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ACKNOWLEDGEMENTS. V.K. thanks the Department of Botany, University of Jammu, Jammu for providing laboratory and library facilities, and the Council of Scientific and Industrial Research, New Delhi for providing financial assistance in the form of Junior and Senior Research Fellowships. Dr Geeta is thanked for reading the manuscript critically.

Received 19 December 2011; accepted 14 June 2012

Biological characterization of lead-resistant bacteria to explore role of bacterial metallothionein in lead resistance

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Lead-resistant bacterial isolates *Salmonella choleraesuis* strain 4A, *Proteus penneri* strain GM10, *Bacillus subtillis* strain GM02, *Pseudomonas aeruginosa* strain 4EA, *Proteus penneri* strain GM03 and *Providentia rettgeri* strain GM04 were isolated from soil contaminated with car battery waste from Goa, India. All the isolates except *Pseudomonas aeruginosa* strain 4EA showed presence of plasmids. Polymerase chain reaction amplification of 507 bp internal fragment of *smtAB* genes encoding bacterial metallothionein and intracellular bioaccumulation of 19 and 22 mg lead per gram dry weight in *Salmonella choleraesuis* strain 4A and *Proteus penneri* strain GM10 respectively revealed presence of metal-binding metallothionein (SmtA) being responsible for lead resistance encoded by genomic DNA. These lead-resistant and lead-bioaccumulating bacterial isolates can be employed for bioremediation of lead present in contaminated environmental sites.

Keywords: Bacteria, bioaccumulation, lead, metallothionein.

LEAD has a wide range of applications in various industries, viz. petroleum, electronics, battery, paints, ceramics, stained glass, biocide preparation and ammunitions with annual global demand of refined lead exceeding 87 lakh tonnes[1]. Lead, mercury and cadmium are biologically non-essential and toxic heavy metals which affect the terrestrial and aquatic biota along with human beings due to their release from industrial effluents directly into terrestrial and estuarine ecosystems[1,2]. Lead is a persistent environmental pollutant with half life of approximately 5000 years and biomagnifies through the trophic levels. It is important to note that lead causes neurodegenerative diseases, reproductive impairments and renal failure in humans[3,4]. Long-term exposure of humans to lead causes anaemia, cancer, interferes with vitamin D metabolism and causes coma and death if blood level exceeds 70 μg/dl[5–7].

Heavy metal contamination is a major environmental threat worldwide due to their adverse effects (toxicity) on natural biota and humans which is manifested as DNA

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damage, lipid peroxidation, binding to –SH groups of essential proteins and enzymes and generation of reactive oxygen species[8–10]. Elevated levels of heavy metals, viz. Hg, Cd, Pb and Zn in the environment adversely affect the function and structure of microbial community[11]. Interestingly, some natural microbes tolerate very high levels of heavy metals although they are toxic to non-target organisms present in the environment. It is important to note that several genetic mechanisms are known in bacteria which maintain intracellular homeostasis of essential metals and regulate resistance against toxic metals. Metal efflux, intracellular sequestration by metal binding metallothioneins, extracellular sequestration by exopolysaccharides, cell surface biosorption by negative groups, bioprecipitation and redox reactions are the resistance mechanisms which are present in microorganisms to counteract heavy metal stress[12–17]. Metal-induced metallothioneins are small cystein-rich metal-binding proteins synthesized under heavy metal stress and are involved in intracellular metal sequestration and homeostasis in bacteria[12,16].

The present communication reports on isolation, identification and characterization of lead-resistant bacteria from car battery waste with reference to growth in presence of lead, lead tolerance limit, molecular detection of metallothioneins and lead bioaccumulation.

Lead-contaminated soil samples collected from a battery manufacturing company from Goa, India were plated on PYT80 agar plates containing 100 μM lead nitrate using dilution plating technique and incubated at room temperature[18,19]. Lead-resistant discrete colonies were further spot inoculated on PYT80 agar plates with different levels of lead nitrate and bacterial colonies which appeared at the highest lead level were selected for further biological characterization.

Identification of highly lead-resistant bacterial strains from car battery waste was done by biochemical tests according to the *Bergey's Manual of Systematic Bacteriology* which was substantiated by fatty acid methyl ester (FAME) profile using Sherlock version 6.0B[20].

Lead resistance limit of bacterial strains was determined using tris-minimal medium (TMM) amended with different concentrations of lead nitrate with 0.4% glucose as carbon source. Bacterial cultures were grown at pH 7.2 and 30°C with constant shaking at 150 rpm and growth was monitored as absorbance at 600 nm after regular time interval using UV-Vis spectrophotometer (Shimadzu, UV-2450, Japan). Stock of 1 M lead nitrate (SRL, India) was prepared and filter-sterilized before use.

