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Bacterial diversity in Cr(VI) and Cr(III)-contaminated industrial wastewaters

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Abstract The bacterial community structure of a chromium water bath, a chromium drainage waste system, a chromium pretreatment tank, and a trivalent chromium precipitation tank from the Hellenic Aerospace Industry S.A. was assessed using 16S rRNA libraries and a high-density DNA microarray (PhyloChip). 16S rRNA libraries revealed a bacterial diversity consisting of 14 distinct operational taxonomic units belonging to five bacterial phyla: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, and *Bacteroidetes*. However, employing a novel microarray-based approach (PhyloChip), a high bacterial diversity consisting of 30 different phyla was revealed, with representatives of 181 different families. This made it possible to identify a core set of genera present in all wastewater treatment stages examined, consisting of members of *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria*, *Deltaproteobacteria, Epsilonproteobacteria*, and *Bacteroidetes.* In the chromium pretreatment tank, where the concentration of Cr(VI) is high (2.3 mg/l), we identified the presence of *Pseudomonadales*, *Actinomycetales*, and *Enterobacteriales* in abundance. In the chromium precipitation tank, where the concentration of Cr(III) is high, the dominant bacteria consortia were replaced by members of *Rhodocyclales* and *Chloroflexi*. The bacterial community structure changed significantly with changes in the chromium concentration. This in-depth analysis should prove useful for the design and development of improved bioremediation strategies.

Keywords Bacterial diversity · 16S rRNA gene · Cr(VI) · PhyloChip · Chromium treatment plant

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Introduction

Chromium, a steel-gray, lustrous, hard and brittle metal, occurs in several oxidation forms ranging from −2 to +6, the trivalent and hexavalent states being the most stable (Cheung and Gu 2007). Cr(VI) enters the environment through anthropogenic activities such as chromite ore processing, electroplating, corrosion control, wood preservation, and leather-tanning processes (Chuan and Liu 1996). Hexavalent chromium is water soluble, highly toxic, mutagenetic to most organisms, and carcinogenic to humans (Costa and Klein 2006; Wise et al. 2008). In contrast, trivalent chromium is at low concentrations essential for

human, plant and animal nutrition (Anderson 1997; Cefalu and Hu 2004), as it has an important role in sugar and fat metabolism; however, in excess, it can cause allergic skin reactions and cancer (Kotas and Stasicka 2000; Viti et al. 2003). Cr(III) is water insoluble and about three hundred times less toxic than Cr(VI), since it does not permeate eukaryotic and prokaryotic membranes (Poljsak et al. 2010; Viti et al. 2003).

Removal or reduction of Cr(VI) to Cr(III) serves as a key process for removal of Cr(VI)-contaminated water and wastewater. Different technologies are available: chemical precipitation, coagulation, ion exchange, membrane technologies, adsorption, and reverse osmosis (Owlad et al. 2009). The most common conventional method for Cr(VI) removal is reduction to Cr(III) at low pH and precipitation of $Cr(OH)_3$ by increasing pH to 9–10 with lime. The disadvantage of the precipitation method is the need to dispose of the solid waste. In addition, the methodologies mentioned above become ineffective with Cr(VI) concentrations below 2 mM (Garavaglia et al. 2010). Recently, microbial-based technologies to remove metal pollutants have attracted much attention.

Bacteria detoxify chromium mainly by reducing Cr(VI) to Cr(III), through Cr(V) and Cr(IV) intermediates (Cheung and Gu 2007); it is a potentially a useful process for remediation of Cr(VI)-affected environments. Reduction of Cr(VI) to Cr(III) can be performed by a wide range of bacteria, including *Pseudomonas aeruginosa* (Aguilera et al. 2004), *P. synxantha* (Gopalan and Veeramani 1994; McLean et al. 2000), *P. putida* (Park et al. 2002), *P. ambigua* (Suzuki et al. 1992), *P. fluorescens* (Lovley 1993), *P. dechromaticans*, and *P. chromatophila* (Cheung and Gu 2007). Bacteria from other genera that have been shown to reduce Cr(VI) include *Acinetobacter lwoffii* (Tekerlekopoulou et al. 2010), *Bacillus megaterium* (Cheung et al. 2006), *Shewanella alga* (Guha et al. 2001, 2003), *Aeromonas dechromatica* (Lovley 1993), and *Escherichia coli* ATCC 33456 (Shen and Wang 1995). Sulfate-reducing bacteria like *Desulfovibrio desulfuricans* and *D. vulgaris* have also been also reported to reduce Cr(VI) (Lovley 1993). Finally, some extremophiles have been found to reduce Cr(VI); among them are the radiation-resistant *Deinococcus radiodurans* (Fredrickson et al. 2000), *Thermoanaerobacter ethanolicus* isolated from millions of years old subsurface sediments (Roh et al. 2002), and *Pyrobaculum islandicum,* which is capable of reducing Cr(VI) at high temperatures (Kashefi and Lovley 2000). Resistance to Cr(VI) has been investigated in *Pseudomonas aeruginosa*, which has been attributed to the decreased uptake and/or enhanced efflux of Cr(VI) by the cell membrane (Aguilera et al. 2004). A similar resistance mechanism has been attributed to *Cupriavidus metallidurans* CH34 (formerly known also as *Ralstonia metallidurans*, *Waustersia eutropha*, and *Alcaligenes eutrophus*), a well-studied strain that is resistant to eight metals including CrO_4^{-2} (Mergeay et al. 2003; Vaneechoutte et al. 2004).

