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Mercury resistance in *Sporosarcina* sp. G3

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Abstract Mercuric reductase (MerA) enzyme plays an important role in biogeochemical cycling and detoxification of Hg and recently, has also been shown to be useful in clean up of Hg-contaminated effluents. Present study describes isolation of a heavy metal-resistant isolate of *Sporosarcina*, which could tolerate up to 40, 525, 210, 2900 and 370 μM of Cd, Co, Zn, Cr and Hg respectively. It was found to reduce and detoxify redox-active metals like Cr and Hg. The chromate reductase and MerA activities in the crude cell extract of the culture were 1.5 and 0.044 units/mg protein respectively. The study also describes designing of a new set of highly degenerate primers based on a dataset of 23 Firmicute *merA* genes. As the primers encompass the known diversity of *merA* genes within the phylum *Firmicutes*, they can be very useful for functional diversity analysis. They were successfully used to amplify a 787 bp *merA* fragment from the current isolate. A 1174 bp *merA* fragment was further cloned by designing an additional downstream primer. It was found to show 92% similarity to the putative *merA* gene from *Bacillus cereus* AH820. To the best of our knowledge, this is the first report of mercury resistance and *merA* gene sequence from *Sporosarcina*.

Keywords Mercuric reductase · *merA* · *Firmicutes* · Gram positive · Degenerate primer

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Introduction

Heavy metals are released into the environment as a result of both natural processes as well as anthropogenic activities. They can have many adverse effects such as loss of ecosystem and agricultural productivity, economic losses, contamination of food chain, and health hazards. They are also toxic to all forms of life and can cause several disorders, cancer or even death in animals, including humans (Nies 1999). Hence, it is very important to remove heavy metals from wastes or contaminated environments. Traditionally, physico–chemical processes, such as adsorption, ion exchange, immobilization, precipitation, and chemical oxidation, reduction or leaching, are used for this purpose. However, with the advancements in biotechnology, focus is shifting toward bioremediation techniques (Pathak et al. 2009). Microbial processes such as bioaccumulation, bioleaching and biochemical transformations, and plant-mediated phyto extraction, phyto stabilization and rhizo filtration, have been found to be promising in this regard (Ruta et al. 2010). Microbial metal removal processes take advantage of their metal resistance systems.

Frequently, these metal resistance systems are encoded on R plasmids, which may also carry antibiotic resistance genes. Thus, although metal resistance genes have bioremediation potential, their widespread presence may be of serious concern.

Mercury (Hg) is one of the most toxic elements on earth. Due to certain desirable properties like high surface tension, flow behaviour and electrical conductivity, it has found wide use in industries, which in turn has resulted in heavy environmental pollution. Hg can exist in three forms in the environment—metallic Hg(0), inorganic Hg(I)/Hg(II), and organic Hg—which can be inter converted by different biological and chemical processes (Jan et al. 2009). Current Hg removal technologies are very costly. The cost of Hg removal has been estimated to be 2.5–1100 thousand US$/Kg of Hg depending on the nature of the contaminated site (Hylander and Goodsite 2006). Bioremediation may provide a cost-effective and efficient alternative in this scenario. Microbial resistance to Hg is mediated through *mer* operon. The key enzyme in this operon is mercuric reductase (MerA), which catalyzes reduction of soluble Hg(II) to insoluble and volatile Hg(0). MerA has been successfully used for removal of Hg through several approaches such as construction of bioreactors containing immobilized MerA enzyme or Hg-resistant bacteria, and overexpression of MerA in bacteria, algae or plants (Lyyra et al. 2007).

The *mer* operon structure differs considerably between Gram positive and Gram negative bacteria. While *merA* gene diversity is well-characterized in Gram negative bacteria, fewer studies are available in Gram positive bacteria. Majority of the Gram positive *merA* sequences are putative sequences resulting from whole genome sequencing efforts. A few studies used degenerate primers, but the primers were based on limited Gram positive *merA* sequences and, hence, might have missed some sequence diversity (Narita et al. 2003; Oregaard and Sørensen 2007). Broadly, there are three Gram positive MerA variants based on absence, or presence of a single or duplicate

