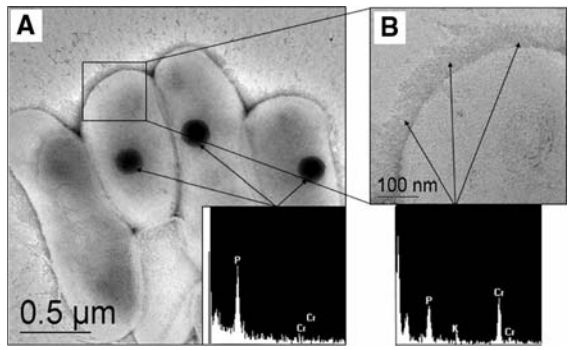

Fig. 2 TEM photograph of PTX1 grown with NAD^+-Cr(III) complex for four months. (**A**) Arrows point to phosphate granules inside the cells. (**B**) Arrows point to areas of Cr(III) precipitates around the cells

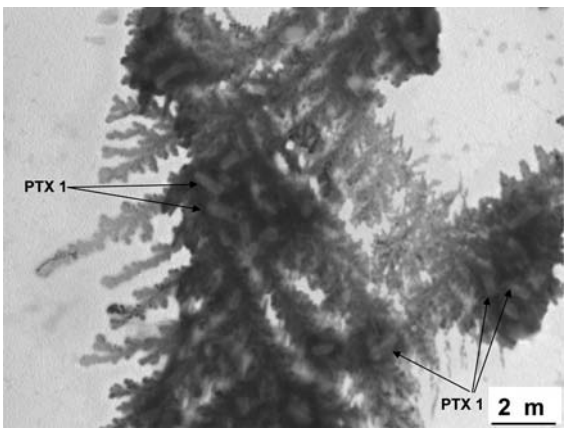

Fig. 3 TEM photograph of PTX1 grown on NAD^+-Cr(III) for 1 year. Arrows indicate cells surrounded by extensive Cr(III) precipitates

PTX1 growth on NAD^+ and the NAD^+-Cr(III) complex

PTX1 and PTX2 were analyzed for their ability to use NAD^+ as the sole carbon and energy source. PTX2 did not grow on NAD^+. When PTX1 cells from a single colony were inoculated into 4 ml of MM medium with 2 mM NAD^+ and incubated at 30°C, it took the culture 1–2 weeks before growth was evident. The NAD^+-grown culture was transferred at 1% inoculum into fresh MM medium containing either 2 mM NAD^+ or 2 mM NAD^+-Cr(III), incubated at 30°C and monitored daily. PTX1 grew slowly on NAD^+ with a doubling time of 17 ± 1 h, calculated from growth between 70 and 95 h (Fig. 4 A). On the other hand, the bacterium showed minimal growth on the NAD^+-Cr(III) complex (Fig. 4A). The initial small, apparent increase in turbidity (within the first 5 days) is likely due to growth on trace amount of free NAD^+ initially present in the NAD^+-Cr(III) preparation (Fig. 4A). Close examination of the growth between 7 and 24 days revealed the increase in cell density (Fig. 4B), but the growth was very slow. A linear regression was fitted into the data, and an upward slope (0.001 turbidity per day) was obtained, which was used to estimate a doubling time of 35 days. Prolonged incubation showed further increase in turbidity to higher than 0.1 and the formation of visible precipitates, consisting of PTX1 cells and Cr(III) (Fig. 3). No growth was detected in cultures without NAD^+, or the NAD^+-Cr(III) complex. PTX1 consumed NAD^+ without apparent accumulation of nicotinamide or adenine as a dead-end product (Fig. 5).