The aim of this study was to investigate the bacterial diversity and community structure of: (a) the chromium water bath from the Hellenic Aerospace Industry S.A. using a 16S rRNA library approach and (b) of the wastewater treatment plant from the same industry, using 16S rRNA libraries and a high-density oligonucleotide microarray that permits simultaneous monitoring of the population dynamics of almost 9000 distinguishable prokaryotic taxa/operational taxonomic units (OTUs). The results of this study provide a detailed description of the succession of the microbial communities in the stages of a chromium wastewater treatment plant, starting from the chromium water bath with a concentration of 248 g/l of chromic acid and 2.5 g/l of sulfuric acid, down to the trivalent wastewater chromium precipitation tank with a total chromium concentration of below 50 μg/l.

Materials and methods

Sampling

Samples were collected at the Hellenic Aerospace Industry located in the region of Schimatari (Viotia, Greece) in January 2009 from: (1) a chromic acid bath (CB), where metal objects are immersed to be coated with a thin layer of chromium, (2) a chromium drainage waste system (CD), (3) a chromium pretreatment tank (CPR), and (4) a trivalent chromium precipitation tank (CP). All samples were collected in sterilized glass bottles using routine microbiological precautions. Four samples (~4 l each) were collected from each sampling site.

The chromic acid bath displayed an extremely low pH of 1.3. The temperature of the bath was 50°C. Salinity in the chromium acid bath was 254.5 psu with a dissolved oxygen value of 4.4 mg/l. No natural light was reaching the CB tank. The pH values of CD, CPR, and CP were 6.8, 6.5 and 9.6, while the temperatures were 9.6, 9, and 9°C, respectively. Salinity of CD, CPR, and CP was 0.37, 0.6, and 0.41 psu, the dissolved oxygen was measured to 2.4, 7.4, and 3.87 mg/l, the Cr(VI) concentration was measured at 0.84, 2.3, and 0.08 mg/l, while the Cr(III) concentration was measured at 1.5, 2.3, and 0.05 mg/l respectively. All measurements were performed using the Multi-Parameter Troll 9000 WQP-100 (In Situ Inc.), except the Cr(VI) concentration which was determined by the 3500-Cr D Colorimetric method according to the "Standard Methods for the Examination of Water and Wastewater" (APHA 1985), using a spectrophotometer. Trivalent chromium was

estimated as the difference between total and hexavalent chromium.

DNA extraction, and PCR amplification and sequencing of 16S rRNA genes

For the isolation of genomic DNA, 4 l each from the three industrial wastewater samples were filtered first through a sterile 3 μm glass fiber filter (Whatman, Florham Park, NJ, USA) and then through a sterile 0.2 μm of membrane filter (Whatman, Florham Park, NJ, USA). For the chromium plating bath due to the extremely high concentration of Cr(VI) and its high toxicity, 2 l out of the 4 l were filtered following the above procedure. Total DNA was extracted as was previously described (Tekerlekopoulou et al. 2010). The DNA was quantified with a Qubit fluorometer (Invitrogen, USA) according to the manufacturer's instructions.

Near full-length 16S rRNA gene amplification for library construction and PhyloChip analysis was carried out using universal 16S primers for bacteria (27F and 1492R) and archaea (4Fa and 1492R) (Lane 1991). A reaction mixture (20 μl) containing the PCR buffer supplied by the polymerase manufacturer, 1.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, the appropriate primers at 0.3 mM each, and 1 U Taq polymerase (Takara Mirus Bio Inc., WI, USA), was prepared. PCR reactions were performed using a PTC-200 thermocycler (MJ Research Inc., USA) with a denaturation step of 10 min at 94°C, followed by 35 cycles for library construction and 30 cycles for the PhyloChip analysis of: 1 min denaturation at 94°C, 1 min primer annealing at 52°C for the library construction; for the PhyloChip analysis, eight annealing temperatures between 48 and 58°C (48, 48.3, 49.7, 52.3, 54, 56.5, 57.8, 58°C) were used, and finally 90 s extension at 72°C. The PCR was completed by a final extension at 72°C for 10 min.

The size of the PCR products was determined by agarose gel electrophoresis using appropriate size markers. No PCR products were generated using the universal archaeal primers. The bacterial PCR products were polyethylene glycol (PEG) precipitated (Hartley and Bowen 2003). Purified DNA was cloned into pGEM-T, according to the manufacturer's protocol (Promega, Madison, WI, USA), and transformed into competent DH5α cells. White colonies on ampicillin/X-gal plates were screened for inserts of correct length by PCR with the pGEM-T compatible primers T7 and SP6. Inserts were fully sequenced with the same primers and with internal 16S rRNA gene-specific primers (Tsiamis et al. 2008). Sequencing was performed using an ABI310 analyzer according to the manufacturer's instructions (Applied Biosystems, USA). The 16S rRNA gene sequences reported in this study have been deposited in GenBank with accession numbers JN697380 to JN697571.