Internal fragment *smtAB* genes was PCR-amplified using following primers: smt1 (5′ GAT CGA CGT TGC AGA GAC AG 3′) and smt2 (5′ GAT CGA GGG CGT TTT GAT AA 3′). Plasmid and genomic DNAs of lead-resistant bacterial strains were used as template[21]. Genomic DNA of lead-sensitive *E. coli* HB101 was taken as negative control. Reaction mixture (50 μl) contained 0.2 mmol each dNTPs (dATP, dCTP, dGTP and dTTP), 20 pmol each primer, 10 ng template DNA and 0.25 U *Taq* DNA polymerase. Plasmid DNA of bacterial strains was extracted using alkaline lysis method[22] whereas genomic DNA was isolated using DNA isolation kit (Bangalore Genei, India).

Two bacterial isolates 4A and GM10 were grown separately in 0.2 and 0.4 mM lead nitrate respectively, and harvested using refrigerated centrifuge at 8000 rpm for 10 min. Cell pellet was washed with 20 mM ethylene dianine tetra acetic acid (EDTA) to remove lead bound to the cell surface. Cell pellets were dried at 100°C and 0.1 g of dried cell pellet was then digested with concentrated HNO_3 using microwave digestion system. Lead

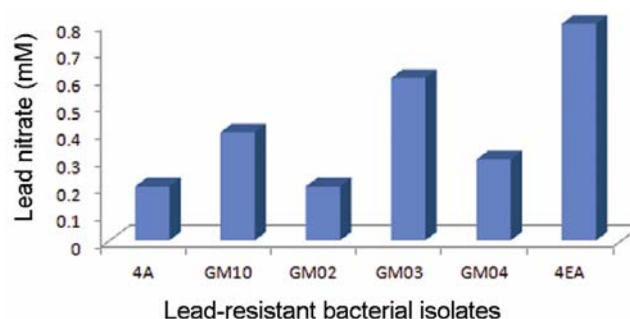

Figure 1. Lead-resistance limit of *Salmonella choleraesuis* strain 4A, *Proteus penneri* strain GM-10, *Bacillus subtillis* strain GM02, *Proteus penneri* strain GM03, *Providentia rettgeri* strain GM04 and *Pseudomonas aeruginosa* strain 4EA.

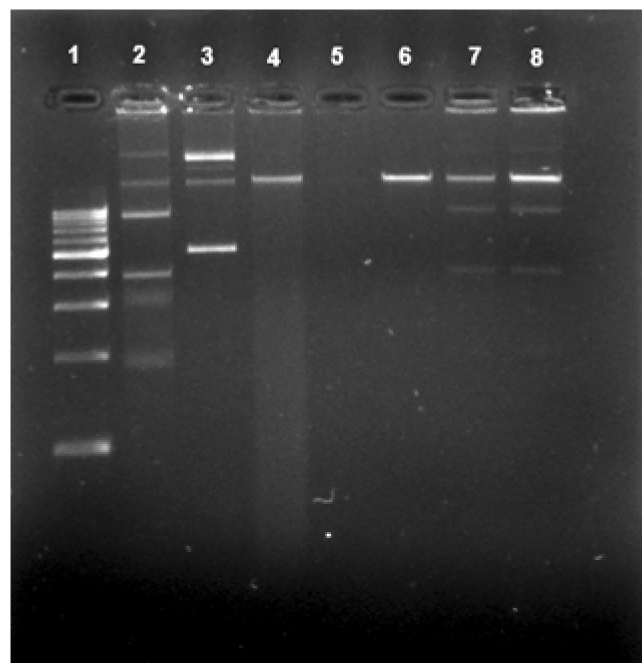

Figure 2. Plasmid profile of lead-resistant bacteria. Lane 1, 1 kb DNA ladder (marker); Lane 2, *Salmonella choleraesuis* strain 4A; Lane 3, *Proteus penneri* strain GM-10; Lane 4, *Bacillus subtillis* strain GM02; Lane 5, *Pseudomonas aeruginosa* strain 4EA; Lane 6, *Proteus penneri* strain GM03 and Lanes 7, 8, *Providentia rettgeri* strain GM04.

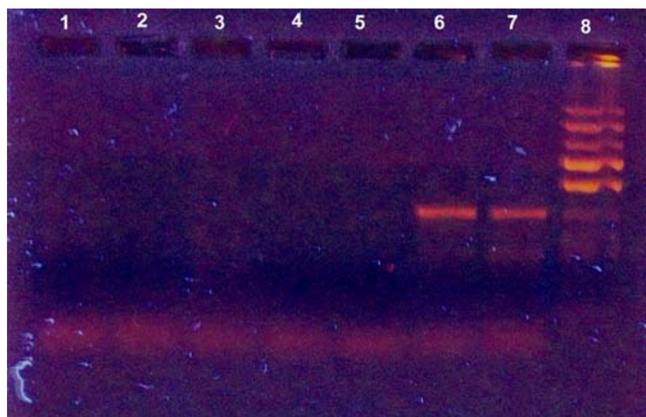

Figure 3. PCR amplification of internal fragment of *smtAB* gene using genomic DNA as template. Lane 1, *Providentia rettgeri* GM04; Lane 2, *Proteus penneri* GM03; Lane 3, *Pseudomonas aeruginosa* 4EA; Lane 4, *Bacillus subtillis* GM02; Lane 5, *E. coli* HB101; Lane 6, *Proteus penneri* GM-10; Lane 7, *Salmonella choleraesuis* 4A; Lane 8, 100 bp DNA ladder.

