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Proteogenomic and functional analysis of chromate reduction in *Acidiphilium cryptum* JF-5, an Fe(III)-respiring acidophile

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Abstract *Acidiphilium cryptum* JF-5, an acidophilic iron-respiring Alphaproteobacterium, has the ability to reduce chromate under aerobic and anaerobic conditions, making it an intriguing and useful model organism for the study of extremophilic bacteria in bioremediation applications. Genome sequence annotation suggested two potential mechanisms of Cr(VI) reduction, namely, a number of *c*-type cytochromes, and a predicted NADPH-dependent Cr(VI) reductase. In laboratory studies using pure cultures of JF-5, an NADPH-dependent chromate reductase activity was detected primarily in soluble protein fractions, and a periplasmic *c*-type cytochrome (ApcA) was also present, representing two potential means of Cr(VI) reduction. Upon further examination, it was determined that the NADPH-dependent activity was not specific for Cr(VI), and the predicted proteins were not detected in Cr(VI)-grown cultures. Proteomic data did show measureable amounts of ApcA in cells grown with Cr(VI). Purified ApcA is reducible by menadiol, and in turn can reduce Cr(VI), suggesting a means to obtain electrons from the respiratory chain and divert them to Cr(VI). Electrochemical measurements confirm that Cr reduction by ApcA is pH dependent, with low pH being favored. Homology modeling of ApcA and comparison to a known Cr(VI)-reducing *c*-type cytochrome structure revealed basic amino acids which could interact with chromate ion. From these studies, it can be concluded that *A. cryptum* has the physiologic and genomic capability to reduce Cr(VI) to the less toxic Cr(III). However, the expected chromate reductase mechanism may not be the primary means of Cr(VI) reduction in this organism.

Keywords Acidophile · Chromium reduction · Cytochrome *c* · Proteomics

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Introduction

In addition to radioactive fuels and fission products, toxic metals, such as Hg, Pb, Ni, and Cr, are widely used by domestic industries. These metals have subsequently entered the soil and both ground and surface waters. Many microorganisms are known to transform metals, often converting highly toxic and environmentally mobile metal species to less toxic and relatively immobile products (Cervantes et al. 2001). Metal-reducing bacteria are therefore attractive for potential use in bioremediation strategies, and metal-reducing bacteria that can live in

extreme conditions are particularly useful because metal-contaminated sites are often characterized by temperature and/or pH extremes (Ginder-Vogel et al. 2005; He et al. 2005; Horton et al. 2006; Zayed and Terry 2003). Thus, the understanding of these extremophilic, metal-transforming organisms will contribute to remediation situations where mesophilic microbes simply cannot survive, let alone remediate the contamination.

Chromate (Cr(VI)) reduction in bacteria involves several documented mechanisms, such as *c*-type cytochromes (Assfalg et al. 2002; Barton et al. 2007), hydrogenases (Chardin et al. 2003), quinone reductases (Sedlacek et al. 2009), and NAD(P)H-dependent flavin oxidoreductases (Opperman and van Heerden 2008; Opperman et al. 2008). Even though these components are linked to electron transport chains, it is generally observed that Cr(VI) does not serve as a terminal electron acceptor, although there are exceptions (Francis et al. 2000; Tebo and Obraztsova 1998). Chromium(VI) detoxification is the most likely scenario, where toxic aqueous Cr(VI) is reduced to non-toxic Cr(III) precipitates (Francisco et al. 2002; Viti et al. 2003). Generally, the cellular location of Cr(VI) reduction dictates the mechanisms. For example, extracellular and periplasmic Cr(VI) reduction is cytochrome-mediated, membrane Cr(VI) reduction is via hydrogenases and components of the respiratory chain, and intracellular Cr(VI) reduction is catalyzed by flavin-dependent reductases. Thus, a wide physiologic variety of bacteria, such as sulfate- and iron-respiring bacteria (Barton et al. 2007), Gram-positive bacilli (Desai et al. 2008a, b), thermophiles (Kieft et al. 1999), and acidophiles such as *Acidiphilium cryptum* (Cummings et al. 2007), can reduce Cr(VI).

Acidiphilium cryptum JF-5 is an acidophilic Fe(III)-respiring bacterium that is also capable of reducing Cr(VI) over a pH range of 1.7–4.7 (Cummings et al. 2007). Members of the genus *Acidiphilium* are found in a wide variety of acidic environments, although few actually have a capacity for Fe(III) respiration. The genome sequence of *A. cryptum* JF-5 was determined and initial annotation revealed it to contain genes for many metabolic processes relevant to mineral colonization, biofilm formation, metal transport, and Fe(III) respiration. Initial studies on the physiology of this organism demonstrated that *A. cryptum* could reduce Cr(VI) at low pH, and it was proposed that two mechanisms existed for Cr(VI) reduction in vivo; one being dependent on cytochrome *c*-mediated Fe(III) reduction, with subsequent abiotic reduction of Cr(VI) by Fe(II), and one based on a specific protein that reduced Cr(VI) under both aerobic and anaerobic conditions (Cummings et al. 2007). Further study was required, however, to clarify the details of Cr(VI) transformation, in particular the enzymes and proteins involved in the reduction process.