Sequence and phylogenetic analysis

Sequencing analysis was performed using the Mothur software package (Schloss et al. 2009). All clones were first screened for potential chimeric structures using CHIMERA-CHECK (http://rdp.cme.msu.edu) and BELLEROPHON (Huber et al. 2004). After sequencing, clustering analysis was performed and the closest relative was assigned using the Ribosomal Database Project (Cole et al. 2007, 2009). Alignment of sequences was carried out using the program MUSCLE (Edgar 2004). A phylogenetic tree, based on the distance matrix method, was constructed using the software package Phylip 3.69. Evolutionary distances were calculated using the F84 model, and topology was inferred using the "neighbor-joining" method. A phylogenetic tree calculated by maximum parsimony, using the PAUP phylogenetic package, was also generated. Sequences with 1110-bp length were used for tree constructions. Bacterial diversity was estimated by abundance-based coverage estimator (ACE), Shannon–Weaner index, and Chao1 index of species richness (Chao 1984, 1987). Statistical analyses of all samples were performed on the genetic distance matrices using LIBSHUFF (Schloss et al. 2004) to determine whether variability in library composition between treatments was due to chance or real biological effects.

Microarray sample preparation and analysis

16S rRNA gene amplification and 16S rRNA microarray sample preparation were performed as previously described (Tsiamis et al. 2008). To identify significantly different pairs of samples, the UniFrac significance test was used (Hamady et al. 2010). UniFrac distances are based on the fraction of branch length shared between two communities within a phylogenetic tree inferred by PhyloTrac (http://www.phylotrac.org), based on the PhyloChip data from all the communities. We used unweighted UniFrac, in which only the presence or absence of lineages is considered (community membership) and weighted UniFrac, which also accounts for relative abundance (community structure).

Results

16S rRNA libraries

PCR products were only generated with the universal bacterial primers; the archaeal universal primers produced no PCR fragments.

A 16S rRNA gene bacterial library was constructed from each of the chromic acid bath (CB), the chromium

Table 1 Biodiversity indices and statistics among the 16S rRNA libraries

Library	Clones sequenced	OTUs	Chao1 (95% CI)	% coverage (genus level-95%)	H′ (95% CI)
CB	15	3	4	86.7	0.48
CD	51	3	3	100	0.77
CPR	45	2	2	100	0.18
CP	81	6	6	100	1.2

drainage waste system (CD), the chromium pretreatment tank (CPR), and the trivalent chromium precipitation tank (CP). A total of 192 independent sequences were obtained. Based on the coverage estimates (Table 1), clone libraries of CD, CPR, and CP were completely sampled, whereas CB was relatively well sampled (Table 1). The CP library had the highest community richness (Shannon–Weaver H′) and Chao1 index, followed in this order by CD, CB, and CPR (Table 1). Based on ∫-LIBS-HUFF analyses, the chromium pretreatment (CPR) 16S rRNA library is significantly different from the other three (Table 2).

Table 2 ∫-LIBS-HUFF significance of a chromic acid bath (CB), a chromium drainage waste system (CD), a chromium pretreatment tank (CPR) and a trivalent chromium precipitation tank (CP) based on 16S rRNA libraries

Sample source	∫-LIBS-HUFF			
	CB	CD	CPR	CP
CB				
CD	0.4831			
CPR	<0.0001	<0.0001		
CP	0.7539	0.0018	<0.0001	

Sequence analysis

The four 16S rRNA libraries covered 14 different microbial OTUs, with the *Betaproteobacteria* (43%), *Alphaproteobacteria* (30%), and Gammaproteobacteria (23%) being the dominating classes. *Bacteroidetes* (3%), *Actinobacteria* (1%) were also represented.

The CB library contained bacteria from three classes, dominated by *Betaproteobacteria* (87%), while members of Alphaproteobacteria (6%) and *Actinobacteria* (6%) were also detected. All members of the *Betaproteobacteria* were closely related to *Ralstonia* sp., while the *Alphaproteobacteria* were related to *Sphingomonas* sp. and the *Actinobacteria* to members of the *Propionibacteriaceae* (Fig. 1). The CD library was populated exclusively by *Alphaproteobacteria* and *Betaproteobacteria* grouped in the bacterial orders of *Rhizobiales*, *Burkholderiales*, and *Rhodospirillales*. In contrast, the CPR library was dominated by *Gammaproteobacteria,* with all forty-five members closely related to *Acinetobacter* spp., the closest relatives being *A. johnsonii* and *A. beijerinckii* (Fig. 1). Finally, a total of six clusters were represented in the CP library, which was dominated by *Alphaproteobacteria* (50%) and *Betaproteobacteria* (43%) and to a lesser degree by members of the *Bacteroidetes* (7%).

Classes exclusive to the chromium bath tank (CB) were the *Actinobacteria,* with members of the *Propionibacteriaceae* family, while the *Bacteroidetes* were found only in the CP library and *Gammaproteobacteria* only in the CPR library (Fig. 1).

PhyloChip analysis

For the PhyloChip analysis, sufficient amount (500 ng) of 16S rRNA gene PCR product was generated for the chromium drainage waste system (CD), the chromium pretreatment tank (CPR), and the trivalent chromium precipitation tank (CP). The chromium plating bath (CB) did not produce satisfactory amount of 16S rRNA PCR product and it was excluded from the PhyloChip analysis.

The microarray-based approach revealed a significant bacterial diversity related to 181 families from 30 different phyla out of the 63 that are currently represented in the PhyloChip. Bacterial taxa include members of the bacterial phyla of *Acidobacteria*, *Actinobacteria, Bacteroidetes*, *Chlorobi*, *Chloroflexi*, *Coprothermobacteria*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Lentisphaerae, Planctomycetes*, all *Proteobacteria* sub-divisions, *Spirochaetes*, *Synergistetes*, *Thermodesulfobacteria*, *Verrucomicrobia*, candidate bacterial phyla BRC1, NC10, OP10, OP3, SPAM, TM7, marine group A, and unclassified bacteria (Table 3; Figure S1A, S1B).