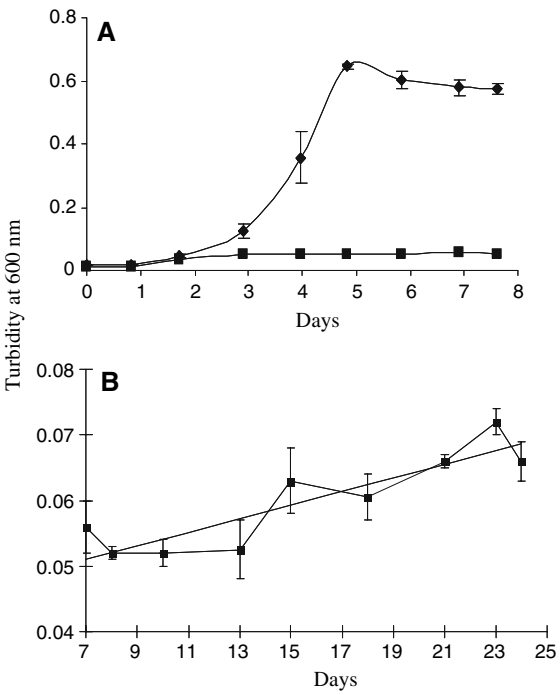

Fig. 4 Growth of PTX1 in MM medium with NAD^+ (◆) or NAD^+-Cr(III) (■) as the carbon and energy source. (**A**) The growth for the first 8 days; (**B**) for the growth on NAD^+-Cr(III) from day 7 to day 24. Data are averages of triplicate experiments with standard deviations

PTX1 characterization

PTX1 grew aerobically in LB broth and did not grow under anaerobic conditions. It showed similar growth rate in LB broth between 30 and 37°C with a doubling time of 2.1 ± 0.2 h. Microscopic analysis identified PTX1 as a gram positive rod, typically less than 1 μm in length. PTX1 was further characterized based on 16S rRNA gene sequence analysis. PCR amplified 1,388 base pairs of PTX1 16S rRNA gene, which was sequenced. The sequence was compared to others in the NCBI database. The 10 most similar sequences were selected and aligned to create a phylogenetic tree. Phylogenetic analysis identified PTX1 as a member of the *Microbacteriaceae* family, most closely related to *Leifsonia* species with 98–99% sequence identity. The constructed phylogenetic

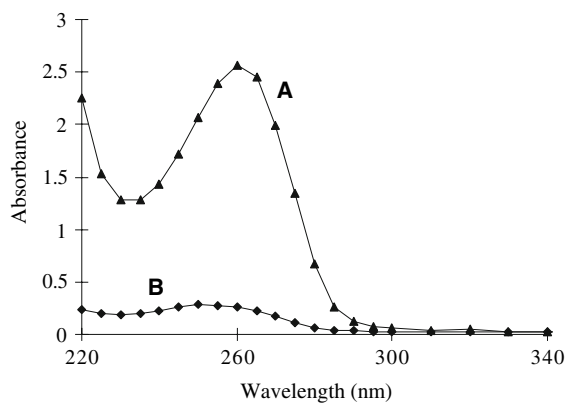

Fig. 5 Absorption spectrum analysis of NAD^+ consumption by PTX1. The medium contained 2 mM NAD^+ with an absorption peak at 260 nm (**A**), and culture supernatant of PTX1 after 8 days of growth (**B**). All samples were diluted 14-fold to allow the absorbance readings within detection limits

tree placed PTX1 on a distinct branch beside a naphthalene-utilizing bacterium IS1, which is tentatively assigned to *L. xyli* (Fig. 7).

Discussion

Bacterium PTX1 consumed NAD^+ and drove Cr(III) precipitation from the soluble NAD^+-Cr(III) complex

Chromate reduction by NADH-dependent enzymes produced the NAD^+-Cr(III) complex as the only identified product (Puzon et al. 2002; Kwak et al. 2003). Under sterile conditions, the complex is stable, suggesting that the complex does not readily dissociate. This is in agreement with the slow ligand exchange of the Cr(III) complexes, which have dissociation half lives in the order of weeks (Beattie and Haight 1972). However, *Leifsonia* sp. strain PTX1 transformed the soluble NAD^+-Cr(III) complex to Cr(III) precipitates. The extreme slow growth (doubling time of 35 days) with the complex as the substrate implies that PTX1 did not use the complex directly, but rather used trace NAD^+ dissociated from the complex. The data presented in Fig. 4 demonstrate that PTX1 grew on NAD^+ with a doubling time of 17 h, which was slow, but much faster than its growth on the complex. Since dissociation of the NAD^+-Cr(III) complex was not detected in the absence of PTX1, the overall precipitation process appeared to be actively driven by bacterial consumption of NAD^+.