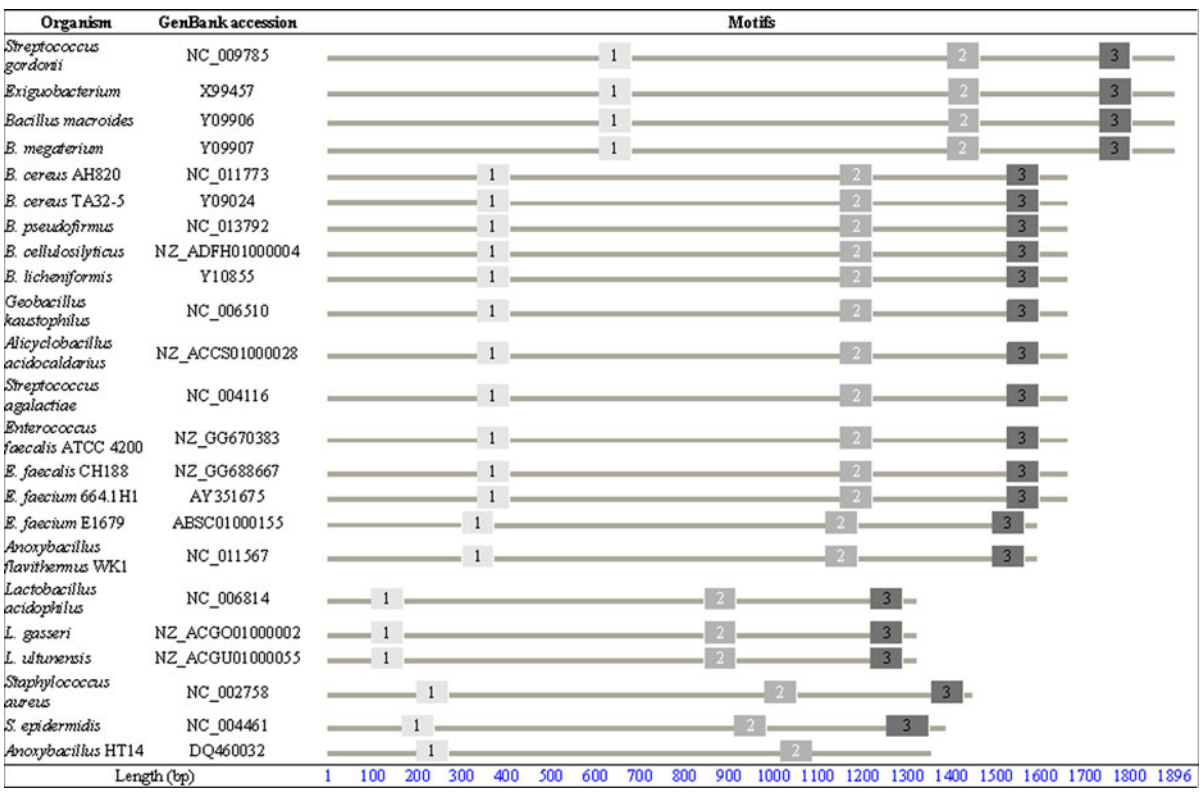

Fig. 1 Firmicute *merA* genes used in the present study, showing relative positions of the motifs used for primer designing and variation in the protein N-terminal domain. All three motifs are represented by *differentially shaded boxes*

N-terminal domain (Fig. 1). However, much of the sequence variation still remains unexplored. Present study describes characterization of a heavy metal-resistant isolate of *Sporosarcina*. The isolate was found to bioaccumulate cadmium (Cd), cobalt (Co) and zinc (Zn), and possess MerA and chromate reductase activities. Thus, it might be useful for removal or detoxification of heavy metals from metal-contaminated sites. A set of degenerate primers was designed to amplify partial *merA* gene from the isolate. The primer set covers all the variation in *merA*-annotated *Firmicutes* sequences available in the database, and hence, can potentially amplify any *merA* gene from this phylum. The partial *merA* gene from the present isolate was found to show some sequence variation as compared to previously described Gram positive *merA* sequences. To the best of our knowledge, this is the first report of Hg resistance in *Sporosarcina*.

Materials and methods

Isolation of the culture

Mercuric salt-contaminated soil was obtained from an industrial site in India and analyzed for Hg content by acid digestion followed by cold vapour atomic absorption spectrometry (APHA 1989). It was enriched for Hg-resistant bacteria by incubating first in nutrient broth (composition in g/l: peptone-10, NaCl- 5, beef extract-3) containing 18.5 μM mercuric chloride ($HgCl_2$), and then transferring to nutrient broth containing 92 μM $HgCl_2$. Resulting suspension was plated on nutrient agar plates containing different concentrations of $HgCl_2$ and the colony showing the highest Hg tolerance was selected for further study. For identification, its genomic DNA was isolated and 16S rRNA gene was amplified using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Edwards et al. 1989). The amplified product was then cloned in T-vector (Bangalore Genei, India) using *Escherichia coli* XL1-blue as host. Sequencing was carried out with M13 primer on ABI 3100 automated DNA sequencer (Applied Biosystems, USA). A Hg-sensitive *Sporosarcina ureae* strain was used as negative control during the study.