The purpose of this study was to determine the genomic capacity and the biochemical entities involved in Cr(VI) reduction in *A. cryptum*. The genomic capacity for the Cr(VI) reduction process was confirmed by discovery of genes encoding Cr(VI) reductase and Cr(VI) transporters, as well as a suite of genes encoding Class I monoheme *c*-type cytochromes. Examination of cell extracts and subcellular fractions revealed cytochrome *c*-mediated reduction of Cr(VI).

Materials and methods

Genome sequence analysis

The complete genome sequence of *A. cryptum*, including the main chromosome (GenBank accession number NC_009484) and eight plasmids (GenBank accession numbers NC_009467-472 and NC_009474), was determined by the U.S. Department of Energy Joint Genome Institute. Genes corresponding to putative *c*-type cytochromes and Cr(VI) transformation-related genes were identified by bioinformatic analysis of the *A. cryptum* genome sequence. The software package Integrated Microbial Genomes (IMG) was used for determining location of genes and relatedness of selected genes to those of similar function in other organisms (via Basic Local Alignment Search Tool or BLAST).

Cultivation of organisms

Acidiphilium cryptum JF-5 (provided by Dr. K. Küsel, University of Beyreuth) was cultivated in Acidiphilium Minimal Medium (AMM) containing (per liter) 0.5 g tryptic soy broth, 5 ml vitamin solution (Wolin et al. 1963), 5 ml mineral solution (Wolin et al. 1963), 1 g $(NH_4)_2SO_4$, 0.01 g KCl, 0.05 g K_2HPO_4, 0.25 g $MgSO_4$, 0.1% glucose and 0.03% yeast extract. Cells

were harvested by centrifugation and stored at −80°C until used for protein extraction. Chromium-grown cultures were supplemented with 30 or 100 μM Cr(VI) as K_2CrO_4.

Preparation of cell extracts and subcellular fractions

Cell pastes were resuspended in 50 mM Tris–HCl pH 7.5 (1:1 ratio of cell paste to buffer) and subjected to mechanical disruption using a BioSpec Bead-Beater homogenizer/blender with 0.1 mm glass beads (1:1 ratio of glass beads to cell suspension). The crude cell extract was clarified by centrifugation at 5,000*g* for 20 min to remove cell debris. The cell extract was then fractionated by ultracentrifugation (100,000*g* for 1 h) to separate membranes from soluble proteins. The resultant fractions were then assayed for Cr(VI) reductase activity as described below.

Purification of *c*-type cytochromes

The monoheme Class I *c*-type cytochrome ApcA (10.1 kDa) was purified by the following procedure: Cells were grown in 10 l AMM at pH 3.2 for 24 h under aerobic conditions after which oxygen sparging was stopped and 10 mM ferric sulfate was added as an electron acceptor. After 48 h cells were subjected to aerobic conditions for an additional 24 h and harvested via centrifugation (6000×*g*, 20 min). Cell pellets were washed twice with 20 mM citric acid buffer pH 3.0 and stored at −20°C. 20 mM citric acid buffer pH 3.0 containing 2% 3-(*N,N*-dimeth ylmyristyl-ammonio)propanesulfonate (Zwittergent 3-14, Sigma–Aldrich Chemical, St. Louis MO) and 60 mM Na_2EDTA was added to frozen cell pellets at 3.5 ml/g cells. Cells were incubated in the lysis buffer for 24 h at 4°C, after which the cell suspension was centrifuged (8,000×*g*, 20 min) and the supernatant was retained as the crude protein fraction. The lysis buffer/protein extraction was repeated 6 times.