The mechanism for NAD^+ utilization by PTX1 is unknown, but may occur via an NAD^+ glycohydrolase (NADase). NADases, present in both prokaryotes (Dassy and Alouf 1983) and eukaryotes (Han et al. 1995), hydrolyze NAD^+ into ADP-ribose and nicotinamide. *Streptococcus pyogenes* and *Vibrio fischeri* both secrete NADases extracellularly to hydrolyze exogenous NAD^+ (Dassy and Alouf 1983; Stabb et al. 2001). PTX1 likely uses a similar mechanism to digest NAD^+ before the breakdown products are transported into the cell for metabolism. Complexation of NAD^+ to Cr(III) may block the enzymatic reaction, hindering the consumption of the complexed NAD^+ by PTX1. PTX2 did not grow on NAD^+. It was identified as a *Rhodococcus* sp. on the basis of its 16S rDNA sequence, but its role in the original enrichment was uncharacterized.

PTX1 characterization

Gram staining and phylogenetic analysis of the 16S rRNA gene confirmed PTX1 as a gram-positive rod assigned to the genus of *Leifsonia*. PTX1 shows 98–99% sequence identity to *Leifsonia* species. Phylogenetic alignment placed PTX1 on a distinct branch separate from other known *Leifsonia* species (Fig. 6),

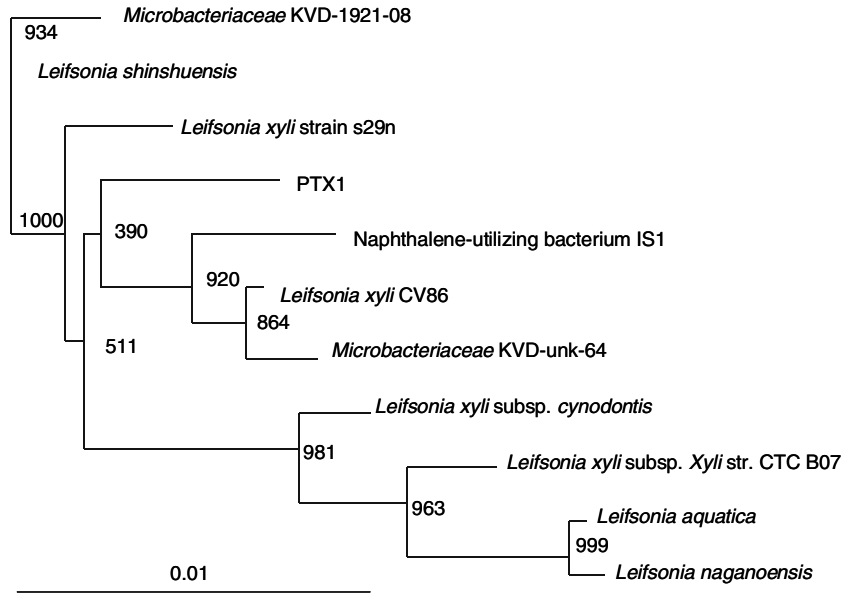

Fig. 6 Phylogenetic tree of PTX1 bacterium. Bootstrap values determined from 1000 replicates are listed at the branch points. The bar infers 0.01 nucleotide substitutions per site

which are often soil bacteria. Some of them have phytopathogenic traits (Davis et al. 1984), and others can degrade pollutants (Dore et al. 2003).