Determination of metal and antibiotic resistance profile

Resistance of the isolate toward different heavy metal salts (cadmium acetate, cobalt chloride, zinc sulfate, potassium dichromate and $HgCl_2$), and antibiotics (ampicillin and tetracycline) was determined. Phenylmercuric acetate (PMA) and methylmercuric chloride (MMC) were also used to detect resistance toward organic mercuric compounds. Briefly, nutrient agar plates containing different concentrations of above metals (1–1500 μM) or antibiotics (1–10 mg/l) were plated with OD_{600} 0.1 culture of the isolate. The plates were incubated at 25°C for 48 h before recording the maximum tolerated concentration (MTC) i.e. the maximum concentration at which culture could grow. All further experiments were carried out at sublethal concentration, which was considered to be 50% of MTC for Cd, Co, Zn and Cr, and 20% of MTC for Hg (Table 1). Lower concentration was used for Hg as it inhibited the culture growth more profoundly in broth (MTC of 110 μM in nutrient broth). Firstly, effect of above metals on growth curve of the culture was studied at sublethal concentrations. Nutrient broth flasks containing appropriate concentrations of different metal salts were inoculated with OD_{600} 0.1 culture of the isolate. The flasks were incubated on shaker at 25°C and growth was quantified in terms of OD_{600}.

Table 1 Resistance profile of *Sporosarcina* sp. G3

Metal/Antibiotic	MTC[a, b] (μM)	Sublethal concentration (μM)
Cd acetate	40 ± 1.7	20
$CoCl_2$	525 ± 1.7	262.5
$ZnSO_4$	210 ± 2.9	105
$K_2Cr_2O_7$	1450 ± 2.9[c]	725
$HgCl_2$	370 ± 2.9	74
PMA	S	–
MMC	S	–
Ampicillin	S	–
Tetracycline	S	–

[a] *S* Sensitive, i.e. no growth at 1 mg/l concentration

[b] Values represent mean ± standard error of triplicate experiment

[c] It corresponds to 2900 μM of Cr

Bioaccumulation of Cd, Co and Zn

Overnight culture of the isolate was diluted to OD_{600} of 0.1 in nutrient broth flasks containing sublethal concentrations of different metal salts (Table 1). After 24 h of growth, the cultures were centrifuged and cell pellets washed with saline. The cells were then digested with acid for metal content analysis by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (APHA 1989). Results were expressed as % of the initial amount of metals in the medium, which was bioaccumulated by the cells.

Resistance to chromium (Cr)

Both Cr and Hg are redox-active metals that can be reduced to less toxic oxidation states by bacterial resistance systems (Nies 1999). Hence, the isolate was tested for chromate reductase (Cr(VI) reduction) and MerA activities. Chromate reductase activity is generally demonstrated by reduction in the level of Cr(VI) by the culture (Park et al. 2000). However, it could not be shown in situ during growth of the culture, as components of nutrient broth interfered with Cr(VI) estimation. To demonstrate chromate reductase activity, the culture was induced by growth in presence of 51 μM $K_2Cr_2O_7$, and the resulting biomass was re-suspended in 100 mM phosphate buffer (pH 7) followed by disruption by sonication. Cells debris was removed by centrifugation and supernatant was used as the crude cell extract in chromate reductase assay. The assay mixture consisted of 100 mM phosphate buffer (pH 7), 0.1 mM $K_2Cr_2O_7$, 0.1 mM NADH and 0.1 ml of crude cell extract in a total volume of 1 ml. Two controls containing all the reagents, except the cell extract in one tube and except $K_2Cr_2O_7$ in another tube, were kept to measure abiotic and non-chromate reductase-mediated reduction. A chromate reductase-negative *S. ureae* strain was also processed as above as a negative control. After incubating for 30 min, Cr(VI) content of the samples was determined by 1,5-diphenylcarbazide method (APHA 1989). One unit of enzyme activity was defined as the amount of enzyme that reduces 1 nmol of Cr(VI)/min at 25°C (Park et al. 2000).

Resistance to Hg

Mercuric reductase activity was determined by measuring the rate of loss of Hg from the culture. The culture was diluted to OD_{600} of 0.1 in nutrient broth containing sublethal concentration of $HgCl_2$ (Table 1) and samples were removed after 1, 2 and 3 days. A control flask, containing $HgCl_2$ but not the culture, was also maintained to determine abiotic volatilization. Hg content was analyzed in both biomass and supernatant medium fractions by acid digestion followed by cold vapour atomic absorption spectrometry (APHA 1989). % Hg reduction was determined by comparing total Hg content of the sample with that of control. Results were expressed as % of the initial amount of Hg in the medium, which was volatilized by the cells. Hg volatilization in the control flask was subtracted from the sample reading to estimate the culture-mediated Hg reduction.

