ISSN : 0974 - 7532 Volume 9 Issue 3

Research & Reviews in

BioSciences

Regular Paper

RRBS, 9(3), 2014 [98-103]

Isolation and transformation of plasmid DNA of *Streptococcus sp.* (MTCC No. 9724) and assessment of mercuric reductase activity for mercury (II) detoxification

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ABSTRACT

The objective of the present study was to find out the molecular basis for and mercury resistance and mechanism of mercury detoxification by isolated *Streptococcus sp.* (MTCC No. 9724). For this purpose a bacterial strain was isolated from the soil of solid waste dumping site of Kolkata, India and identified at IMTEC Chandigarh, India. Plasmid DNA of the strain was isolated and was detected by horizontal electrophoresis in 0.7% agarose gel using TAE buffer (1X). Transformation of isolated plasmid DNA of *Streptococcus sp.* (MTCC No. 9724) was carried out at room temperature with heating at 42°C(shock therapy). *E.coli* HB101which was used as competent cells for isolated plasmid and the transformation efficiency was measured by dividing colonies observed with mass of plasmid introduced to the competent cells. Both bacterial cells i.e. isolated *Streptococcus sp.* (MTCC No. 9724) and transformed *E.coli* HB101 used for assay mercuric reductase enzyme. Here NADPH was used as substrate of the test enzyme and magnesium acetate, EDTA, and β-mercaptaethanol was added for enzymatic reaction. The whole experimental set up was kept in dark place for one hour and reading was taken spectrophotometrically at 340 nm wavelength. A single band of plasmid DNA with molecular weight of 3000 base pair was isolated from *Streptococcus sp.* (MTCC No. 9724). The transformation efficiency was 20% and the MIC for mercury of transformed *E.coli* HB101 was 44.5 mg/l. This study ultimately identifies the molecular basis of the mercury removal mechanism of isolated *Streptococcus sp.* (MTCC No. 9724). The responsible genetic material and mercuric reductase enzyme activity of the wild organism was confirmed through this study.

KEYWORDS

Mercury;
Plasmid;
Mercuric reductase;
Streptococcus sp. (MTCC No. 9724);
Mercury (II) detoxification.

INTRODUCTION

Many bacterial strains contain genetic determinants of resistance to heavy metals such as mercury, silver, arsenic, bismuth, cadmium, chromium, nickel, and lead and undoubtedly others. These resistance determinants are often found on plasmids and transposons, which facilitate their analysis by molecular genetics technique[1,2]. In the frequent absence of any obvious source of direct selection, these resistances occur with surprisingly high frequencies. It has been suggested that heavy metal resistances may have been selected in earlier times, and that they are merely carried along today for a free ride with selection for antibiotic resistances. For example, in Tokyo in the late 1970s both heavy metal resistances and antibiotic resistances were found with high frequencies in *Escherichia coli* isolated from hospital patients, where as heavy metal resistance plasmids without antibiotic resistance determinants were found in *E.coli* from an industrially polluted river. Selection occurs for resistances to both types of agents in the hospital, but only for resistance to toxic heavy metals in the river environment. Mercury resistance microbes are available in agricultural soil with no known mercurial input. In such settings, resistance microbes may be very rare, but they may come into much greater quantitative prominence after industrial or agricultural pollution. These major recent progresses have consisted of the cloning and DNA sequence analysis of determinants for mercury, arsenic, cadmium and tellurium resistances and initial reports of still additional resistances[3].

Mercury resistance (Hg) is a common plasmid-mediated property in gram-negative and gram-positive bacteria. This may be related to the use of mercurial compounds in industry, agriculture, and hospitals. Two classes of mercury resistance have been described: (i) narrow-spectrum resistance involves the enzymatic reduction by mercuric reductase (mer-A) of Hg2+ to Hg°, which is released as a vapor into the surrounding medium; and (ii) broad-spectrum resistance involves the cleavage of C-Hg bonds of organomercuric compounds by organomercurial lyase (merB) and the subsequent reduction of Hg2e by the reductase. Narrow-spectrum resistance (reductase alone) protects the cell from Hg2+ and some organomercuric compounds, whereas broad-spectrum resistance (reductase and lyase) provides protection from additional organomercuric compounds[4,5]. Although both merA- and merB-type genes are found in various species, there is only limited relatedness between gram-positive and gram-negative genes and approximately 60% sequence identity between the *Staphylococcus aureus* and *Bacillus* genes[6]. The genes specifying resistance to mercury are organized on mer operon, including merR, merT, merP and merA[7].