Environmental implications

The role of microorganisms in the biogeochemical cycle of chromium has long been focused on the reduction of soluble Cr(VI) to insoluble Cr(III). However, microbial, enzymatic, and chemical reduction of Cr(VI) has been shown to produce significant amounts of soluble Cr(III), e.g., (Shen and Wang 1993; Campos et al. 1995; Buerge and Hug 1998; McLean and Beveridge 2001; Assfalg et al. 2002; Puzon et al. 2002, 2005; Bencheikh-Latmani et al. 2007). The soluble Cr(III) forms are likely organo-Cr(III) complexes. Little is known about the fate of the soluble organo-Cr(III) complexes. Since some organo-Cr(III) complexes could be soluble and mobile in groundwater (James and Bartlett 1983), they may have frequent contact with manganese oxide, making re-oxidization of Cr(III) to Cr(VI) by manganese rich soils possible (Bartlett and James 1979; Murray et al. 2005). Another possible fate is biodegradation with the formation of Cr(III) precipitates as reported here. PTX1 likely consumed trace NAD^+ dissociated from the organo-Cr(III) complex, thereby facilitating continuous dissociation of the complex and formation of Cr(III) precipitation. However, the process was slow limited by the intrinsic stability of the organo-Cr(III) complexes. The slow process predicts the relative recalcitrance of organo-Cr(III) complexes, correlating well with the observation of soluble organo-Cr(III) complexes in the environment (Mattuck and Nikolaidis 1996; Farmer et al. 2002; Icopini and Long 2002). Biodegradation of dissociated organics may represent a general microbial activity towards organo-Cr(III) complexes. However, other microorganisms may also produce specific enzymes and reactive small molecules to directly attack the complexes or enhance the dissociation. Thus, a modified biogeochemical cycle of chromium is proposed here to include the formation and degradation of organo-Cr(III) complexes (Fig. 7).

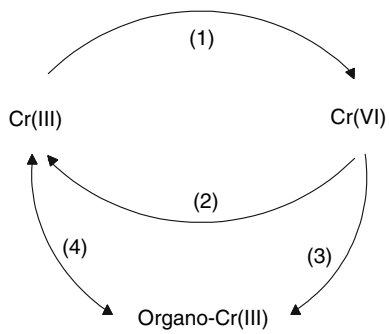

Fig. 7 Proposed new Cr(III) biogeochemical cycle. Cr(III) is oxidized to Cr(VI) by chemical reactions (1); Cr(VI) is reduced either chemical or biologically to Cr(III) precipitates (2) and soluble organo-Cr(III) complexes (3); and microorganisms can further transform the soluble organo-Cr(III) to Cr(III) precipitates (4). Some chelating agents can also bind Cr(III) to form organo-Cr(III) (4)

Acknowledgments We thank Chris Davitt for her help with the TEM and Charles Knaack for his help with the ICP/MS analysis. This research was funded by the Environmental Remediation Science Program (formerly NABIR program), Biological and Environmental Research (BER), US Department of Energy (Grant #DE-FG02-04ER63727).

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Assfalg M, Bertini I, Bruschi M, Michel C, Turano P (2002) The metal reductase activity of some multiheme cytochromes *c*: NMR structural characterization of the reduction of chromium(VI) to chromium(III) by cytochrome c_7. PNAS 99:9750–9754

Barnhart J (1997) Chromium chemistry and implications for environmental fate and toxicity. J Soil Contam 6:561–568

Bartlett RJ (1991) Chromium cycling in soil and water: links, gaps, and methods. Environ Health Perspect 92:17–24

Bartlett R, James B (1979) Behavior of chromium in soils: III. Oxidation. J Environ Qual 8:31–35

Beattie JK, Haight GPJ (1972) Chromium(VI) oxidations of inorganic substrates. Prog Inorg Chem 17:93–145

Bencheikh-Latmani R, Obraztsova A, Mackey M, Ellisman M, Tebo BM (2007) Toxicity of Cr(III) to *Shewanella* sp. strain MR-4 during Cr(VI) reduction. Environ Sci Technol 41:214–220

Buerge IJ, Hug SJ (1998) Influence of organic ligands on chromium(VI) reduction by iron. Environ Sci Technol 32:2092–2099

Campos J, Martinez-Pacheco M, Cervantes C (1995) Hexavalent-chromium reduction by a chromate-resistant *Bacillus* sp. strain. Antonie van Leeuwenhoek 68:203–205

Dassy B, Alouf JE (1983) Growth of *Streptococcus pyogenes* and streptolysin O production in complex and synthetic media. J Gen Microbiol 129:643–651

Davis MJ, Gillaspie AG Jr, Vidaver AK, Harris RW (1984) *Clavibacter*: a new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and bermudagrass stunting disease. Int J Syst Bacteriol 34:107–117

Dore SY, Clancy QE, Rylee SM, Kulpa CF (2003) Naphthalene-utilizing and mercury-resistant bacteria isolated form an acidic environment. Appl Microbiol Biotechnol 63:194–199

