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Molecular characterization of chromium (VI) reducing potential in Gram positive bacteria isolated from contaminated sites

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ABSTRACT

Hexavalent chromium [Cr(VI)] is highly toxic, teratogenic and carcinogenic to man and other animals. Some bacterial species have the ability to reduce Cr(VI) to a stable speciation state of trivalent chromium [Cr(III)], which is insoluble and comparatively less toxic. Therefore, the reduction of Cr(VI) thus provides potential as a means for environmental bioremediation of Cr(VI) pollution. In the present study bacteria isolated from chromium and diesel contaminated sites were found to have the ability to rapidly reduce highly toxic concentrations of Cr(VI) to Cr(III) when grown in minimal medium supplemented with glucose as the sole carbon source. Partial chromate reductase gene sequences were retrieved after PCR amplification of genomic DNA extracted from three Gram positive isolates which were highly similar (>99% sequence similarity) to chromate reductase genes found in Gram negative bacteria, more specifically those identified from *Escherichia coli* and *Shigella* spp. whole-genome studies. The isolated bacteria were putatively identified by 16S rRNA gene sequencing as *Arthrobacter aurescens* strain MM10, *Bacillus atrophaeus* strain MM20, and *Rhodococcus erythropolis* strain MM30.

1. Introduction

Contamination of the soil, surface water and groundwater with hexavalent chromium [Cr(VI)] is an issue of potential concern due to its toxicity (DEFRA, 2002). It is well known for its toxic, mutagenic, carcinogenic and teratogenic effects on human beings and other living organisms and is classified under priority pollutants in many countries (Ye and Shi, 2001; Avudainayagam et al., 2003). The toxicity of chromium to non-tolerant soil microorganisms inhibits the bioremediation of organic pollutants in contaminated soils (Kourtev et al., 2009). Chromate is generated as a by-product of a large number of industries including those engaged in welding, paper and pigment production, leather-tanning, chrome plating and thermonuclear weapons manufacturing. Cr(VI) bears structural similarity to sulphate (SO_4^{2-}), and is readily taken up by bacterial and mammalian cells through the sulphate transport system (Singh et al., 1998; Cervantes et al., 2001). It is more toxic than its reduced trivalent form [Cr(III)] which is rather considered essential for some biological functions (Krishna and Philip, 2005).

Metal pollutants, unlike organic contaminants, cannot be degraded. So, their detoxification can be achieved either by adsorption/accumulation or by conventional physicochemical treatments but they are quite expensive and cumbersome (Malik, 2004). Thus, biological detoxification of Cr(VI), transforming it to a less toxic oxidation state Cr(III), is considered as an ecofriendly and cost-effective technique for the environmental clean-up of this heavy metal contaminant (Camargo et al., 2003; Megharaj et al., 2003). A number of microorganisms have been reported to resist/tolerate Cr(VI) by periplasmic biosorption, intracellular bioaccumulation, and/or biotransformation to a less toxic speciation state through direct enzymatic reaction or indirectly with metabolites, and include members within the genera *Pseudomonas*, *Aeromonas*, *Streptomyces*, *Microbacterium*, *Desulfovibrio*, *Enterobacter*, *Escherichia*, *Shewanella*, and *Bacillus* (Cervantes and Silver, 1992; Camargo et al., 2003; Thacker et al., 2007) and have attracted considerable interest for their potential use in the bioremediation of chromate-containing industrial waste waters. Thus, biotransformation of Cr(VI) to the non-toxic trivalent form by chromium-reducing bacteria (CRB) therefore offers an option for Cr(VI) detoxification to achieve bioremediation of contaminated environment (Pal et al., 2005). Most of the studies carried out so far on

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Cr(VI) reduction by environmental isolates or microbial cultures describe the enzyme kinetics in different media (Bo et al., 2009; Thacker et al., 2007), location of the enzyme (Dhakephalkar et al., 1996), influence of physicochemical or cultural factors (Parameswari et al., 2009), or nutrient supplementation (Bhide et al., 1996) etc. but lack details of possible genetic mechanisms responsible for the Cr(VI) reduction.