The bacterial diversity in the chromium drainage waste system (CD) was found to be dominated mainly by *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Cyanobacteria*, *Bacteroidetes*, and *Planctomycetes* (Figure S1A). Members from candidate divisions and also from some other phyla were also detected, but to a lesser degree (Table 3; Figure S1B). The bacterial diversity in the chromium pretreatment tank (CPR) was limited compared to the diversity encountered

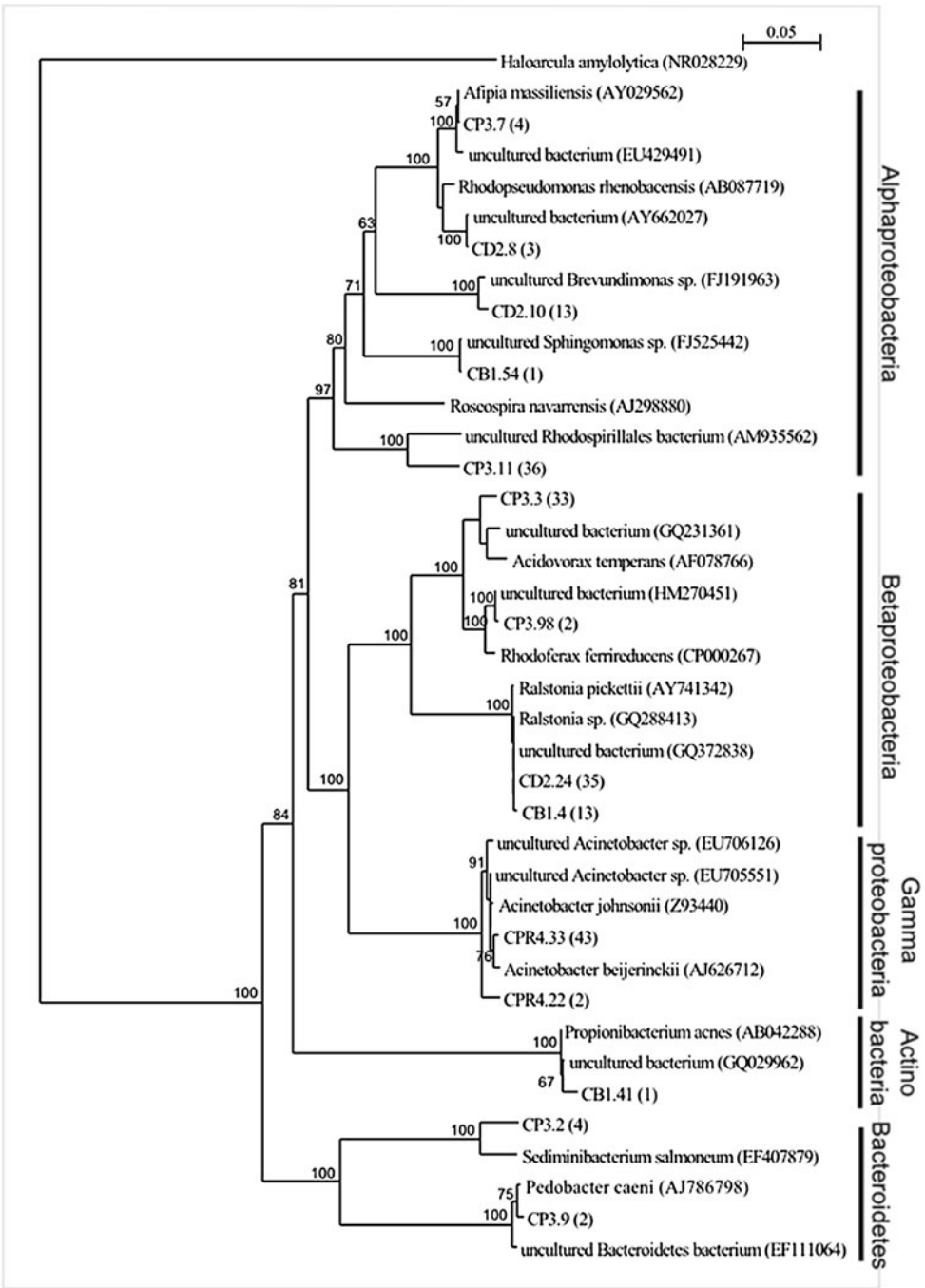

Fig. 1 Phylogenetic relationships based on 16S rRNA gene sequence analysis from a chromic acid bath (CB), a chromium drainage waste system (CD), a chromium pretreatment tank (CPR), and a trivalent chromium precipitation tank (CP). Evolutionary distances were calculated using the F84 model, and the topology was inferred using the neighbor-joining method. *Numbers* on the nodes represent % bootstrap values based on 1000 replicates. *Scale bar* represents 0.05 substitutions per site. The 16S rRNA gene sequences of the archeae *Haloarcula amylolytica* was arbitrarily chosen as an outgroup

in the chromium drainage waste system (CD). It was dominated by *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*, *Firmicutes*, and *Bacteroidetes*. Finally, the trivalent chromium precipitation tank (CP) exhibited a limited bacterial diversity comprising mainly *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria, Actinobacteria*, *Bacteroidetes,* and unclassified bacteria (Figure S1A and S1B).