To demonstrate MerA activity in cell free extract, the culture was induced by growth in presence of 55 μM $HgCl_2$, and the resulting cells were re-suspended in buffer A (20 mM phosphate buffer pH 7.4, 0.5 mM EDTA, 0.1% β-mercaptoethanol) before disruption by sonication. Cell debris was removed by centrifugation and the crude cell extract was assayed for MerA activity. The assay mixture consisted of 80 mM phosphate buffer (pH 7.4), 1 mM β-mercaptoethanol, 0.1 mM $HgCl_2$, 0.2 mM NADPH and 0.1 ml of crude cell extract in a total volume of 1 ml. Oxidation of NADPH was followed spectrophotometrically at 340 nm. A control containing all the reagents except $HgCl_2$ was also maintained to determine Hg-independent oxidation of NADPH. A Hg-sensitive *S. ureae* strain was also processed as above as a negative control. One unit of enzyme activity was defined as the amount of enzyme capable of catalyzing Hg-dependent oxidation of 1 μmol of NADPH/min at 25°C (Fox and Walsh 1982).

Detection of *merA* gene

Degenerate primers specific for *Bacillus* or Firmicute *merA* sequences were derived from previous studies (Table 2). They were used according to the PCR programs published with them. For designing new *Firmicutes*-specific primers, full length *merA*-annotated Firmicute sequences were downloaded from GenBank. Sequences showing more than 95% identity were removed to reduce redundancy. This resulted in selection of 23 sequences (Fig. 1). The sequences were analyzed for conserved motifs by the

Table 2 *merA*-specific primers used in this study

Primer	Sequence[a]	Specificity	Reference
1F	5′-ATG AAA AAR TAT CGA GTR RAC G-3′	*Bacillus*	Narita et al.
1890R	5′-TTA WCC WGC ACA RCA RGA TA-3′	*Bacillus*	2003
Fw	5′-GTT TAT GTW GCW GCY TAT GAA GG-3′	*Firmicutes*	Oregaard and
Rv_{1832}	5′-CCT TCW GCC ATY GTT ARA TAW GG-3′	*Firmicutes*	Sørensen 2007
F_1	5′-TGY RTI AAY RTH GSI TGY DTI CC-3′	*Firmicutes*	This study
R_1	5′-GMI AYA TAI RYR AAY TGI RRD CC-3′	*Firmicutes*	This study
R_2	5′-CCY TCI GCC ATI GTI ARR TA-3′	*B. cereus*	This study

[a] *D* A/G/T, *H* A/C/T, *I* Inosine, *M* A/C, *R* A/G, *S* G/C, *W* A/T, *Y* C/T

Fig. 2 Regions of motifs 1 and 2 corresponding to the primers F_1 and R_1 respectively. The names of the organisms are as in Fig. 1