Farmer JG, Thomas RP, Graham MC, Geelhoed JS, Lumsdon DG, Paterson E (2002) Chromium speciation and fractionation in ground and surface waters in the vicinity of chromite ore processing residue disposal sites. J Environ Monit 4:235–243

Felsenstein J (1993) PHYLIP Inference Package. Department of Genetics, University of Washington, Seattle, WA

Garbisu C, Alkorta I, Llama MJ, Serra JL (1998) Aerobic chromate reduction by *Bacillus subtilis*. Biodegradation 9:133–141

Han MK, Kim J, Lee D, Kim U (1995) Immunohistochemical localization of NAD glycohydrolase in human and rabbit tissues. Histochem Cell Biol 104:185–189

Icopini GA, Long DT (2002) Speciation of aqueous chromium by use of solid-phase extractions in the field. Environ Sci Technol 36:2994–2999

James BR, Bartlett RJ (1983) Behavior of chromium in soils: V. Fate of organically complexed Cr(III) added to soil. J Environ Qual 12:169–172

Kwak YH, Lee DS, Kim HB (2003) *Vibrio harveyi* nitroreductase is also a chromate reductase. Appl Environ Microbiol 69:4390–4395

Langard S (1990) One hundred years of chromium and cancer: a review of epidemiological evidence and selected case reports. Am J Ind Med 17:189–215

Lebedeva EV, Lyalikova NN (1979) Reduction of crocoite by *Pseudomonas chromatophila* sp. nov. Mikrobiologiya 48:517–522

Lovley DR, Philips EJP (1994) Reduction of chromate by *Desulfovibrio vulgaris* and its c_3 cytochrome. Appl Environ Microbiol 60:726–728

Mattuck R, Nikolaidis NP (1996) Chromium mobility in freshwater wetlands. J Contam Hydrol 23:213–232

McLean J, Beveridge TJ (2001) Chromate reduction by a Pseudomonad isolated from a site contaminated with chromated copper arsenate. Appl Environ Microbiol 67:1076–1084

Megharaj M, Avudainayagam S, Naidu R (2003) Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. Curr Microbiol 47:51–54

Murray KJ, Mozafarzadeh ML, Tebo BM (2005) Cr(III) oxidation and Cr toxicity in cultures of the manganese(II)-oxidizing *Pseudomonas putida* strain GB-1. Geomicrobiol J 22:151–159

Page R (1996) Tree View: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Park CH, Keyhan M, Wielinga B, Fendorf S, Matin A (2000) Purification to homogeneity and characterization of novel *Pseudomonas putida* chromate reductase. Appl Environ Microbiol 66:1788–1795

Puzon GJ, Petersen JN, Roberts AG, Kramer DM, Xun L (2002) A bacterial flavin reductase system reduces chromate to a soluble chromium(III)-NAD^+ complex. Biochem Biophys Res Commun 294:76–81

Puzon GJ, Roberts AR, Kramer DM, Xun L (2005) Formation of soluble organo-chromium(III) complexes after chromate reduction in the presence of cellular organics. Environ Sci Technol 39:2811–2817

Schmieman EA, Yonge DR, Rege MA, Petersen JN, Turick CE, Johnstone DL, Apel WA (1998) Comparative kinetics of bacterial reduction of chromium. J Environ Eng 124:449–455

Shen H, Wang Y (1993) Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. Appl Environ Microbiol 59:3771–3777

Stabb EV, Reich KA, Ruby EG (2001) *Vibrio fischeri* genes *hvnA* and *hvnB* encode secreted NAD^+-glycohydrolases. J Bacteriol 183:309–317

Stocchi V, Cucchiarini L, Magnani M, Chiarantini L, Palma P, Crescentini G (1985) Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. Anal Biochem 146:118–124

Suzuki T, Miyata N, Horitsu H, Kawai K, Takamizawa K, Tai Y, Okazaki M (1992) NAD(P)H-dependent chromium(VI) reductase of *Pseudomonas ambigua* G-1: a Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III). J Bacteriol 174:5340–5345

Tebo BM, Obraztova AY (1998) Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors. FEMS Microbiol Lett 162:193–198

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. Nucleic Acids Res 22:4673–4680