The weighted UniFrac was calculated, which weighs the branches based on abundance information, since the relative abundance of different bacteria can be critical for describing community changes. UniFrac analysis indicates that the bacterial community composition in the three Cr(VI) treatment stages tested were not significantly different. At least 40% of the OTUs detected with the PhyloChip were present in all three samples examined. Common members from ten bacterial orders include *Bradyrhizobiales*, *Rhizobiales*, *Sphingomonadales*, *Caulobacterales* of *Alphaproteobacteria*, *Burkholderiales* of *Betaproteobacteria, Pseudomonadales and Alteromonadales* of *Gammaproteobacteria*, *Campylobacterales* of

Table 3 Phylogenetic linkages between bacteria identified with a high-density phylogenetic DNA microarray (PhyloChip) from the three wastewater treatment stages of a Cr(VI) processing facility

Phylogenetic linkage	CD	CPR	CP
Acidobacteria			
Acidobacteria, Acidobacteriaceae	+[13]	+[2]/−[11]	+[3]/−[10]
Other *Acidobacteria*	+[2]/−[2]	+[2]/−[2]	+[4]
Actinobacteria			
Acidimicrobiales:Acidobacteriaceae	+[2]/−[2]	+[3]/−[1]	−[4]
Actinomycetales:Actinomycetaceae	+[2]	+[1]	−[2]
Actinomycetales:Corynebacteriaceae	+[6]	+[1]/−[5]	−[6]
Actinomycetales:Gordoniaceae	+[3]	−[3]	−[3]
Actinomycetales:Microbacteriaceae	−[4]	+[4]	−[4]
Actinomycetales:Micrococcaceae	+[3]	+[2]/−[1]	−[3]
Actinomycetales:Micromonosporaceae	+[4]	−[4]	−[4]
Actinomycetales:Mycobacteriaceae	+[6]	+[5]/−[1]	−[6]
Actinomycetales:Nocardiaceae	+[2]	−[2]	−[2]
Actinomycetales:Propionibacteriaceae	+[2]	−[2]	−[2]
*Actinomycetales:*unclassified	+[5]	+[4]/−[1]	+[1]/−[4]
Other *Actinomycetales*	+[4]/−[1]	+[2]/−[3]	−[5]
Bifidobacteriales:Bifidobacteriaceae	+[6]	+[3]/−[3]	−[6]
Rubrobacterales:Rubrobacteraceae	+[1]	−[1]	−[1]
Other Actinobacteria			
Bacteroidetes			
Bacteroidetes:Bacteroidales	+[4]/−[1]	+[4]/−[1]	+[4]/−[1]
Flavobacteria:Flavobacteriales	−[1]	−[1]	+[1]
KSA1	+[1]	+[1]	+[1]
Sphingobacteria:Sphingobacteriales	+[7]/−[8]	+[6]/−[9]	+[11]/−[4]
Caldithrix	−[1]	−[1]	+[1]
Chlorobi	+[2]	+[2]	−[2]
Chloroflexi			
Anaerolineae (*class*)	+[4]/−[1]	+[1]/−[4]	+[1]/−[4]
Dehalococcoidete	+[1]/−[1]	−[2]	+[2]
Thermomicrobia	−[1]	+[1]	−[1]
Chloroflexi (unclassified)	+[2]/−[2]	+[1]/−[4]	+[3]/−[1]
Coprothermobacteria	−[1]	−[1]	+[1]
Cyanobacteria			
Cyanobacteria (class)	+[12]	+[11]/−[1]	+[2]
Cyanobacteria (unclassified)	+[4]	+[1]/−[3]	−[4]
Deinococcus-Thermus	+[3]	+[1]/−[2]	+[1]/−[2]
Firmicutes			
Bacillales	+[1]/−[13]	+[13]/−[1]	+[1]/−[13]
Lactobacillales	+[4]	+[3]/−[1]	+[1]/−[3]
Clostridiales	+[15]/−[1]	+[3]/−[13]	−[16]
Catabacter (class)	+[2]	+[1]/−[1]	+[1]/−[1]
Desulfotomaculum (class)	+[3]	+[3]	+[1]/−[2]
Mollicutes (class)	+[3]	−[3]	+[1]/−[2]
Other *Firmicutes*	+[6]/−[1]	+[4]/−[3]	+[3]/−[4]
Gemmatimonadetes	+[7]	+[1]/−[6]	+[3]/−[4]
Lentisphaerae	+[2]/−[1]	+[2]/−[1]	+[3]