Cell-free mercury volatilization activity (mercuric reductase) was obtained from a mercury-volatilizing *Thiobacillus ferrooxidans* strain, and the properties of intact-cell and cell-free activities were compared with those determined by plasmid R100 in Escherichia coli. Intact cells of *T. ferrooxidans* volatilized mercury at pH 2.5, whereas cells of E. coli did not. Cell-free enzyme preparations from both bacteria functioned best at or above neutral pH and not at all at pH 2.5. The T. ferrooxidans mercuric reductase was a soluble enzyme that was dependent upon added NAD(P)H. The enzyme activity was stable at 80 degrees C, required an added thiol compound, and was stimulated by EDTA. Antisera against purified mercuric reductases from transposon Tn501 and plasmid R831 (which inactivated mercuric reductases from a wide range of enteric and pseudomonad strains) did not inactivate the enzyme from *T. ferrooxidans*[8,9].

The heart of the mercury reduction mechanism is mercuric ion reductase (MerA), an enzyme that catalyzes the conversion of the thiol-avid Hg(II) to volatile, uncharged Hg(0) that lacks significant affinity for any liganding functional groups. The enzyme utilizes NADPH as source of electrons and is located in the cytoplasm where this substrate is plentiful[10]. However, thiols of proteins and smaller molecules that are the primary target for tight binding by Hg (II) are also plentiful in this location. Consequently, the efficiency of the reductase at competing with these cellular thiols to scavenge and reduce the incoming metal ion is critical to the survival of the cell, and significant research has focused on understanding the features of the protein that are essential for this process. In enzymology, a mercury(II) reductase is an enzyme that catalizes the chemical reaction.$Hg(II) + (H+) + NADPH \rightarrow Hg(0) + NADP^+$. The three substrates of this enzyme are Hg, NADPH and H^+ whereas its two products are Hg (II) and NADPH[11].

This enzyme belongs to the family of oxidoreductases, specifically those oxidizing metal ion with NAD+

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or NADP+ as acceptor. The systematic name of this enzyme class is Hg:NADP+ oxidoreductase. Other names in common use include mercuric reductase, mercurate(II) reductase, mercuric ion reductase, mercury reductase, reduced NADP+:mercuric ion oxidoreductase, and mer A[12,13]. Mercury (Hg) resistance (mer) by the reduction of mercuric to elemental Hg is broadly distributed among the Bacteria and Archaea and plays an important role in Hg detoxification and biogeochemical cycling. MerA is the protein subunit of the homodimeric mercuric reductase (MR) enzyme, the central function of the mer system[14]. The objective of the present study was to find out the molecular basis for and mercury resistance and mechanism of mercury detoxification by isolated *Streptococcus sp.* (MTCC No. 9724).The bacteria was isolated and identified as *Streptococcus sp.* (MTCC No. 9724). The potentiality of mercury detoxification was also standardized and quantified in a separate study[15].

MATERIALS AND METHOD

Isolation of plasmid DNA

Plasmid DNA was isolated by the method of Kado and Liu[16]. Plasmid DNA was detected by electrophoresis in horizontal 0.7% agarose gel using TAE buffer (1X). 15 µl samples were loaded in each well and electrophoresis was carried out for two hours at 60 volts. Plasmid free culture of *E.coli* HB101 was used as markers and the gel was stained by 0.5µg/ml ethidium bromide and observed under UV-transilluminator.

Transformation of isolated plasmid DNA in a competent vector HB 101 and determination of minimum inhibitory concentration (MIC) of transformant

Transformation of isolated plasmid DNA of *Streptococcus sp.* (MTCC No. 9724) was carried out at room temperature with heating (at 42°C) shock therapy. 50mM Calcium Chloride was used for suspending pellets of *E.coli* HB101which was used as competent cells for isolated plasmid[17]. 10 µl of plasmid (0.002µg/µl) was added for transformation. Transformation efficiency was measured by dividing colonies observed with mass of plasmid introduced to the competent cells.

Assay of mercuric reductase

Cells of *Streptococcus sp.* (MTCC No. 9724) was harvested at log phase, washed carefully by phosphate buffer for removing all mercury. After centrifugation the pellets was subjected to treat with 20mM Tris-HCl, 1 mM Dithio-thritol and 10 µl of PNSF (to block proteolytic activity). The whole cell suspension was sonicated for 5 minutes. After centrifugation the supernatant was used studying mercuric reductase activities. The detail assay procedure is given in the TABLE 1. Here NADPH was used as substrate of the test enzyme and magnesium acetate, EDTA, and β-mercaptaethanol was added for enzymatic reaction. The whole experimental set up was kept in dark place for one hour and reading was taken spectrophotometrically at 340 nm wavelength[18].