The crude protein mixture was desalted against 20 mM citric acid containing 1.5% Zwittergent 3-14 (Buffer A) using a HiPrep™ desalting column (Amersham Biosciences Corp., Piscataway, NJ). The crude fraction was then run on a HiPrep™ 16/10 SP FF cation exchange column (Amersham Biosciences Corp., Piscataway, NJ) equilibrated with Buffer A and eluted with a linear gradient of 0–1 M NaCl in the same buffer. Fractions were pooled based on absorbance at 409 nm, and were analyzed for cytochrome *c* using a Tricine SDS–PAGE system (Schagger and von Jagow 1987). Fractions containing the 10.1 kDa cytochrome *c* were diluted with an equal volume of Buffer A, concentrated on the same column and eluted with 1 M NaCl. The concentrated fraction was run on a HiPrep™ 26/60 Sephacryl™ S-200 size exclusion column (Amersham Biosciences Corp., Piscataway, NJ) equilibrated with the sample buffer containing 0.5 M NaCl at a flow rate of 1.5 ml/min. Cytochrome containing fractions (A409) were pooled and desalted on a HiPrep™ desalting column (Amersham Biosciences Corp., Piscataway, NJ) against Buffer A. As a final polishing step the 10.1 kDa cytochrome was run on a UNO™ S1 ion exchange column (Bio-Rad Laboratories, Inc., Hercules, CA) and eluted with a linear gradient of 0.25–0.75 M NaCl in Buffer A. Samples were analyzed for purity using Tricine SDS–PAGE as described above.

Enzyme assays

Chromate reductase activity was measured using the Cr(VI) capture reagent 1,5-diphenylcarbazide (DPC) (Chakraborty and Mishra 1992). Menadiol-mediated reduction of ApcA was performed by titrating ApcA with 10 mM menadiol in 50 mM Tris–HCl pH 7.5 until reduction was observed spectrophotometrically by appearance of the heme gamma absorption band at 552 nm. Reduction/reoxidation experiments were carried out by first titrating the cytochrome solution (50 μM protein) with 10 mM sodium dithionite just to the point of reduction, as monitored by UV–Visible spectrophotometry (Shimadzu 2400 UV–Vis spectrophotometer). The reduced protein was then back-titrated with 10 mM K_2CrO_4 until reoxidation was observed spectrophotometrically. Reactions were carried out in either 50 mM citrate buffer pH 3.0, or 50 mM MOPS–NaOH buffer, pH 6.8.

Electrochemistry of ApcA

Purified ApcA was analyzed by cyclic voltammetry (CV) using a Gamry Model 600 potentiostat and a Dr. Bob's electrochemical cell (5 ml working volume). The working electrode was glassy carbon,

which was cleaned and polished with alumina before each experiment. Experiments were performed at pH 3.0 (50 mM citrate–NaOH/0.1 M NaCl) or pH 6.8 (50 mM MOPS/0.1 M NaCl). Protein sample (100 μM, buffer exchanged into the proper buffer system) was layered onto the glassy carbon surface and air-dried. The electrolyte was thoroughly deoxygenated with nitrogen before each experiment. CV was performed at 200 mV/s scan speed over a −700 to +700 mV range, and the current response recorded. For experiments with Cr(VI), K_2CrO_4 was added to 0.5 mM final concentration in the reaction mixture.

Proteomic analysis of Cr(VI)-exposed cells

Acidiphilium cryptum was cultivated aerobically at 30°C in 10 ml AMM broth with 1% glucose in screw-cap tubes. Duplicate cultures were grown with no Cr(VI), 30 μM Cr(VI), or 100 μM Cr(VI). Cultures were grown to late log phase, after which a 1.0 ml aliquot of cells was harvested by centrifugation at 10,000*g* for 10 min. Cell pellets were washed with high purity water, resuspended in 100 μl 20% formic acid, and heated at 90°C for 30 min. Insoluble materials were removed by centrifugation (10,000 × *g*, 10 min.), and the supernatant was dried by vacuum centrifugation. The pellet was dissolved in 100 μl 0.1 M $NaHCO_3$ buffer, and trypsin-digested using 20 U trypsin (Sequencing Grade, Sigma–Aldrich Chemical, St. Louis MO) for 16 h at 37°C. The digest was dried using vacuum centrifugation, and then subjected to liquid chromatography–mass spectrometric peptide analysis using the methods of Bansal et al. (Bansal et al. 2009).

Homology modeling of ApcA

The sequence of ApcA, after signal sequence cleavage, was used to generate a homology model of the protein using the SWISS-MODEL (Bordoli et al. 2009) and PHYRE (Kelley and Sternberg 2009) web-based software packages. The template 1HROA (cytochrome c_2 from *Rhodopila globiformus*) was consistently the closest match and was used to generate the model. The software package VMD 1.8.7 (Humphrey et al. 1996) was used to visualize the models and render both ribbon and surface charge structures.