Table 3 continued

Phylogenetic linkage	CD	CPR	CP
Planctomycetes			
Planctomycetacia:Planctomycetales	+[7]/−[1]	+[5]/−[3]	+[3]/−[5]
Proteobacteria: Alphaproteobacteria			
Acetobacterales	+[6]/−[1]	+[1]/−[6]	+[4]/−[3]
Azospirillales	+[4]	+[1]/−[3]	+[2]/−[2]
Bradyrhizobiales	+[42]/−[6]	+[28]/−[20]	+[42]/−[6]
Caulobacterales	+[8]/−[5]	+[5]/−[8]	+[8]/−[5]
Consistiales	+[4]	−[4]	+[2]/−[2]
Rhizobiales	+[29]/−[9]	+[13]/−[25]	+[29]/−[9]
Rhodobacterales	+[7]/−[1]	+[4]/−[4]	+[6]/−[2]
Rickettsiales	+[4]/−[2]	+[3]/−[3]	+[4]/−[2]
Sphingomonadales	+[26]/−[6]	+[27]/−[5]	+[18]/−[14]
Other *α-Proteobacteria*	+[16]/−[4]	+[8]/−[12]	+[17]/−[3]
Proteobacteria: Betaproteobacteria			
Burkholderiales	+[70]/−[18]	+[45]/−[43]	+[75]/−[13]
Methylophilales	−[2]	+[2]	+[1]/−[1]
Neisseriales	+[1]/−[2]	+[2]/−[1]	+[2]/−[1]
Nitrosomonadales	+[4]/−[4]	+[6]/−[2]	+[7]/−[1]
Rhodocyclales	+[5]/−[8]	−[13]	+[12]/−[1]
Other *β-Proteobacteria*	+[5]/[5]	+[2]/−[8]	+[10]
Proteobacteria: Deltaproteobacteria			
Bdellovibrionales	+[1]	+[1]	+[1]
Desulfobacterales	+[1]/−[2]	+[1]/−[2]	+[1]/−[2]
Desulfovibrionales	+[6]/−[1]	+[4]/−[3]	+[2]/−[5]
Myxococcales	+[6]/−[2]	+[4]/−[4]	+[3]/−[5]
Syntrophobacterales	+[2]/−[1]	−[3]	+[2]/−[1]
Other *δ-Proteobacteria*	+[3]/−[1]	−[4]	+[1]/−[3]
Proteobacteria: Epsilonproteobacteria			
Campylobacterales	+[9]/−[2]	+[8]/−[3]	+[8]/−[3]
Nautiliales	−[1]	−[1]	+[1]
Proteobacteria: Gammaproteobacteria			
Acidithiobacillales	+[3]	+[1]/−[2]	−[3]
Aeromonadales	+[1]/−[1]	+[1]/−[1]	+[1]/−[1]
Alteromonadales	+[9]/−[10]	+[7]/−[12]	+[14]/−[5]
Chromatiales	+[1]/−[1]	+[1]/−[1]	−[2]
Enterobacteriales	+[16]/−[4]	+[14]/−[6]	+[2]/−[10]
Legionellales	+[4]/−[2]	+[3]/−[3]	+[3]/−[3]
Methylococcales	+[1]	+[1]	−[1]
Oceanospirillales	+[5]/−[2]	+[2]/−[5]	+[1]/−[6]
Pasteurellales	+[1]/−[1]	+[1]/−[1]	+[1]/−[1]
Pseudomonadales	+[29]/−[9]	+[32]/−[6]	+[9]/−[29]
Symbionts	+[1]	−[1]	−[1]
Thiotrichales	+[3]	+[3]	+[3]
Vibrionales	+[1]	−[1]	−[1]
Xanthomonadales	+[6]	+[3]/−[3]	+[5]/−[1]
Unclassified	+[5]/−[5]	+[3]/−[7]	+[6]/−[4]
Other *γ-Proteobacteria*	+[4]/−[1]	+[3]/−[2]	+[2]/−[3]

Table 3 continued

Phylogenetic linkage	CD	CPR	CP
Proteobacteria (unclassified)	+[4]/−[1]	+[2]/−[3]	+[1]/−[4]
Bacteria (unclassified)			
Spirochaetes	+[11]/−[1]	+[2]/−[10]	+[4]/−[8]
Synergistes	+[2]/−[1]	+[2]/−[1]	+[2]/−[1]
Thermodesulfobacteria	+[1]	+[1]	+[1]
Unclassified	+[7]/−[1]	+[6]/−[2]	+[4]/−[4]
Verrucomicrobia	+[4]/−[6]	+[7]/−[3]	+[4]/−[6]
Candidate division			
AD3	+[1]	+[1]	−[1]
BRC1	+[1]	−[1]	+[1]
DSS1	+[1]	−[1]	−[1]
NC10	+[1]	−[1]	−[1]
OP10	+[2]	+[1]−[1]	+[1]/−[1]
OP3	+[1]	+[1]	+[1]
OP9/JS1	−[1]	−[1]	+[1]
TM6	+[1]	−[1]	−[1]
TM7	+[2]	+[2]	+[2]
Marine group A	+[2]	+[2]	+[1]/−[1]

Numbers in brackets report members of bacteria with positive (+, high hybridization intensity phylogenetic relatives) and negative (−, hybridization intensity) 16S rRNA hybridization signals

CD chromium drainage waste, *CPR* chromium pretreatment tank, *CP* trivalent chromium precipitation tank

Epsilonproteobacteria, Myxococcales of *Deltaproteobacteria*, and *Sphingobacteriales* of *Bacteroidetes* (Figure S2).

A major change in the bacterial diversity was detected in the chromium precipitation tank (CP) with representatives from the orders *Pseudomonadales*, *Actinomycetales*, and *Enterobacteriales* being highly restricted, while members of the order *Rhodocyclales* are more abundant (Figs. 2, S1 and S2).

Discussion

16S rRNA library analysis

The percent library coverage based on Good's estimate for all samples indicate that a high degree of coverage was achieved for all libraries. The bacterial diversity in all samples examined was restricted, with the trivalent chromium precipitation tank exhibiting the highest diversity (Table 1).