TABLE 1 : Protocol for mercury reductase estimation

Experiment	Ingredients were added in cuvtte and reading was measured at 340 nm separately		
Set I (Test)	Supernatant	$HgCl_2$	NADPH
Set II (Substrate blank)	Sterile water	$HgCl_2$	NADPH
Set III (Enzyme blank)	Supernatant	Sterile water	NADPH
Set IV (Control)	Supernatant	Sterile water	$HgCl_2$

RESULTS

Isolation of plasmid DNA

A single band of plasmid DNA with molecular weight of 3000 base pair was isolated from *Streptococcus sp.* (MTCC No. 9724) while no plasmid was detected in from *E.coli* HB101 strain Plate 1.

Transformation efficiency determination and MIC of trasformant -*E.coli* HB101

The transformation efficiency was 20% and the MIC for mercury of transformed *E.coli* HB101 was 44.5 mg/l Figure 1; Plate 2.

Estimation of mercuric reductase activity

NADPH was rapidly oxidized by the crude extract obtained from *Streptococcus sp.* (MTCC No. 9724) in presence of Hg (II). Essentially no oxidation of

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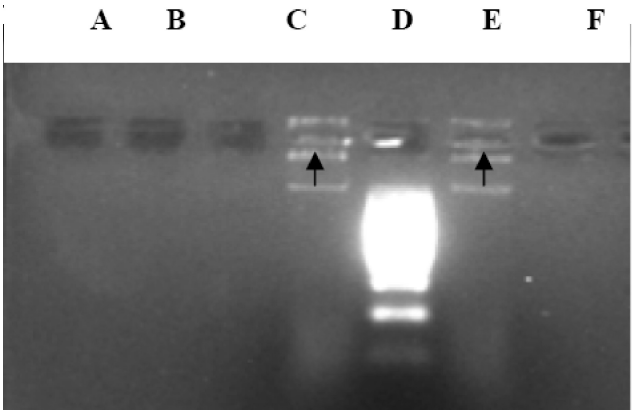

Plate 1 : Plasmid DNA isolated from *Streptococcus sp* (MTCC No. 9724) (Lane D and F)

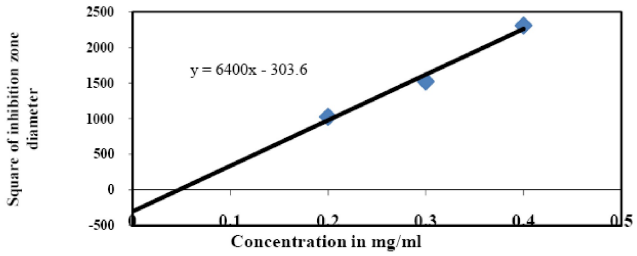

Figure 1 : MIC for mercury of transformed *E.coli* HB101

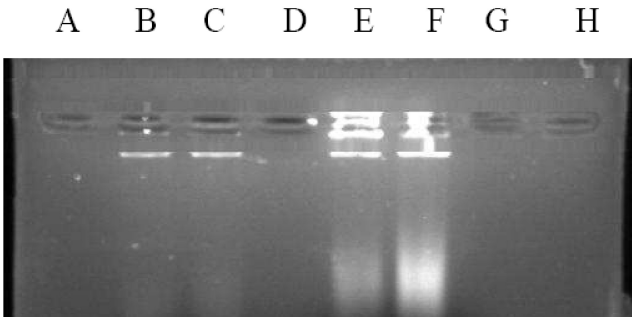

Plate 2 : Plasmid DNA isolated from *E.coli* HB101 (Lane A), transformed *E.coli* HB101 (Lane B and C) and *Streptococcus sp*. (MTCC No. 9724) (Lane E and F)

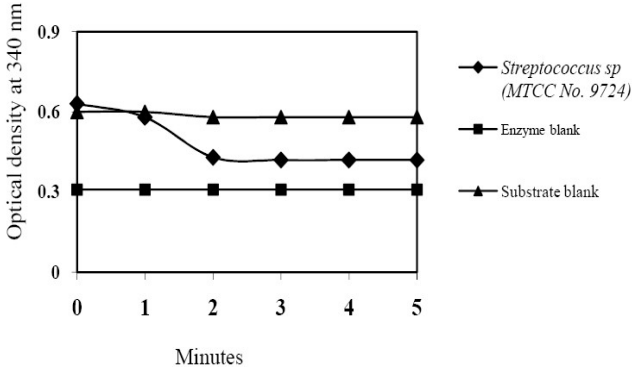

Figure 2 : Mercuric reductase activity of *Streptococcus sp.* (MTCC No. 9724)

NADPH was observed in absence of crude enzyme, whereas oxidation of NADPH in absence of Hg^{2+} was insignificant Figure 2.