Several mechanisms of Cr(VI) reduction have been identified in bacteria and these include reduction by DT-diaphorase, aldehyde oxidase in the cell cytoplasm, Cr(VI) reductase and cytochrome P450 on cell membrane as well as nitroreductase (Kwak et al., 2003; Cheung and Gu, 2007). Although Cr(VI) reductase from *Pseudomonas ambigua* has been purified and characterized (Suzuki et al., 1992), the identity of the genes involved in Cr(VI) reductase, and its expression, has not been published with respect to an individual organism or environmental consortia. This paper describes Cr(VI) reducing potential of bacteria isolated from contaminated environments, the method developed for identification of specific genes responsible for Cr(VI) reduction and their characterization. The standardized protocol could be applied to screen the bacteria collected from contaminated environments to verify if the organism or consortium has the gene of interest for remediation of Cr(VI) contaminated environments.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

Bacterial isolates were grown from single colonies using the methods of Eisenstadt et al. (1994), from surface soil samples (0–15 cm depth) collected from a long term tannery waste contaminated site at Mount Barker, South Australia and sediment samples (0–5 cm depth) from a diesel contaminated site from Perth, Western Australia. Isolation and subsequent growth experiments were carried out with a minimal salt medium (Megharaj et al., 2003) supplemented with Cr(VI) (100 mg/l chromium as $K_2Cr_2O_7$) and glucose as the sole carbon source (0.5% w/v). All three isolates were Gram positive, strains MM10 and MM20 were isolated from long term tannery waste contaminated soil and strain MM30 from diesel contaminated sediment.

2.2. Bacterial growth and Cr(VI) reduction

Bacterial isolates were cultured overnight in Tryptic soy broth (TSBA) (BBL, Becton Dickinson, Microbiology Systems, Cockeysville, MD) and the cells were harvested by centrifugation (6000 *g* for 10 min at 10 °C) followed by washing of the cell pellet in phosphate buffer (0.1 M NaH_2PO_4–Na_2HPO_4; pH, 7.1). After two washes, the cells were resuspended in the same buffer. Culture flasks (150-ml Erlenmeyer flasks with a final liquid volume of 30 ml) containing minimal salts medium supplemented with 10 mg of Cr(VI)/l medium and 0.5% glucose were inoculated with the isolates. Media without Cr(VI) but inoculated with bacteria, and uninoculated media containing Cr(VI) served as controls. All the cultures including controls (in triplicate) were incubated for 72 h with shaking at 100 rpm in a temperature controlled room maintained at about 21 ± 1 °C. The density of the bacteria was monitored at definite time intervals, by measuring optical density of the cultures at 600 nm. To measure the Cr(VI) reduction by growing cells, a 1 ml culture from each of the above flasks ($n = 3$) was centrifuged (6000 rpm for 10 min at 10 °C) and the supernatant analyzed for Cr(VI) by reacting with 1 ml of 1 N H_2SO_4, and 0.4 ml of colour developing reagent prepared by dissolving 4.0 g of phthalic acid and 0.25 g of 1,5-diphenylcarbazide (DPC) in 100 ml of 95% ethanol (Bartlett and James, 1979). The optical density (OD) of the developed colour was measured using a spectrophotometer at 540 nm and the OD converted to Cr(VI) concentration using the calibration curve developed with known concentrations of Cr(VI).

2.3. DNA extraction from bacterial culture

DNA was extracted from each bacterium by growing the culture in a 10 ml volume of minimal broth medium containing Cr(VI) as described before. The cultures were grown for 5 days in a shaking incubator (80 rpm) at 25 °C and 2 ml of each culture was centrifuged in a sterile microcentrifuge at 13 000 rpm. The supernatants were discarded and the pellet was transferred to Mo Bio UltraClean Soil DNA Kit (Laboratories Inc, CA, USA) and DNA extracted according to the manufacturer's instructions. The extracted DNA was electrophoresed on 1% agarose gel in TAE buffer and visualized under UV (Gel Doc, Bio-Rad Laboratories, USA) to check for integrity. The DNA was stored at −20 °C until further use.

2.4. Amplification of 16S rRNA and Cr(VI) reductase gene

Primers E8F and 1541R (Lane, 1991) were used to amplify the 16S rRNA gene. Primers designed in this study to amplify the Cr(VI) reductase gene were designed from the complete genome sequence of *Escherichia coli* strain K-12 (GenBank accession no. NC_000913) that target nucleotide positions 3892910–3893178 of *E. coli*, and are given in Table 1. PCR amplifications were performed in a total reaction volume of 50 μL containing 1 μL of DNA extract, 0.2 mM of each deoxynucleotide triphosphate (Promega, Madison, USA), 1.25 U of *Taq* polymerase (Promega), 200 mM betaine (Sigma), 2 mM magnesium chloride (Promega), 1× buffer (Promega) and 25 pmol each of forward and reverse primers.