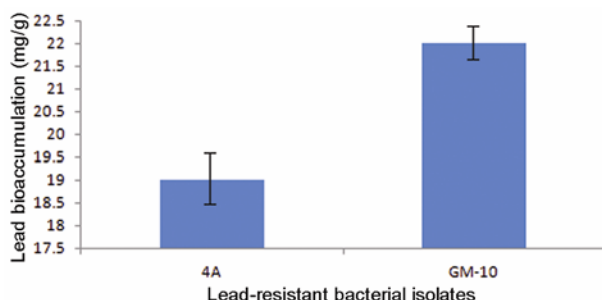

Figure 4. Lead bioaccumulation by *Salmonella choleraesuis* strain 4A and *Proteus penneri* strain GM10.

content was analysed using atomic absorption spectroscopy (AAS, Varian AA240 FS, Australia)[23].

The lead-resistant bacterial isolates were identified as *Salmonella choleraesuis* strain 4A, *Proteus penneri* strain GM-10, *Bacillus subtillis* strain GM02, *Pseudomonas aeruginosa* strain 4EA, *Proteus penneri* strain GM03 and *Providentia rettgeri* strain GM04 based on biochemical characteristics and FAME profile.

It is interesting to note that *Salmonella choleraesuis* strain 4A, *Proteus penneri* strain GM-10, *Bacillus subtillis* strain GM02, *Pseudomonas aeruginosa* strain 4EA, *P. penneri* strain GM03 and *Providentia rettgeri* strain GM04 could resist lead nitrate up to 0.2, 0.4, 0.2, 0.8, 0.6 and 0.3 mM respectively (Figure 1). Plasmid profile of these lead-resistant bacterial strains clearly demonstrated/revealed presence of large plasmids and some of them possessed multiple plasmids (Figure 2). However, *Pseudomonas aeruginosa* strain 4EA was devoid of plasmids, indicating that lead resistance in this isolate may be governed by genes located on chromosomal genome.

PCR amplification of 507 bp internal fragment of *smtAB* genes using genomic DNA of *Salmonella choleraesuis* strain 4A and *Proteus penneri* strain GM-10 as the template revealed involvement of metal-binding metallothionein (SmtA) in lead resistance (Figure 3). But, no amplicon of *smtAB* internal region was found using plasmid DNA as template. This evidently demonstrated that *smtA* gene encoding lead-binding metallothionein is located on chromosomal genome, although these strains possess plasmids. AAS spectroscopic analysis revealed significant intracellular bioaccumulation of lead 19 and 22 mg per gram dry weight in case of *Salmonella choleraesuis* strain 4A and *Proteus penneri* strain GM-10 respectively (Figure 4). Significant intracellular accumulation of lead is due to sequestration of lead by SmtA metallothionein. These results also go hand in hand with our earlier studies where we have demonstrated that *Pseudomonas aeruginosa* strain WI-1 resists 0.6 mM lead nitrate by metallothionein (BmtA)-mediated intracellular sequestration of 26.5 mg lead/g dry cell[16].

We have thus, demonstrated that the presence of *smtA* gene encoding bacterial metallothionein (SmtA) in *Salmonella choleraesuis* strain 4A and *Proteus penneri* strain GM10 is responsible for lead resistance, but there may be other lead resistance mechanisms in *Bacillus subtillis* strain GM02, *Pseudomonas aeruginosa* strain 4EA, *Proteus penneri* strain GM03 and *Providentia rettgeri* strain GM04, which could be plasmid or genomic DNA-mediated. *Salmonella choleraesuis* strain 4A and *Proteus penneri* strain GM10 may be employed as potential strains for bioremediation and recovery of lead from terrestrial sites contaminated with lead since these bacteria have potential for significant intracellular bioaccumulation of lead with metal-binding metallothioneins.

Bioremediation and recovery of heavy metals including lead from contaminated terrestrial sites and industrial effluents is a major challenge. Therefore, several approaches have been adopted for lead bioremediation. One of these approaches is based on microorganisms, as the technique is economically viable and ecofriendly. The two highly lead-resistant bacterial isolates bioaccumulating significant amount of lead may be employed for bioremediation of lead in contaminated environmental sites.

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ACKNOWLEDGEMENT. M.M.N. gratefully acknowledges UGC, New Delhi for financial support as JRF.

Received 25 January 2012; revised accepted 28 June 2012