Results

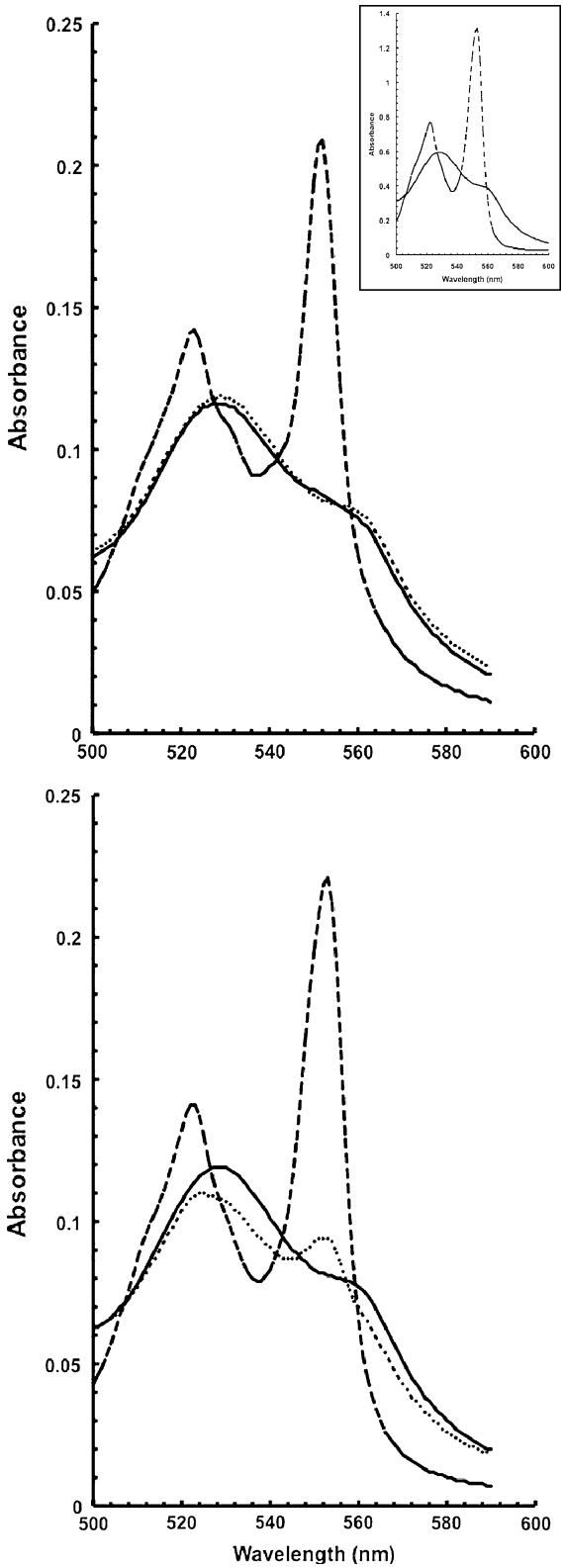
Enzymes and proteins involved with Cr(VI) reduction

NADPH-dependent chromate reductase activity was detected primarily in the soluble fraction, with activities in the soluble and membrane fraction being 43.9 and 19.0 nmol Cr(VI) reduced/min/mg protein, respectively. This is consistent with the localization of several other described Cr(VI) reductase activities (Desai et al. 2008b; Gonzalez et al. 2005). Our results indicate that there was measureable enzymatic NADPH-dependent Cr(VI) reductase activity, which was eliminated upon boiling the enzyme. Control reactions containing only NADPH and Cr(VI) showed significant background Cr reduction, however (data not shown).

In addition to the NADPH-dependent reductase activity, we also identified a *c*-type cytochrome (ApcA) that has the ability to reduce Cr(VI). This protein is redox-active towards reduced menadione (Fig. 1 inset), with the initial oxidized state (solid line) shifting to a reduced state (dashed line) upon reduction by menadiol. ApcA also shows a characteristic change in absorption spectra after reoxidation with Cr(VI) (Fig. 1). Oxidized protein (solid line) is readily reduced by dithionite (dashed line), and addition of Cr(VI) reoxidizes the protein (dotted line). This appears to be pH-dependent, with lower pH being favored for complete reoxidation of the protein. Additional evidence for Cr(VI) reduction capability is shown in Fig. 2, where cyclic voltammetry results show that ApcA can transfer electrons to Cr(VI), demonstrating catalytic reduction of Cr(VI), again favored at low pH. The protein-only sweep (thin line) clearly shows the reduction and oxidation potentials of ApcA, while addition of Cr(VI) (thick line) results in a significant increase in the reduction peak, and disappearance of the oxidation peak, indicating electron transfer from reduced cytochrome to Cr(VI).