The primers reported earlier to amplify Cr(VI)-reducing gene such as nitroreductase gene (Kwak et al., 2003) were also used for amplification of desired product in our isolates following the respective protocol.

PCR amplification of 16S rRNA gene was carried out in an icycler thermalcycler (Bio-Rad) subjected to denaturation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min and a final extension of 72 °C for 10 min. Cr(VI)-reducing gene was amplified with a similar thermal cycle except that annealing was performed at 50 °C for 30 s. The PCR products were separated on 1% agarose gels in TAE buffer stained with ethidium bromide (0.5 μg/mL) and visualized with UV light.

The PCR products were purified using Mo Bio UltraClean (Laboratories Inc) PCR clean-up kit (Laboratories Inc) according to the manufacturer's instructions and were sequenced using Big Dye Terminator v3.1 cycle sequencing ready reactions (Applied Biosystems, Foster City, CA, USA) at the DNA Sequencing Facility, Flinders Medical Centre, Adelaide. The nucleotide sequences and the deduced amino acids were edited by using Chromas software (version 2.3). 16S rRNA gene sequences were assembled with SeqMan (DNASTAR) free trial version (http://www.dnastar.com/forms/demo_request.php). Homology searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), ribosomal database project (http://rdp.cme.msu.edu/) and greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi).

Nucleotide sequences of Cr(VI) reductase and 16S rRNA genes determined in this study were submitted to GenBank under the accession nos. EU729733–EU729735 and EU729736–EU729738, respectively.

Table 1
Primers used for amplification of chromate reductase and nitroreductase.

Method used		Sequence primers/probe	Genome	Remarks
ChRF	Forward	5′-TCACGCCGGAATATAACTAC-3′	Chromate reductase	Designed in the present study
ChR	Reverse	5′-CGTACCCTGATCAATCACTT-3′		
EcNfsA1	Forward	5′-GTAGGATCCACGCCAACCATTGAAC-3′	*nfsA* gene (nitroreductase)	Kwak et al., 2003
EcNfsA2	Reverse	5′-ACTGAATTCTTAGCGCGTCGCCCAAC-3′		
EcNfsB1	Forward	5′-GTAGGATCCGATATCATTTCTGTCGC-3′	*nfsB* gene (nitroreductase)	
EcNfsB2	Reverse	5′-ACTGAATTCTTACACTTCGGTTAAGGTG-3′		

2.5. Construction of phylogenetic tree

16S rRNA gene sequences from isolates were aligned with the NAST alignment tool (DeSantis et al., 2006a), and chimera formation was checked with the Bellerophon program. Both NAST and Bellerophon were accessed through the Greengenes website (DeSantis et al., 2006b). Bootstrapped neighbour-joining relationships were estimated with MEGA, version 4.0 (Kumar et al., 2004).

3. Results

3.1. Cr(VI) reducing ability of bacterial strain

Two Gram positive Cr(VI) resistant bacterial strains (MM10 and MM20) were isolated from long term tannery waste contaminated soil and another Gram positive strain (MM30) from diesel contaminated sediment in Western Australia by selection on minimal medium agar plates amended with 50 mg/l Cr(VI) with 0.5% glucose as the sole carbon source. Fig. 1 shows the graphical representation of the density of the bacterial isolates and % Cr(VI) reduction at different time intervals. The increase in bacterial density was almost similar in three different isolates but the Cr(VI) concentration in the culture supernatant reduced by more than 94% in isolates MM20 and MM30 after 50 h of incubation whereas reduction was about 65% by this time in isolate MM10. The rate of Cr(VI) reduction was steady from 16 to 74 h of incubation in isolate MM10 whereas an accelerated reduction of Cr(VI) was recorded between 16 and 50 h of incubation in isolates MM20 and MM30, suggesting that MM20 (*Bacillus atrophaeus*) and MM30 (*Rhodococcus erythropolis*) have better Cr(VI) reduction potential when compared to MM10 (*Arthrobacter aurescens*) at the initial growth period.