16S rRNA library analysis indicates that the main cluster of *Ralstonia* sp. that was present in the chromium bath (CB) was also present in the chromium drainage system (CD) (Fig. 1). Interestingly, members of *Ralstonia* spp., identified in sample CB and CD, have been previously found in industrial and polluted biotopes like degreasing systems, arsenic, and radioactivity-contaminated soils (Sarret et al. 2005; Turpeinen et al. 2004). Members of *Ralstonia* spp. have also been found to survive and prosper in oligotrophic environment and to use a variety of compounds as energy and carbon sources, with a capacity to degrade a number of toxic substances (Ryan et al. 2007). Interestingly, *C. metallidurans* CH34 (formerly known as *Ralstonia metallidurans*) and its related strains are found almost exclusively in anthropogenic, industrial biotopes mostly bound to the activities and wastes of metallurgical activities (Mergeay et al. 2003). The genome of *C. metallidurans* CH34 contains more genes related to resistance to heavy metals than any other genome sequenced. It will be interesting to sequence the *Ralstonia* sp. strain that is present in the chromium bath capable of enduring concentrations of Cr(VI) up to 248 g/l at 1.3 pH. Due to the extremely high toxicity of this sample, a single cell genomic approach for deciphering the genome of this highly resistant *Ralstonia* sp. strain through a combination of small volume filtering and cell sorting using FACS will be appropriate.

The other two clusters from the chromium bath (CB), consisting of *Sphingomonas* sp. and *Propionibacterium* sp., have closest relative strains, which were isolated from contaminated sediments, metal-contaminated biotopes, and chromium-contaminated sites (Abulencia et al. 2006; Sarret et al. 2005; Thorenoor et al. 2009; Turpeinen et al. 2004). Abulencia et al. (2006) reported that *Sphingomonas* sp. in low biomass contaminated subsurface sediment cores, with several of those strains exhibiting a minimal inhibitory concentration of up to 0.5 mM of Cr(VI).

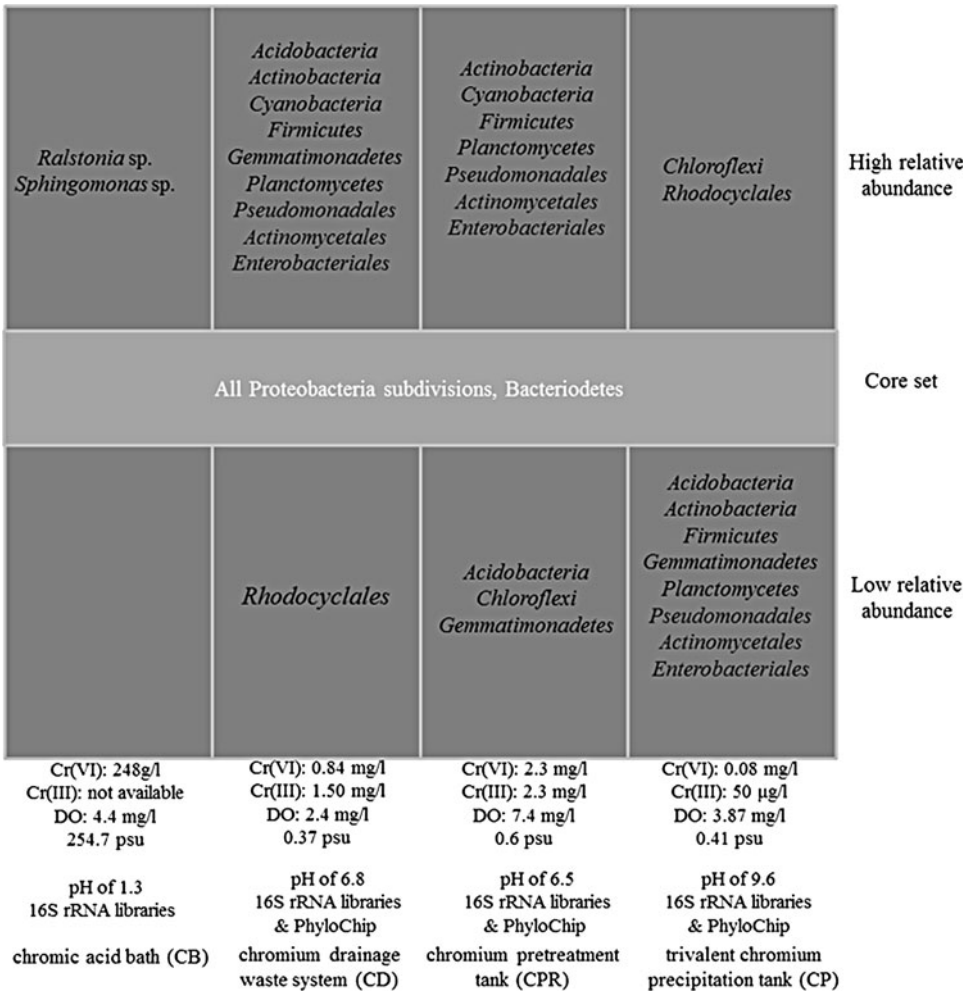

Fig. 2 Graphical representation of the bacterial community succession in a chromium water bath (CB) and the three stages of a wastewater treatment plant. Note that CB data are based only on 16S rRNA libraries. CD, CPR, and CP data are based on 16S rRNA libraries and PhyloChip data

In the chromium drainage system (CD) with a Cr(VI) concentration of 0.84 mg/l, the second and third most dominant groups were those of *Brevundimonas* sp. and *Rhodopseudomonas* sp. The closest relative of these two clusters were isolated from iron mines, sewage systems, swine effluents, and groundwater contaminated with high levels of nitric acid and uranium waste (Okabe et al. 2007). Among the vast array of pollutants that *Rhodopseudomonas* strains can reduce is Cr(VI) to Cr(III) with a rate of 10 mg/l per 2 h and 80 mg/l per 2 days at a cell density equivalent to optical density reading of 1.5 at 600 nm (Mehrabi et al. 2001).