GenBank accession	Location	Motif 1	Location	Motif 2
NC_009785	619	TGCGTTAATGTCGGATGCGTTCC	1426	GGTCCCCAATTTGTTTATGTAGC
X99457	619	TGCGTTAATGTCGGATGCGTTCC	1426	GGTCCCCAATTTGTTTATGTAGC
Y09906	619	TGTGTTAACGTCGGATGCGTTCC	1426	GGTCCCCAATTTGTTTATGTAGC
Y09907	619	TGCGTTAATGTCGGATGCGTTCC	1426	GGTCCACAATTTGTTTATGTAGC
NC_011773	364	TGCGTGAACATCGGATGTGTTCC	1171	GGACCTCAATTTGTATATGTGGC
Y09024	364	TGTGTAAATATTGGTTGTGTTCC	1171	GGACCCCAATTTGTGTATGTGGC
NC_013792	364	TGTGTAAATATTGGTTGTGTGCC	1171	GGACCTCAATTTGTCTATGTAGC
NZ_ADFH01000004	364	TGCGTAAATATTGGTTGTGTTCC	1171	GGACCCCAATTTGTATATGTAGC
Y10855	364	TGCGTGAACATCGGCTGTGTTCC	1171	GGTCCACAATTTGTATATGTAGC
NC_006510	364	TGCGTCAATATCGGATGCGTGCC	1171	GGCCCGCAGTTCGTCTATGTCGC
NZ_ACCS01000028	388	TGTGTAAACATCGGTTGTGTTCC	1195	GGACCACAGTTTGTATATGTGGC
NC_004116	364	TGTGTGAATATTGGCTGTGTTCC	1171	GGACCACAATTTGTATATGTAGC
NZ_GG670383	382	TGCGTTAATGTCGGATGCGTTCC	1189	GGTCCCCAATTTGTTTATGTAGC
NZ_GG688667	367	TGCGTTAACATCGGTTGTGTACC	1174	GGTCCGCAATTCGTTTATGTTGC
AY351675	364	TGTGTAAATATTGGTTGTGTGCC	1171	GGACCTCAATTCGTTTATGTAGC
ABSC01000155	349	TGTGTGAACATTGGTTGTATCCC	1156	GGACCTCAGTTTGTCTATGTAGC
NC_011567	340	TGCGTGAATATCGGCTGTGTTCC	1147	GGTCCACAATTTGTCTATGTCGC
NC_006814	133	TGTATTAACATTGCGTGCTTACC	895	GGTCCTCAATTCACCTATATTTC
NZ_ACGO01000002	127	TGTATTAATATCGCCTGCTTACC	901	GGTCTTCAATTTACATATATTTC
NZ_ACGU01000055	127	TGTATTAATATCGCTTGTTTGCC	889	GGTCTTCAATTCACCTATATTTC
NC_002758	253	TGTATAAATATAGGATGTATACC	1015	GGACTTCAATTTACGTATATATC
NC_004461	163	TGCATTAATATCGGATGTATACC	928	GGATTGCAATTTACGTATATTTC
DQ460032	237	TGCGTCAATATCGGCTGCGTGCC	1044	GGCCCGCAGTTTGTCTATGTCGC

program MEME version 4.3 (Bailey and Elkan 1994). Out of the resulting motifs, two motifs of 50 bp, having information content of 67.15 and 69.02 bits, showed satisfactory conservation and were selected for primer designing (Fig. 2; Table 2). The constraints applied to control the degeneracy of primers were: at least 50% of the bases should be the regular bases, at least one base on each end should be a regular base (preferably guanine or cytosine), and inosine content should not be more than 15%. Motif 1 corresponding to the forward primer F_1 was found to be part of PROSITE's (Sigrist et al. 2010) pyridine nucleotide disulphide reductase class I pattern (PS00076) and corresponding fingerprint (PR00411) in PRINTS database (Attwood et al. 2003). It contained the characteristic pattern G-G-X-C-[LIVA]-X(2)-G-C-[LIVM]-P, involving a pair of cysteine residues which transfer reducing equivalents from FAD to the substrate. Surprisingly, motif 2 corresponding to the reverse primer R_1 was not part of any conserved domain or pattern in any of the protein databases. The primer pair was expected to give an amplification product of about 800 bp. PCR was performed in 50 µl reaction volume containing 1X PCR buffer, 0.2 mM of each deoxynucleoside triphosphate, 5 µM of each primer, 50 ng of template and 3 units of Taq polymerase. The optimized PCR program consisted of 30 cycles of denaturation at

94°C for 30 s, annealing at 50°C for 45 s and extension at 72°C for 1 min, followed by a final extension of 72°C for 15 min.

A reverse primer was further designed based on a conserved region of *Bacillus cereus* AH820 *merA* gene (GenBank accession NC_011773), which corresponded to motif 9 of PRINTS' PR00411 fingerprint. This region was part of motif 3, which was detected in all, but one, of the analyzed sequences by MEME program (Fig. 1). Motif 3 contained the conserved histidine/Tyrosine and Glutamate diad found at positions 16 and 21 of the fingerprint. However, its overall sequence conservation was not strong enough to design a primer. Hence, the amino acid sequence of corresponding region of NC_011773 was reverse translated to construct a degenerate primer R_2 (Table 2). This primer agreed perfectly with the profile of motif 3, but was far less degenerate, and hence, satisfied the constraints for degeneracy control as described above. PCR was performed using the R_2 and F_1 primers as above, except that the extension time was increased to 1.5 min as the expected product size was about 1200 bp. All PCR products were cloned in T-vector using *E. coli* XL1-blue as host and sequencing was carried out with M13 primer on ABI 3100 automated DNA sequencer. Contaminating sequences corresponding to cloning vector or degenerate primers were removed to get the finished sequences for GenBank submission. Sequence similarity searches were carried out using the BLASTN program (Altschul et al. 1997).