3.2. Identification of bacterial strains

16S rRNA gene sequence nucleotide BLAST analysis of the three different isolates MM10, MM20 and MM30 showed that the bacteria belong to genus *Arthrobacter*, *Bacillus* and *Rhodococcus*, respectively. Phylogenetic analyses of the isolates based on 16S rRNA gene sequence, are given in Figs. 2–4, respectively and BLAST analysis is presented in Table 2.

3.3. Molecular characterization of Cr(VI) reducing potential

The primers designed (Table 1) from whole-genome sequence of *E. coli* strain K-12 (GenBank accession no. NC_000913) in our study, for the partial amplification of chromate reductase gene, amplified the 268 bp fragment in each of the three Gram positive bacterial isolates. DNA nucleotide sequence analysis carried out from the amplified products in those isolates showed high degree of homology with various class 1 flavoproteins from Gram negative bacteria available in GenBank. It is noteworthy that no amplification could be achieved for nitroreductase gene.

The sequencing and BLASTN analysis of the sequence matched around 100% to the chromate reductase gene of *E. coli*. All three partial chromate reductase sequences (ca. 230 bp) had very high

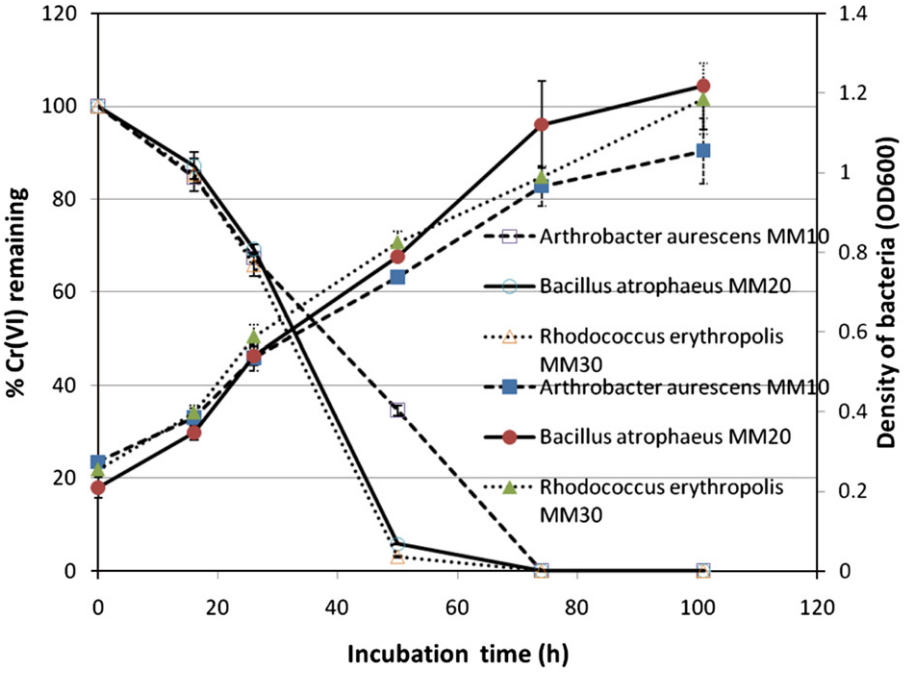

Fig. 1. Density of bacteria in the solution (solid markers) and % Cr(VI) reduction (respective hollow marker) of three different bacterial isolates.