Proteogenomic analysis

The genome of *A. cryptum* JF-5 contains three genes putatively associated with chromium metabolism (Table 1). Among these are genes for Cr(VI) transport, as well as the genes for an NADPH-dependent



◀ **Fig. 1** UV–Visible absorption spectra of purified ApcA reacting with Cr(VI) at pH 3.0 (*top panel*) and pH 6.8 (*bottom panel*); Inset shows reduction of ApcA by menadiol

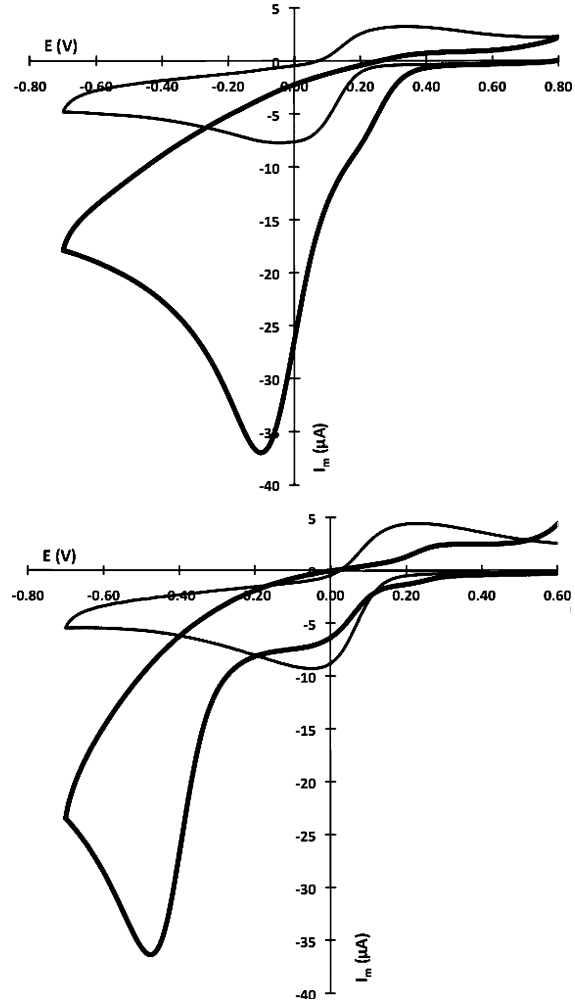


Fig. 2 Cyclic voltammetry analysis of ApcA at pH 3.0 (*Top*) and pH 6.8 (*Bottom*)

FMN reductase. In addition, the genome also harbors the genes for a number of monoheme cytochromes *c* (Table 2). Proteomic analysis demonstrated that ApcA (Acry_2099) is expressed in higher amounts in Cr(VI)-grown cells than in Cr(VI)-free cells (Table 3).

Homology modeling of ApcA

The Fold and Function Assignment System (FFAS, October 2009) gave a z score of −70.3, and protein

Table 1 *Acidiphilium cryptum* predicted proteins hypothetically involved in Cr(VI) metabolism

Locus ID	Function	Closest Match	Source of Match	BLAST E-value	Comments
Acry_1913[a]	Chromate Transport	ChrA	*Burkholderia* ZP_02377597	7e-37	Paired with Acry_1914
Acry_2828[b]	Reduction	ChrR	*Comamonas* ZP_01521939	4e-29	Most likely involved in peroxide generation and regeneration
Acry_1073[c]	Reduction	ArsH	*Phenylobacterium* YP_002130282	4e-95	Next to phosphate transporter

[a] Acry_1913 encodes a full length protein, Mr 17400, ChrA. Acry_1914 has no start codon, most likely not expressed

[b] Acry_2828 encodes full-length NADPH-dependent FMN reductase, Mr 20400, More hits with ChrR than Acry_1073

[c] Acry_1073 encodes a full-length NADPH-dependent flavin reductase. Mr 27868, ArsH-like

Table 2 *Acidiphilium cryptum* predicted proteins encoding potential Cr(VI) reducing *c*-type cytochromes

Locus ID	Name	Length(aa)	Signal[a]	Functional Description
Acry_2099	ApcA	123	Yes	10.1 kDa monoheme cytochrome c Class I
Acry_3471	ApcB	199	Yes	11.7 kDa monoheme cytochrome c Class I
Acry_2798	OacA	410	Yes	42.0 kDa monoheme cytochrome c Class I

[a] Signal sequence for export through cytoplasmic membrane to periplasm or outer membrane, as predicted by SignalP Version 3.0. Expression of all three proteins has been experimentally verified

alignments also showed a high similarity between ApcA and the template 1HROA (Fig. 3). The associated table describes each hit in detail. Conserved lysine (K) residues are shown in pink, while the heme-binding and -coordinating resides are shown in red. Figure 4 shows a homology model and surface charge rendition for ApcA from *A. cryptum*. Of particular interest is the presence of lysine residues in ApcA that provide a potential binding site for Cr(VI). The heme resides in a pocket containing coordinating Met and His residues, and surface charge representations are positive (blue), negative (red), polar (green), and hydrophobic (white).