Phylogenetic analysis was carried out using the sequence dataset selected in present study (Fig. 1), as it represented majority of the variation in Firmicute *merA* genes. An actinobacterial (*Streptomyces lividans*, GenBank accession X65467) and a Gram negative (*E. coli* Tn*21*, GenBank accession AF071413) *merA* sequences were also included as outgroups. Above sequences were aligned with the partial *merA* gene from present study using CLUSTALW 2.0.12 program with IUB substitution matrix (Larkin et al. 2007). The program DNADIST was used to construct a distance matrix for this alignment using F84 distance matrix (Felsenstein 2009). A neighbour-joining tree was then constructed from the distance matrix by NEIGHBOUR program. One hundred bootstrap alignments were generated using SEQBOOT program and each alignment was also analyzed as above. A consensus bootstrap tree was generated from the resulting trees using the CONSENSE program.

Results

A Gram positive sporulating bacillus was isolated from mercuric salt-contaminated soil containing 37 mg Hg/Kg soil. The isolate was identified as *Sporosarcina* sp. by 16S rRNA gene sequencing (GenBank accession HM162836). It was found to resist several heavy metals apart from Hg, but was sensitive to antibiotics like ampicillin and tetracycline, and organo mercurials like PMA and MMC (Table 1). Thus, the culture displayed narrow-range Hg resistance i.e. resistance toward inorganic Hg only. When the culture was grown in presence of sublethal concentration of above metals, Cr was found to have virtually no effect on the growth curve, while Hg was found to be the most inhibitory (Fig. 3). This is the reason why Hg was used at the concentration of 20% of MTC as compared to 50% of MTC for other metals. Metabolism of these heavy metals by the culture was studied further. While Cd, Co and Zn were bioaccumulated to significant degree (Fig. 4), Hg and Cr could be reduced to less toxic forms by the culture. Chromate reductase activity in the crude cell extract of the culture was found to be 1.5 units/mg protein. Thus, Cr resistance in the culture was very likely

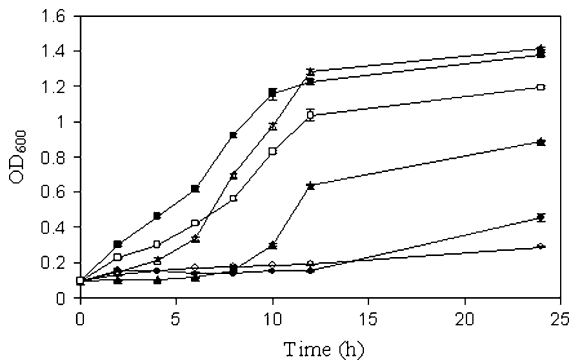

Fig. 3 Growth of *Sporosarcina* sp. G3 at sublethal concentrations of metal salts. (*Closed square*) Control, (*closed triangle*) Cd acetate—20 μM, (*closed circle*) $CoCl_2$—262.5 μM, (*open square*) $ZnSO_4$—105 μM, (*open triangle*) $K_2Cr_2O_7$—725 μM and (*open circle*) $HgCl_2$—74 μM. *Error bars* represent standard error from triplicate experiment

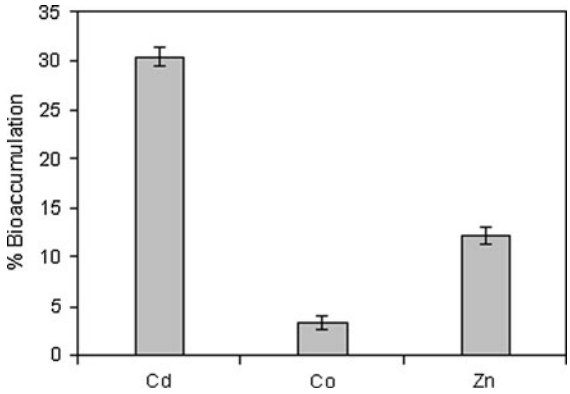

Fig. 4 Bioaccumulation of Cd, Co and Zn in 24 h. Metal salt concentrations are as in Fig. 3. *Error bars* represent standard error from triplicate experiment

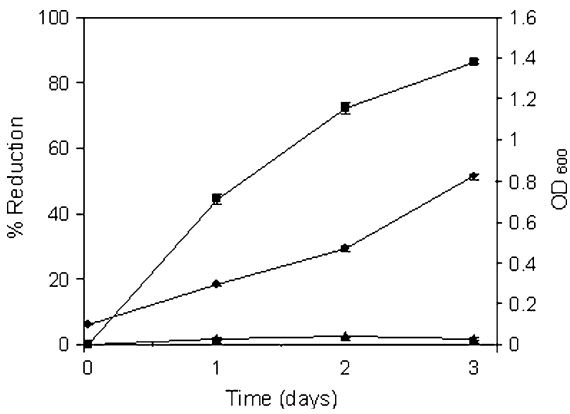

Fig. 5 Growth of *Sporosarcina* sp. G3 (*closed circle*) and % reduction (*closed square*) of Hg(II) in the presence of 74 μM $HgCl_2$ (20% of MTC). *Closed triangle* represents abiotic Hg(II) reduction. *Error bars* represent standard error from triplicate experiment