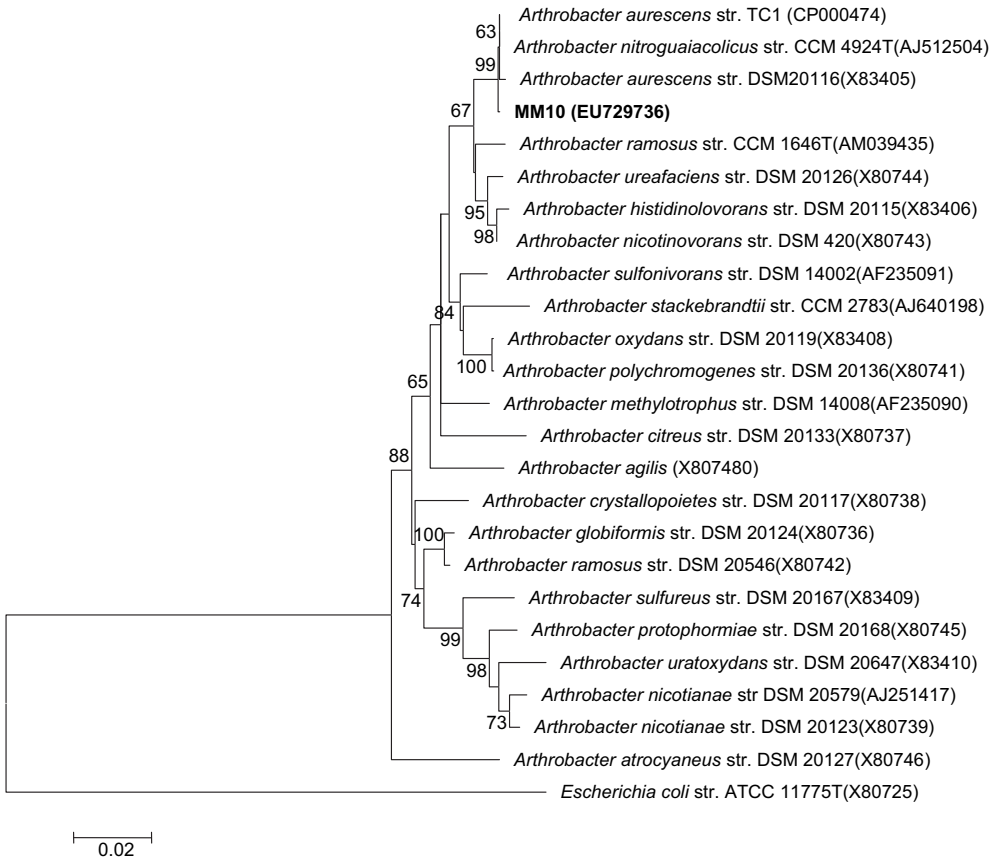

Fig. 2. Phylogenetic tree constructed from the 16S rRNA gene of isolate MM10 (GenBank accession no. EU729736) and related organisms constructed using neighbour-joining algorithm from an alignment of 1253 nucleotides. Accession numbers of corresponding sequences are given in parentheses and the scale bar represents 1 base substitution per 20 nucleotide positions. The bootstrap probabilities calculated from 1000 replications with values greater than 60 are indicated at the nodes. *E. coli* ATCC 11775T was taken as an out-group.

similarity to one another, each having a single nucleotide mismatch with one another. BLASTN analysis revealed very high similarities with chromate reductase sequences identified in Enterobacteria. When our sequences were compared to the *E. coli* chromate reductase sequences, the number of mismatches ranges from one (CP000948) to four (DQ987901). Chromate reductase sequences from *Shigella* spp. also had 98% similarity (ca.), while several sequences from *Salmonella enterica* had 87% similarities (ca.). Theoretical protein sequences derived from the nucleotide sequence, revealed identical amino acid sequence for isolates MM10 and MM20. BLASTP analysis revealed a perfect match with NADPH-dependent FMN reductases from *Shigella* spp. and *E. coli*. Lower identities (94–60%) were observed in many other subclasses of Proteobacteria, while identities of approximately 50% were found with several Archea and Gram positive Firmicutes.

4. Discussion

The ever increasing concern about the toxicity of Cr(VI) and the potential biotransformation technology for detoxification of Cr(VI) through *in situ* microbial reduction of Cr(VI) to less toxic and insoluble Cr(III) that circumvents the limitations posed by physical and chemical treatment methods (Viamajala et al., 2002) coerce the isolation of Cr(VI) reducing bacteria from contaminated sites and characterization of chromate reductase genes in Cr(VI) reducing bacteria (CRB).