Discussion

Acidiphilium cryptum possesses several potential biochemical mechanisms for Cr(VI) reduction. In previous work, we demonstrated that *A. cryptum* not only grows in the presence of significant amounts of Cr(VI), but that it can utilize two distinct pathways for Cr(VI) reduction: direct enzymatic reduction of Cr(VI) and an abiotic reaction involving Fe(II)-mediated Cr(VI) reduction, where Fe(II) is generated via the Fe-respiration pathway (Cummings et al. 2007). The present work extends these earlier findings, indicating the presence of an NADPH-dependent enzyme activity, as well as a *c*-type cytochrome that has Cr(VI) reduction capability. And, although NADPH-dependent Cr(VI) reductase activity can be measured in cell fractions, the genome-predicted proteins (FMN or FAD reductases) are not detected by proteomic analysis.

Upon more detailed investigation, ApcA was shown to have the requisite redox properties to serve as a Cr(VI) reductase. Interestingly, its ability to accept electrons from reduced quinones suggests that it can serve as an intermediary electron transporter between respiratory complexes and electron accepting metals, providing *A. cryptum* access to electron accepting substrate(s) not available to competing microbes. The UV–Visible and electrochemical redox measurements clearly show that ApcA has the proper midpoint potential (+375 mV vs. SHE) to donate electrons from the respiratory chain to Cr(VI) ($E_0 = +500$ at pH 6.8, +1000 mV at pH 2.0). Furthermore, electron transfer to Cr(VI) is observed with both UV–VIS and CV techniques at low pH values, confirming that under a physiologic pH of 3, ApcA can directly conduct electrons from the electrode to soluble Cr(VI). Homology modeling revealed a structure with high confidence based on sequence similarity to the 1HROA template used to

Table 3 Proteins expressed under Cr(VI)-grown conditions, as determined by proteomic analysis

Condition	Acc. Number	Description	Peptides[a]	Coverage (%)[b]
No Cr(VI)				
	YP_001235614.1	Chaperonin Cpn10	4	50.00
	YP_001235073.1	50S ribosomal protein L7 L12	9	49.61
	YP_001235108.1	Histone family protein DNA binding protein	4	48.31
	YP_001235948.1	Phasin family protein	8	45.76
	YP_001235068.1	Elongation factor Tu	23	43.29
	YP_001233321.1	OmpA MotB domain containing protein	12	41.83
	YP_001235613.1	Chaperonin GroEL	23	41.71
	YP_001235069.1	30S ribosomal protein S7	5	36.08
	YP_001233507.1	OmpA MotB domain containing protein	7	35.33
	YP_001235325.1	Homocysteine methyltransferase	22	32.38
30 μM Cr(VI)				
	YP_001235614.1	Chaperonin Cpn10	4	50.00
	YP_001235073.1	50S ribosomal protein L7 L12	10	49.61
	YP_001235108.1	Histone family protein DNA binding protein	5	48.31
	YP_001235068.1	Elongation factor	19	38.99
	YP_001233507.1	OmpA MotB domain containing protein	8	38.32
	YP_001235093.1	Monosaccharide transporting ATPase	12	38.21
	YP_001234058.1	Histone family protein DNA binding protein	4	34.48
	YP_001235217.1	**Cytochrome c class I (ApcA)**	**5**	**31.71**
	YP_001235948.1	Phasin family protein	7	30.51
100 μM Cr(VI)				
	YP_001234755.1	Signal transduction protein	11	55.36
	YP_001235093.1	Monosaccharide transporting ATPase	20	54.47
	YP_001235614.1	Chaperonin Cpn10	4	50.00
	YP_001235073.1	50S ribosomal protein L7 L12	7	49.61
	YP_001234058.1	Histone family protein DNA binding protein	6	43.68
	YP_001235613.1	Chaperonin GroEL	29	43.53
	YP_001235068.1	Elongation factor Tu	19	38.99
	YP_001235948.1	Phasin family protein	7	35.59
	YP_001233507.1	OmpA MotB domain containing protein	7	35.33
	YP_001233321.1	OmpA MotB domain containing protein	10	34.07
	YP_001235217.1	**Cytochrome c class I (ApcA)**	**5**	**31.71**