mediated by chromate reductase activity, catalyzing reduction of highly toxic Cr(VI) to less toxic Cr(III). Similarly, mercuric reductase activity in crude cell extract was found to be 0.044 units/mg protein. The culture was also found to reduce and volatilize Hg(II) during growth in nutrient broth (Fig. 5). Cr(VI) reduction during growth could not be measured as components of nutrient broth interfered with Cr(VI) estimation. On the other hand, absolutely no chromate reductase or mercuric reductase activity could be detected in the crude cell extract of a *S. ureae* strain used as negative control.

As *Sporosarcina* is a Firmicute, primers based on *Bacillus* or Firmicute *merA* sequences were used to

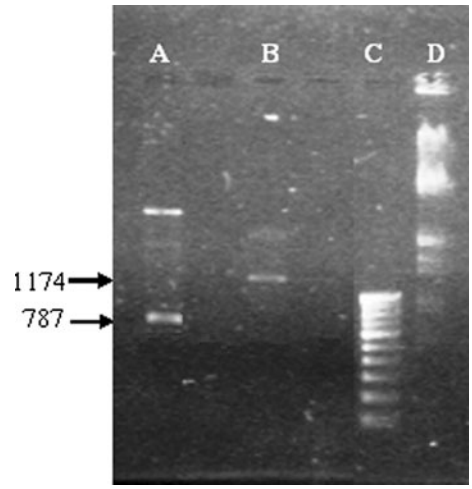

Fig. 6 Agarose gel showing amplification of *merA* gene from *Sporosarcina* sp. G3. A—PCR with F_1 and R_1 primers, B—PCR with F_1 and R_2 primers, C—100 bp ladder, D—*Eco*RI/*Hin*dIII double digest of λ phage. The non-specific amplification products were less intense than the expected bands, but could not be totally eliminated during PCR optimization

amplify its *merA* gene (Table 2). Initially, degenerate primers described for *Bacillus merA* sequences were used but they failed to give any amplification product. Then another set of degenerate primers based on conserved regions of Firmicute *merA* sequences was used. However, this approach also failed. Hence, a new set of degenerate primers F_1 and R_1 was designed based on conserved regions of a wider set of Firmicute *merA* sequences (Figs. 1, 2). PCR with these primers resulted in a 787 bp product (Fig. 6), which showed 91% similarity to *B. cereus* AH820 putative *merA* gene (NC_011773). In spite of such substantial similarity, the *Bacillus*-specific degenerate primers, covering the NC_011773 sequence, had already failed to give any product. This indicated presence of some sequence variation in the current isolate. Hence, another degenerate reverse primer R_2 was designed based on amino acid sequence of NC_011773 in the region corresponding to conserved motif 9 of PRINTS' PR00411. PCR performed using R_2 and F_1 primers gave a 1174 bp product (GenBank accession HM162837; Fig. 6), which showed 92% similarity to NC_011773. At amino acid level the identity was 92% with 99% query coverage. Phylogenetic analysis also revealed that the sequence was most closely related to *merA* genes from different *B. cereus* strains (Fig. 7).