In the present investigation, two bacteria, *Arthrobacter* and *Bacillus*, were isolated from a long term tannery contaminated site with a potential to reduce Cr(VI). Cr(VI) reducing bacteria isolated around five years ago from the same site and identified by fatty acid methyl esters (FAME) as belonging to the same genera (Megharaj et al., 2003) show that Cr(VI) reducing bacterial population is still growing in the heavily Cr(VI) contaminated site. Isolate MM30 (EU729736), which had a 16S rRNA gene sequence most similar to *R. erythropolis* and originally isolated from sediments contaminated with diesel also had the potential to reduce the Cr(VI). Horton et al. (2006) isolated *A. aurescens*, indigenous to the subsurface, that had the potential to be a predominant metal reducer in enhanced, *in situ* subsurface bioremediation efforts involving Cr(VI) and possibly other heavy metals and radionuclides, and demonstrated that indigenous microbial populations present in Cr(VI) contaminated aquifers are able to aerobically catalyze the removal of toxic and soluble Cr(VI) from the media, most likely reducing it to the relatively non-toxic and insoluble Cr(III). Cheng and Li (2009) have isolated eight Cr(VI)-resistant *Bacillus* spp. from soil samples of iron mineral area in China. These bacteria were claimed to be Cr(VI)-resistant based on their isolation and growth on Luria–Bertani (LB) agar supplemented with 500 mg/l Cr(VI). One of these bacteria with high efficiency in detoxification of chromate designated as MDS05 was reported to grow with 2500 mg/l Cr(VI) in LB medium and reduce 100% of 10 mg/l Cr(VI) in 24 h. However, the growth of MDS05 was inhibited by 20% when exposed to 100 mg/l Cr(VI) and slightly affected by 1, 5, 10 and 50 mg/l Cr(VI) in the same LB medium compared to control without Cr. Surprisingly these authors did not include the abiotic control (Uninoculated LB medium with Cr(VI)); hence it is difficult to distinguish the true

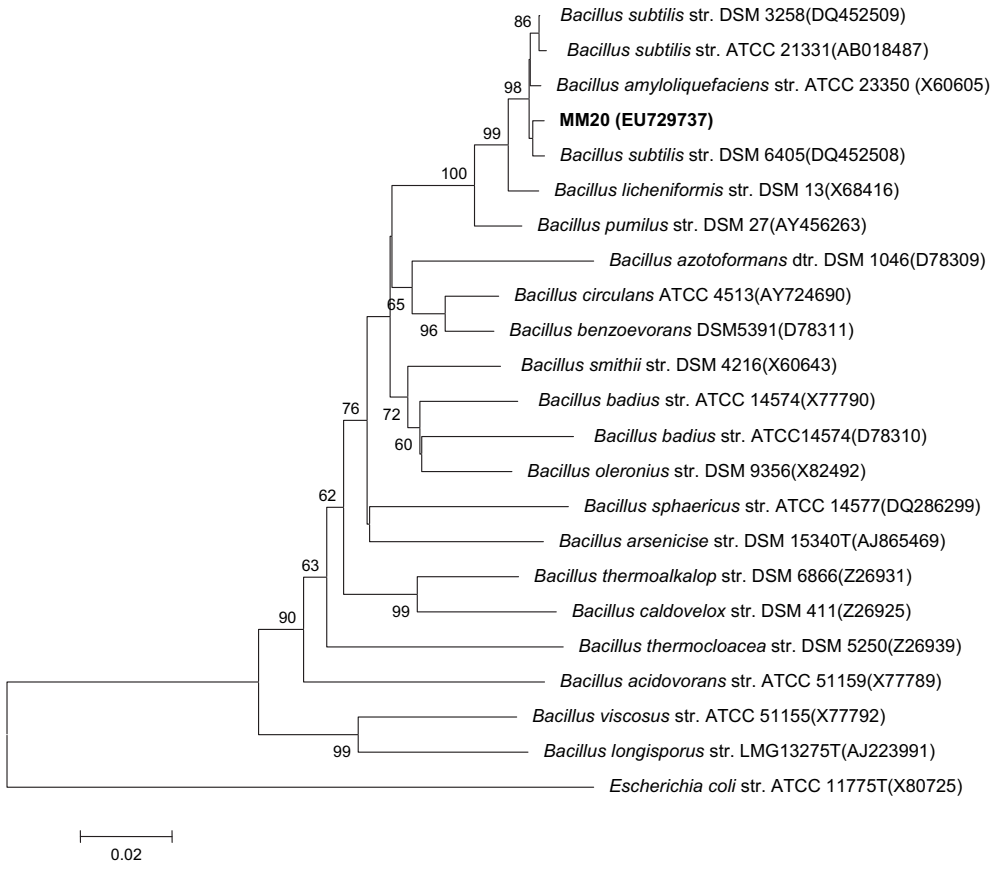

Fig. 3. Phylogenetic tree constructed from the 16S rRNA gene of isolate MM20 (GenBank accession no. EU729737) and related organisms constructed using neighbour-joining algorithm from an alignment of 1243 nucleotides. Accession numbers of corresponding sequences are given in parentheses and the scale bar represents 1 base substitution per 20 nucleotide positions. The bootstrap probabilities calculated from 1000 replications with values greater than 60 are indicated at the nodes. *E. coli* ATCC 11775T was taken as an out-group.