[a] Number of distinct peptides obtained from sample

[b] Percent coverage of the entire protein

create the model. The FFAS03 analysis comparing ApcA to 1HROA gave a value of $z = -70.3$, which substantiates the use of this template for homology modeling. ApcA has a predicted isoelectric point of 9.3, indicating that basic amino acid residues would hold a net positive charge at pH 6.8, facilitating interactions with negatively-charged metal oxyanions such as chromate. The conservation of lysine residues in cytochromes from Cr-reducing genera [e.g., *Desulfuromonas* (Assfalg et al. 2002)] adds strength to this argument, and suggests a utilitarian role for periplasmic and extracellular cytochrome *c* in metal-transforming microorganisms. Basic cytochromes such as ApcA may be adapted for function in acid environments, where charge balance is required for full protein reactivity. While the cytochromes from

Fig. 3 Alignment of ApcA with *D. acetoxidans* cytochrome c_7, the highest BLAST hits for ApcA, and closest experimentally determined structure

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                1        10        20        30        40        50        60
                |        |         |         |         |         |         |
          ApcA  MK-STL-LIA-------VAAGAFCIASAQAANVAHGKALFQAQCAACHS-VSP-GQNGIG
        1HRO_A  G-----------------------SAPPGDPV-EGKHLFHTICITCHTDIK--GANKVG
        1HH5_A  AD---------------VVTYENKKGNVTFDHKAHAEKL---GCDACHE-----GT----
     YP_001166202  MSVRAFMLTAGLSA---VALASGALATPTAD-VARGKALF-ARCAGCHA-VVP-GARSIG
     YP_003061112  MRIPTLPKISFVLATCTLATFAPTLSVAHAD-VEKGEKLFKLQCAVCHY-VEP-AKTKMG
      ZP_06350198  MK--AI-KIAMVGA--ALVWSASAYAA--GDPV-KGEQVFK-QCKICHQ-VGPTAKPGVG

          ApcA  PSLAGVYGAKAAATPGFQFSPA-LKKSGIVWNASTLDKFLANPQADVPGTKMPYMGMANA
        1HRO_A  PSLYGVVGRHSGIEPGYNYSEANIK-SGIVWTPDVLFKYIEHPQKIVPGTKMGYPGQPDP
        1HH5_A  PAKIAI-DKKSA------HKDA-CK---------TCHK--SN-NG--P-TKCG--G----
     YP_001166202  PNLAGVEGRKAASAGSYAYSPA-LARLKITWDRGNLDKFLAGPQKMAPGSKMAFAGLADP
     YP_003061112  PSLFGIVGSKSASSEGFRYSPA-MKEAGLTWDAQTLDEFLASPRKAVPRTSMAFVGQKSE
      ZP_06350198  PVQNNVVGSKAGSRPGFNYSDA-MKNSGLTWDEATLDKYLENPKAVVPGTKMVFVGLKNP