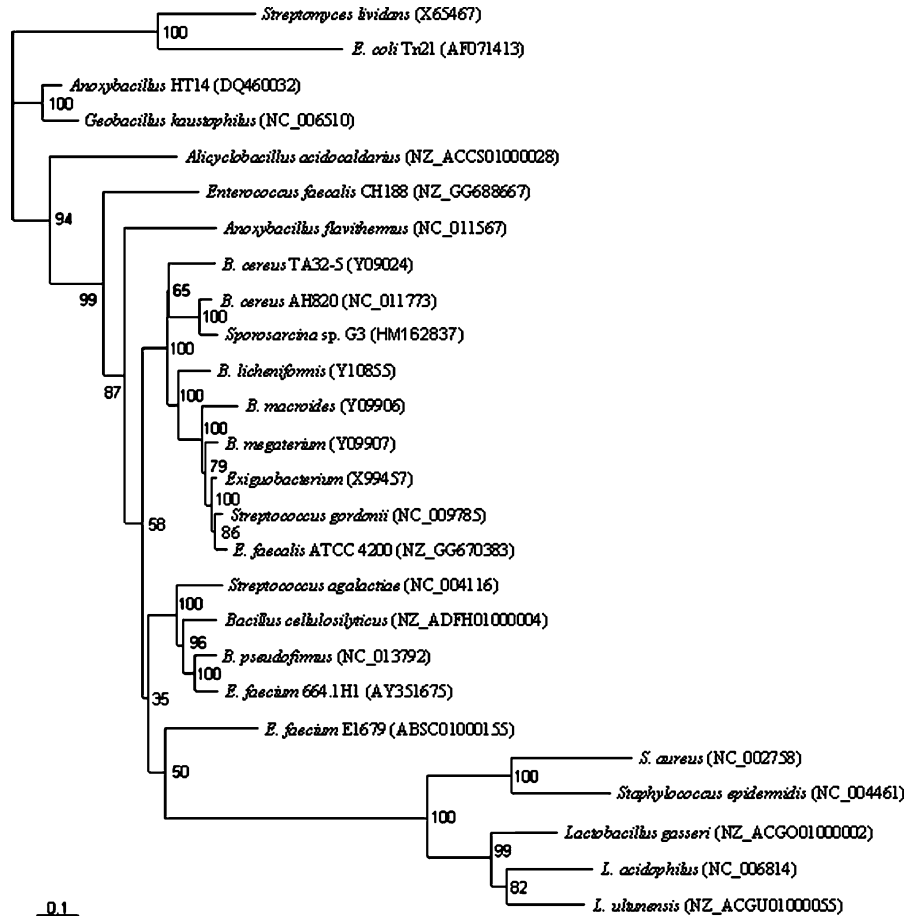

Fig. 7 Neighbour-joining tree showing the phylogeny of partial *merA* gene from *Sporosarcina* sp. G3. GenBank accession numbers are given in parentheses and bootstrap values are shown adjacent to the nodes that they support. Scale bar represents 0.1 changes per nucleotide position

Discussion

Present study describes isolation of heavy metal-resistant *Sporosarcina* strain G3, which exhibited narrow-range Hg resistance i.e. resistance toward inorganic Hg only. In addition to Hg, the culture was found to resist other heavy metals like Cd, Co, Zn and Cr, though MTC of Cd was relatively low (Fig. 3; Table 1). The culture showed promising results in removal or detoxification of metals. It could bioaccumulate significant fraction of Cd, Co and Zn during growth, and detoxify Cr and Hg by reducing them (Figs. 4, 5). Cr(VI) reduction was very likely mediated by soluble cytoplasmic chromate reductase activity, while Hg reduction and volatilization was likely mediated by MerA activity.

Since *merA* gene in Gram positive bacteria is still underexplored as compared to that in Gram negative bacteria, it was further probed in the current study. Previously described group-specific primers for this purpose failed to give any result (Table 2). Hence, a new set of degenerate primers F_1 and R_1 was designed based on a wider set of Firmicute *merA* sequences (Fig. 1). These primers were successfully utilized to amplify partial *merA* gene from the current isolate. Based on the partial sequence, another primer R_2 was designed to amplify downstream region of the *merA* gene (Fig. 6). On database searching, the final partial gene sequence (GenBank accession HM162837) showed 92% identity to *B. cereus* AH820 *merA* sequence (NC_011773). To the best of our knowledge, this is the first report of Hg resistance and *merA* gene in *Sporosarcina*.

Now days culture-independent approaches, based on molecular markers like 16S rRNA gene or specific enzymes, are very commonly used to characterize microbial communities. This frequently requires designing of group-specific primers that cover all

possible sequence variations of the gene under consideration, while avoiding non-specific amplification (Chadhain et al. 2006). Thus, primer designing is central to such analyses. Generally the primers are designed along conserved regions extracted from the gene dataset available in the database. The wider the dataset, the greater is the chance of picking up distantly related homologs. Several studies in the past have described functional diversity assessment of *merA* gene using degenerate primers. Since the *merA* gene sequence differs considerably between Gram positive and Gram negative bacteria, separate primers were frequently used for the two groups (Chatziefthimiou et al. 2007). In the current study, we tested two sets of previously described group-specific primers for the amplification of *merA* gene from *Sporosarcina*, but without any success (Table 2). This clearly indicates presence of a greater sequence diversity than that covered by the earlier primers. Current study describes two primers F_1 and R_1, which are based on conserved motifs extracted from a very wide dataset. F_1 is part of a highly conserved protein pattern, while R_1 is not part of any conserved pattern or domain, but shows very good conservation in all the Firmicute *merA* sequences. We propose that these primers can be very useful for the detection and diversity assessment of Firmicute *merA* genes in both pure culture and culture-independent studies.

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