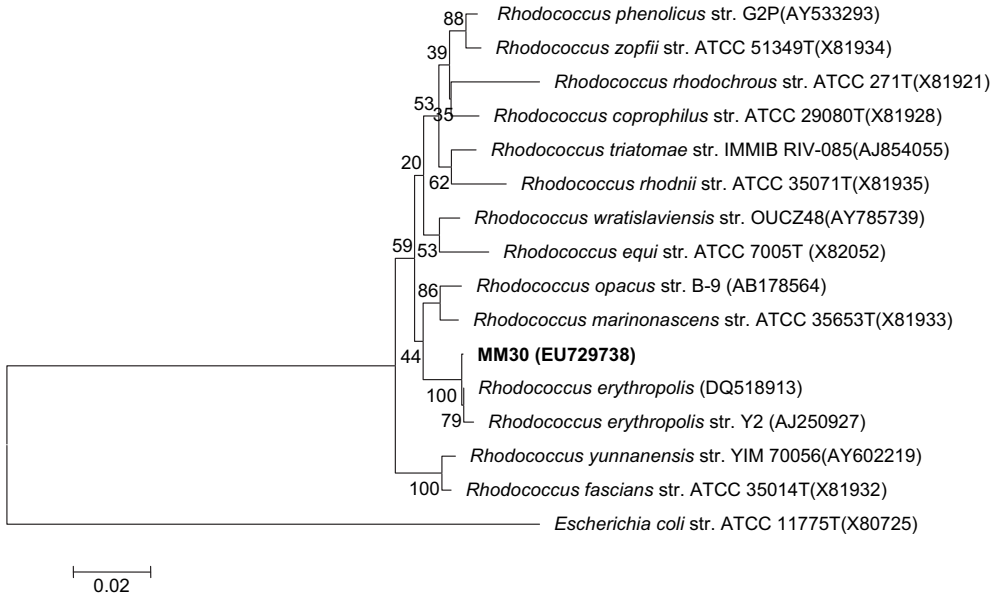

Fig. 4. Phylogenetic tree constructed from the 16S rRNA gene of isolate MM30 (GenBank accession no. EU729738) and related organisms constructed using neighbour-joining algorithm from an alignment of 1239 nucleotides. Accession numbers of corresponding sequences are given in parentheses and the scale bar represents 1 base substitution per 20 nucleotide positions. The bootstrap probabilities calculated from 1000 replications with values greater than 60 are indicated at the nodes. *E. coli* ATCC 11775T was taken as an out-group.

Table 2
BLAST analysis of isolated bacteria.

Isolate	Identification	GenBank accession no.	Similar organism	Accession no.	Sequence similarity (%)
MM10	*Arthrobacter aurescens*	EU729736–EU729738	Nitroaromatic degrading isolate *Arthrobacter nitroguajacolicus*	AJ512504	99.79
MM20	*Bacillus atrophaeus*	EU729737	*Bacillus atrophaeus*	AY881241	99.87
MM30	*Rhodococcus erythropolis*	EU729738	*Rhodococcus erythropolis*	AY168592	99.86

ability of this bacterium to reduce Cr(VI) and requires caution in interpreting the results. Megharaj et al. (2003) have reported that the use of nutrient rich media containing yeast extract, peptone etc. is not suitable to determine bacterial resistance to Cr(VI) since the organic substrates in these media can complex Cr(VI) thereby masking the toxicity leading to overestimation of resistance to Cr(VI).