          ApcA  TDRADVVAYLQTL-GK
        1HRO_A  QKRADIIAYLETL--K
        1HH5_A  -------CHI-----K
     YP_001166202  GQRADVIAYLATLQ-D
     YP_003061112  EKRADLIAYLETL--K
      ZP_06350198  QDRADVIAFLATQHGQ
```

Accession Number	% Identity	BLAST E-Value	Description
1HRO_A	45	3.99e-20	High potential cytochrome c_2 *Rhodopila globiformis*
1HH5_A	21	--	Chain A, Cytochrome c_7 From *Desulfuromonas acetoxidans*
YP_001166202	52	4.15e-24	cytochrome *c*, class I *Novosphingobium aromaticivorans* DSM 12444
YP_003061112	49	5.28e-26	cytochrome *c* class I *Hirschia baltica* ATCC 49814
ZP_063 50198	46	5.42e-22	cytochrome *c* class I *Rhodomicrobium vannielii* ATCC 17100

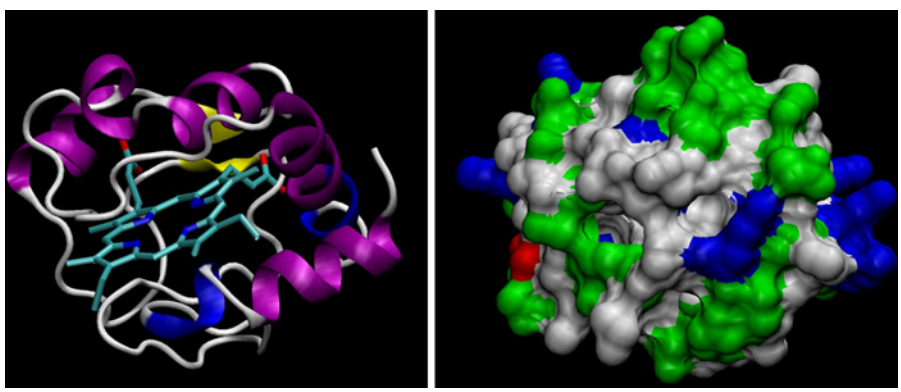

Fig. 4 Homology model of ApcA with secondary structure and heme (*left panel*), and surface charge (*right panel*)

A. cryptum and *D. acetoxidans* differ in heme content, it is plausible that three neighboring molecules of ApcA can deliver the same requisite charge (3 e−) to transform Cr(VI) to Cr(III) that one molecule of triheme cytochrome c_7 can deliver.

A hypothetical model for ApcA-mediated Cr(VI) reduction in *A. cryptum* is shown in Fig. 5. ApcA can be reduced by the quinol pool in the inner membrane (IM), and then react with Cr(VI) in the periplasm (PP). Alternatively, ApcA released by cell lysis or outer membrane (OM) vesicle formation can reduce extracellular Cr(VI). This model is based on several lines of evidence: (1) ApcA is the predominant expressed cytochrome detected by proteomics; (2) ApcA shows reducibility by quinones; (3) reduced ApcA shows reductive activity towards Cr(VI) at both acidic and neutral pH, indicating both intracellular and extracellular roles for it in Cr(VI) reduction;

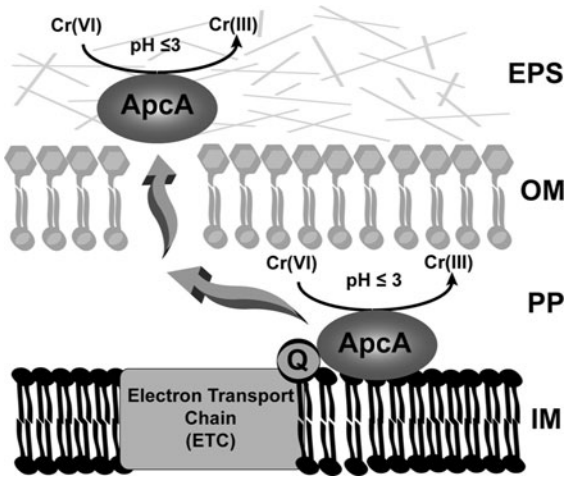

Fig. 5 Hypothetical electron transport model for *A. cryptum* JF-5 chromate reduction

(4) Homology modeling and sequence analysis reveals a putative set of lysine residues very similar in arrangement to the known Cr(VI) binding site of cytochrome c_7 from *D. acetoxidans* (Assfalg et al. 2002). These results are also important in regards to the real nature of chromate reductases, in particular the flavin-dependent proteins found in many representative bacteria. It has been shown (Mazoch et al. 2004) that the flavin-dependent ferric reductase FerB of *Paracoccus denitrificans* also reduces Cr(VI), suggesting that these enzymes can reduce metals non-specifically simply by using reduced NAD(P)H coupled to reduction of protein-bound flavin, which in turn reduces metals (e.g. Cr(VI), Fe(III)) that come into contact with the enzyme.

Many microbial systems have been examined in regards to chromium reduction and the biochemistry of the process, but interestingly, no single mechanism or pattern seems to be conserved for this process to occur. For example, in the sulfate-reducing bacteria, chromium reduction is thought to proceed via periplasmic or outer membrane *c*-type cytochromes (Assfalg et al. 2002), and by hydrogenases that couple oxidation of molecular hydrogen to reduction of Cr(VI) by a partner cytochrome *c* or ferredoxin (Chardin et al. 2003). In Pseudomonads, the flavin-containing chromium reductase system is present in the periplasm or cytosol (Park et al. 2000). Other oxidoreductases can reduce chromium, such as membrane-associated quinone reductases (Sedlacek et al. 2009). The findings reported here with *Acidiphilium* suggest that this metabolically versatile bacterium actually possesses the genomic potential for Cr(VI) reduction by any one of these mechanisms, and proteomic expression analysis confirms that at least one mechanism is active for chromium reduction.

The physiology and biochemistry of acidophilic microorganisms becomes very important when considering remediation options for acidic, contaminated environments such as those found on several nuclear reservations. While neutrophilic organisms such as *Geobacter* and *Shewanella* have been shown to possess many physiologic traits that make them superior bioremediation agents in pH-neutral environs, only the true acidophiles, including *A. cryptum*, will be useful in remediating acidic systems, due to their adaptations to these conditions. In summary, we have demonstrated the physiologic mechanisms and genomic capacity for Cr(VI) reduction in *A. cryptum*, and thus show that this organism, and presumably other acidophiles, do carry out these reductive reactions under acidic conditions. To the best of our knowledge, this is the first description of the biochemistry of this phenomenon in acidophilic bacteria.

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