DISCUSSION

The objective of this part of the work was to confirm the mercury resistance property of *Streptococcus sp.* (MTCC No. 9724) was plasmid mediated. Usually mercury resistance is a common plasmid-mediated property of both gram-negative and gram-positive bacteria[19]. This property may be applied to control the increased use of mercurial compounds in industry, agriculture and hospitals. In gram-positive bacteria systems such as *Staphyllococcus aureus*, mercury resistance is associated with antibiotic resistance plasmids[2]. In the present study, *Streptococcus sp.* (MTCC No. 9724) was found efficient mercury resistance strain and MIC was 44.5 mg/l. similar observation showed that isolated eleven clinical strains of *Enterococcus faecalis* from different geographical regions were resistant to mercuric chlorides and having plasmids[20]. Like present observation Gupta et al.[21] isolated plasmid from *Bacillus cereus* and incorporated into *Escherichia coli*. That transformed *E. coli* successfully volatilize mercury form their media. In this present investigation, after isolating the plasmid from *Streptococcus sp.* (MTCC No. 9724), it was introduced into the competent vector *E.coli* HB101successfully. The newly transformed strain of *E.coli* HB101 had the similar MIC value with respect to *Streptococcus sp.* (MTCC No. 9724) Figure 1. Unlike incorporating mercury resistant gene (Mer-gene) to a competent bacterial cell, De et al[22] had cured the plasmid and after that the bacterial strain had lost their resistance to mercury.

The Bacteria that were resistant to inorganic and organic mercury were first isolated from mercury contaminated soil in Japan in the late 1960s. Since that time mercury resistance has come to be recognized as widespread among prokaryotic species isolated from human and environmental sources[23-25]. The mercury resistant *Streptococcus sp.* (MTCC No. 9724) also isolated from dumping ground of Kolkata, India and also similarly had shown the resistance to inorganic mercury. The mechanism of mercury resistance had been studied extensively by different investigators[26,27,13]. Other researchers reported that most of the isolated mercury resistant bacterial strain have plasmid encoded with a regulatory protein (MerR; for control mRNA synthesis), transport protein (MerT and others), a cell

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surface binding protein (MerP) and the enzyme (mercuric reductase MerA and organomercurial lyase MerB)[28-30]. In this present study it was tried to reveal the mechanism of mercury resistance of *Streptococcus sp.* (MTCC No. 9724). In order to find out the mechanism, mercuric reductase enzyme had been assayed. Both *Streptococcus sp.* (MTCC No. 9724) and transformed *E.coli* HB101 had shown the mercuric reductase activity Figure 2.

Several recent studies had reported that like this working strain *Streptococcus sp.* (MTCC No. 9724), few other strains of *Streptococcus* and *Enterococcus* species had plasmids which is responsible for the production of mercuric reductase[31,32,14]. This enzyme catalyzes the cytoplasmic reduction of inorganic mercuric ions (Hg^{+2}) to elemental mercury (Hg^{0}), which is volatile and is thus automatically removed from their growth media[33]. The reduction of ionic mercury to elemental mercury by the mercuric reductase enzyme (MerA) plays an important role in the biogeochemical cycling of mercury. Like the present *Streptococcus sp.* (MTCC No. 9724), similar aerobic Gram positive bacteria was isolated from sediment of Meadowland, New Jersey could able to detoxify mercury by cytoplasmic mercuric reductase enzyme and the enzymatic activity had observed from soil of mercury contaminated areas of Oak Ridge (USA)[34,35]. A paleobacterial strains carry ancient plasmids and transposons which also contain mercury resistance determine[36].

CONCLUSIONS

This study ultimately identifies the molecular basis of the mercury removal mechanism of isolated *Streptococcus sp.* (MTCC No. 9724). The responsible genetic material and mercuric reductase enzyme activity of the screened organism was confirmed through this study. A single band of plasmid DNA with molecular weight of 3000 base pair was isolated from *Streptococcus sp.* (MTCC No. 9724). The transformation efficiency was 20% and the MIC for mercury of transformed *E.coli* HB101 was 44.5 mg/l. This isolated strain can be used as the end of pipe treatment of mercury removal and the isolated plasmid can also be used in incorporatation of gene for development of transgenic organism.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Department of Environmental Science, University of Kalyani and Department of Biotechnology Heritage Institute of Technology for providing support and necessary facilities for research work.

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