Several chromium-reducing bacteria with biotransformation potential and belonging to several genera such as *Achromobacter*, *Aeromona*, *Agrobacterium*, *Bacillus*, *Desulfovibrio*, *Enterobacter*, *Escherichia*, *Micrococcus* and *Pseudomonas* (Lovley, 1994; Megharaj et al., 2003; Desai et al., 2008; Thacker et al., 2007) have been reported. Park et al. (2000) cloned the chromate reductase-encoding gene, and characterized a novel soluble chromate reductase from *Pseudomonas putida.* Sequence analysis of a novel chromate reductase from *Thermus scotoductus* SA-01, related to old yellow enzyme, showed the chromate reductase to be related to the old yellow enzyme family, in particular the xenobiotic reductases involved in the oxidative stress response (Opperman et al., 2008). Kwak et al. (2003) reported that *Vibrio harveyi* nitroreductase is also a chromate reductase. Whereas the biochemistry of chromium reduction is well understood (Chardin et al., 2003), the molecular mechanism is not. Several bacterial enzymes (De Flora et al., 1985; Puzon et al., 2002; Kwak et al., 2003) in addition to cytochrome P450 (Mikalsen et al., 1991) have been implicated in Cr(VI) reduction depending on the availability of oxygen; however, identifying the mechanism of reduction especially in environmental isolates based on the available literature is limited. This study provides the most efficient means of screening several environmental isolates for chromate reductase (class 1 flavoprotein). The advantages of this method being the speed and culture independence, it would be an excellent technique to use in conjunction with the monitoring of bioremediation methods/technologies.

It is an interesting observation that the chromate reductases in this study are almost identical to one another, but their activities are so different. This could be due to the fact that other genetic elements tied with NADH may be influencing the overall rates of Cr(VI) reduction. Furthermore, the regulatory region of chromate reductase in these isolates remains unknown and the regulation of these almost identical chromate reductase genes could be different. Further studies in characterizing the promoter region of these isolates are therefore needed.

Cr(VI) reduction is different from Cr(VI) resistance. Several microorganisms have the exceptional ability to adapt and colonize environments polluted with the noxious metal by resistant mechanisms. These mechanisms include metal efflux channels, metal resistance plasmids, adsorption, uptake, DNA methylation and metal biotransformation either directly by specific enzymes or indirectly by cellular metabolites (Nies, 2003). However, a more basic understanding of adaptation is possible if the molecular mechanism is also characterized. More recently, Abou-Shanab et al. (2007) reported the presence of *chr* genes that confer resistance to chromium in Gram positive and negative bacteria isolated from Ni-rich serpentine soil. The mechanism of resistance to chromate determined by *ChrA* of *Pseudomonas aeruginosa* has been shown to be associated with active efflux of chromate driven by the membrane potential (Alvarez et al., 1999). Further, Aguilera et al. (2004), based on mutational analysis of *P. aeruginosa ChrA* gene have suggested the involvement of cytoplasmic domains at the N-terminus of the *ChrA* protein coupled with adjacent transmembrane spans in the efflux of chromate ions. The recent work by Rivera et al. (2008) demonstrates that chromate resistance in *P. aeruginosa* PA01 is controlled by diverse genes such as *oprE* (encoding outer membrane), *rmlA* (cell wall LPS) and *ftsK* (cytoplasm). The expression of ChrA protein did not restore chromate resistance in chromate sensitive oprE mutant (outer membrane) of *P. aeruginosa* PA01 strain generated by transposon-insertion mutagenesis indicating the involvement of OprE in the chromate efflux from periplasm to the outside (Rivera et al., 2008). Thus, expression of ChrA protein associated with extrusion of chromate ions from cytoplasm to periplasm leads to accumulation of chromate ions in periplasm causing toxicity unless oprE is also expressed. In the present study we failed to amplify the *chr* gene using oligonucleotide sequences as primers for the partial amplification of the *chrB* loci (Nies et al., 1990) that confer Cr(VI) resistance (Abou-Shanab et al., 2007). There are several mechanisms to evade metal toxicity, and those are encoded by chromosomal genes or on plasmid. The difference in the rate of Cr(VI) reduction among the different isolates in the present investigation could be attributed to the different resistance mechanisms or the difference in regulatory sequences resulting in altered expression of enzymatic activity. However, the designed primer in the present investigation could serve as an effective tool to identify organisms either in culture or in bacterial consortium of having Cr(VI) reducing genetic potential for its exploitation in Cr(VI) bioremediation by its reduction to Cr(III).

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