In the chromium pretreatment tank (CPR) with a Cr(VI) concentration of 2.3 and 2.6 mg/l of Cr(III), the only cluster identified was that of *Acinetobacter* sp., members of which have been reported to be able to reduce Cr(VI) to Cr(III) from industrial sludge from the same electroplating plant (Dermou et al. 2005; Tekerlekopoulou et al. 2010). In the trivalent chromium precipitation tank (CP), the most dominant clusters were those of *Rhodospirillales* sp. and *Acidovorax* sp. Members of the former have a role in the bioremediation of aliphatic hydrocarbon-contaminated soil (Militon et al. 2010), and strains of the second cluster, together with members of the *Sediminibacterium* sp. cluster, are involved in the degradation of polyaromatic hydrocarbons (Humphries et al. 2005; Singleton et al. 2005, 2009). Finally, members of the *Afipia* sp., *Rhodoferax* sp., and *Pedobacter* sp. clusters are generally found in water treatment plants (Li et al. 2010; Shaw et al. 2008; Vanparys et al. 2005).

PhyloChip analysis

Use of PhyloChip, which is currently the most comprehensive microbial microarray, has a great advantage over other molecular approaches, since it permits the detection of a higher number of taxa, including low-abundance species, compared to 16S rRNA libraries (Brodie et al. 2006; DeSantis et al. 2007; Flanagan et al. 2007). It has been estimated that PhyloChip is able to detect more than 30 times as many OTUs as a 16S rRNA library (Van Nostrand et al. 2011). In comparison to previous studies, the use of a high-density oligonucleotide microarray resulted in the identification of one of the highest prokaryotic communities

ever reported for Cr(VI)-contaminated sites (Table 3). The application of a high-density oligonucleotide microarray revealed the presence of representatives of 181 families from 30 different phyla: a detection level that is at least ten times higher at phylum level compared to 16S rRNA libraries.

Interestingly, members of *Pseudomonadales*, *Actinomycetales,* and *Enterobacteriales* that have been previously reported to survive in heavy metal-contaminated sites (Chen and Hao 1998; Ishibashi et al. 1990; McLean et al. 2000; Rajkumar et al. 2005; Viti et al. 2006) and as Cr(VI)-removing bacteria (Martins et al. 2010) were present in abundance in the chromium pretreatment tank, in which the Cr(VI) had the highest concentration from the three chromium treatment tanks (Fig. 2). On the contrary, they were restricted in the trivalent chromium precipitation tank in which the Cr(VI) concentration was the lowest from the three waste treatment plants.

In the CP tank, the dominant bacteria changed to members of *Rhodocyclales* and *Chloroflexi,* species of which are known to be able to resist high concentration of chromium (Battaglia-Brunet et al. 2004; Martins et al. 2010; Zilles et al. 2002). Also, the high pH in CP could limit the growth of *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, *Pseudomonadales,* and *Enterobacteriales* members while supporting the growth of *Chloroflexi* and *Rhodocyclales* members (Fig. 2).

Finally, PhyloChip and 16S rRNA analysis indicate that *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria, and Epsilonproteobacteria*, together with the *Bacteroidetes,* constitute a core set of genera present in all water treatment stages examined (Figs. 2, S2). Members of these genera have been also identified in chromium-contaminated sites in India and USA (Desai et al. 2009; Joynt et al. 2006). Members of the *Bradyrhizobiales*, *Sphingomonadales*, *Caulobacterales*, *Rhizobiales*, *Burkholderiales*, *Alteromonadales*, *Myxococcales*, *Campylobacteriales*, and *Sphingobacteriales* were identified at a similar level in all samples examined (Table 3).

Finally, use of the PhyloChip enabled the report for the first time in chromium-contaminated sites the presence of members of *Gemmatimonadetes, Chloroflexi, Planctomycetes, Lentisphaerae, Spirochaetes, Synergistes, Thermodesulfobacteria, Verrucomicrobia, Chlorobi, Calditrix,* and of the candidate phyla of AD3, BRC1, DSS1, NC10, OP3, OP10, OP9/JS1, SPAM, TM6, TM7, and marine group A in chromium-contaminated sites (Table 3).

Conclusions

Our study characterizes in detail the microbial communities present in a chromium wastewater treatment plant. The findings suggest that a core set of bacterial communities, consisting mainly of all *Proteobacteria* subdivisions and *Bacteriodetes,* is present in all stages of the treatment plant. In those stages where the concentration of Cr(VI) is high, members of the *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes,* and *Planctomycetes* are relatively abundant. In contrast, when the concentration of Cr(III) increases, *Chloroflexi* and *Rhodocyclales* are most abundant. Finally, these findings provide also crucial information on the succession of bacterial communities in the stages of a chromium wastewater treatment plant. This information should aid in the development of improved bioremediation strategies.

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