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ORIGINAL ARTICLE

Lead-resistant *Providencia alcalifaciens* strain 2EA bioprecipitates Pb^{+2} as lead phosphate

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Significance and Impact of the Study: *Providencia alcalifaciens* strain 2EA resist lead nitrate up to 0·0014 mol l^{-1} by precipitating soluble lead (Pb^{+2}) as insoluble light brown solid. Scanning electron microscopy coupled with energy-dispersive X-ray spectrometric analysis (SEM-EDX) and X-ray diffraction spectroscopy (XRD) revealed extracellular light brown precipitate as lead orthophosphate mineral, that is, Pb_9 $(PO_4)_6$ catalysed by phosphatase enzyme. *Providencia alcalifaciens* strain 2EA could be used for bioremediation of lead-contaminated environmental sites, as it can efficiently precipitate lead as insoluble lead phosphate.

Keywords
bacteria, bioprecipitate, bioremediation, lead, phosphatase, resistant.

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Abstract

A lead-resistant bacteria isolated from soil contaminated with car battery waste were identified as *Providencia alcalifaciens* based on biochemical characteristics, FAME profile and 16S rRNA sequencing and designated as strain 2EA. It resists lead nitrate up to 0·0014 mol l^{-1} by precipitating soluble lead as insoluble light brown solid. Scanning electron microscopy coupled with energy-dispersive X-ray spectrometric analysis (SEM-EDX) and X-ray diffraction spectroscopy (XRD) revealed extracellular light brown precipitate as lead orthophosphate mineral, that is, Pb_9 $(PO_4)_6$ catalysed by phosphatase enzyme. This lead-resistant bacterial strain also demonstrated tolerance to high levels of cadmium and mercury along with multiple antibiotic resistance. *Providencia alcalifaciens* strain 2EA could be used for bioremediation of lead-contaminated environmental sites, as it can efficiently precipitate lead as lead phosphate.

Introduction

Pollution of aquatic as well as terrestrial environment due to uncontrolled release of industrial wastes and effluents containing toxic heavy metals viz. Pb, Hg and Cd poses serious threat to natural biota and results in their biomagnifications in the food chain. Lead needs special attention as it is a hazardous pollutant due to its high toxicity, long-term persistence in the environment and is a cumulative poison. It is important to note that prolong exposure of humans to lead can cause anaemia, reproductive impairment, renal failure and neurodegenerative damage (Fowler 1998; Tong *et al.* 2000; Lam *et al.* 2007). But interestingly, micro-organisms have inherent capability to tolerate high concentrations of toxic heavy metals including lead, which involves intracellular or extracellular sequestration, absorption on cell surface, ATPase-mediated efflux, conversion into non-toxic or less toxic forms and precipitation into nonbioavailable forms (Nies 1999; Roane 1999; Blindauer *et al.* 2002; Wang *et al.* 2002; De *et al.* 2008; Taghvi *et al.* 2009; Naik and Dubey 2011; Colin *et al.* 2012; Naik *et al.* 2012). As bioavailability of heavy metals is an important factor regulating their toxicity to micro-organisms and animals (Roane 1999; Pike *et al.* 2002; Park *et al.* 2011), the precipitation mechanism of toxic metals viz. Pb, Cd and Hg to insoluble forms reduces their bioavailability significantly and results in amelioration of toxicity.

Although microbial precipitation of heavy metals is an interesting mechanism, very few reports are available on microbial precipitation of lead. It is interesting to note that scanning electron microscopy and X-ray microanalysis demonstrated that lead was precipitated at the surface of *Citrobacter* sp. as $PbHPO_4$ (Aickin *et al.* 1979), whereas

Levinson *et al.* (1996) reported intracellular accumulation of $Pb_3(PO_4)_2$ is the precipitate catalysed by phosphatase produced by *Staphylococcus aureus*, when grown in the presence of high concentration of soluble lead nitrate. Similarly, *Vibrio harveyi* also showed capability to precipitate lead as an unusual phosphate compound, that is, $Pb_9(PO_4)_6$ (Mire *et al.* 2004), and *Klebsiella* sp. grown in phosphate-limited conditions precipitated lead as PbS (Aiking *et al.* 1985). In *Staph. aureus*, lead precipitation occurred in both lead-sensitive and lead-resistant strains; however, the resistant strains were more effective at expulsion (Levinson and Mahler 1998). Insoluble compound generated by *Pseudomonas* sp. contained both lead and phosphorus, but phosphate-replete cultures were more efficient at expulsion of lead compound (Al-Aoukaty *et al.* 1991). Lead-resistant and phosphate-solubilizing *Enterobacter cloacae* immobilized lead in Pb-spiked soil by forming pyromorphite as indicated by XRD analysis (Park *et al.* 2011). Alkaline phosphatase gene, *phoK*, was isolated from *Sphingomonas* sp. BSAR-1 and cloned in *Escherichia coli*. Overexpressed alkaline phosphatase gene bioprecipitated uranium as uranyl phosphate, that is, $H_2(UO_2)_2(PO4)_2$. $8H_2O$ from alkaline solutions (Nilgiriwala *et al.* 2008). Heavy metal-resistant bacteria (e.g. Cd^{+2}, Cr^{+6}, Pb^{+2} and Hg^{+2}) isolated from radionuclide and metal-contaminated subsurface soils, which produce acid phosphohydrolases (phosphatases), hydrolyses phosphoester substrates in acidic to neutral pH to bioprecipitate uranium (VI) aerobically as uranyl phosphate (Martinez *et al.* 2007). These findings have demonstrated that majority of bacteria precipitate lead and radionuclide typically as a lead phosphate and radionuclide phosphate catalysed by phosphatase enzyme, but very few times chemical nature has been deduced. The precipitate formed can either accumulate at the cell membranes or expel out depending on the bacterial strain. Bioprecipitation of heavy metals as metal phosphate used in reclamation of polluted environment has been effective, affordable and eco-friendly technological solution.

In this investigation, we report isolation, identification and biological characterization of lead-resistant bacteria from soil sample contaminated with car battery waste of Goa, India, with reference to lead tolerance limit, resistance to antibiotics, resistance to cadmium and mercury along with SEM-EDX and XRD analysis of the precipitate to determine its chemical nature.

Results and discussion

Identification of bacterial strain

The bacterial strain was isolated from a soil sample contaminated with 5496 μg Pb g^{-1} of soil, indicating the high resistance of this bacterial strain to lead. It was Gram-negative, rod-shaped, motile and facultative anaerobic. It showed citrate utilization, methyl red and indole tests positive, but Voges–Proskauer's test was negative. It showed presence of enzymes viz. tryptophan deaminase and nitrate reductase, whereas urease was absent. Based on these biochemical tests following *Bergey's Manual of Systematic Bacteriology*, FAME profile and 16S rDNA sequence analysis followed by BLAST search confirmed lead-resistant bacteria as *Providencia alcalifaciens* and were designated as strain 2EA (GenBank accession no. HQ268734). Phylogenetic analysis clearly revealed its evolutionary relatedness with other *Providencia* sp. already reported (Fig. 1).

Lead resistance study and characterization of precipitate

Providencia alcalifaciens strain 2EA can resist lead nitrate up to 0·0014 mol l^{-1} (MIC: 0·0015 mol l^{-1}) (Fig. 2). This bacterial strain also tolerated cadmium and mercury

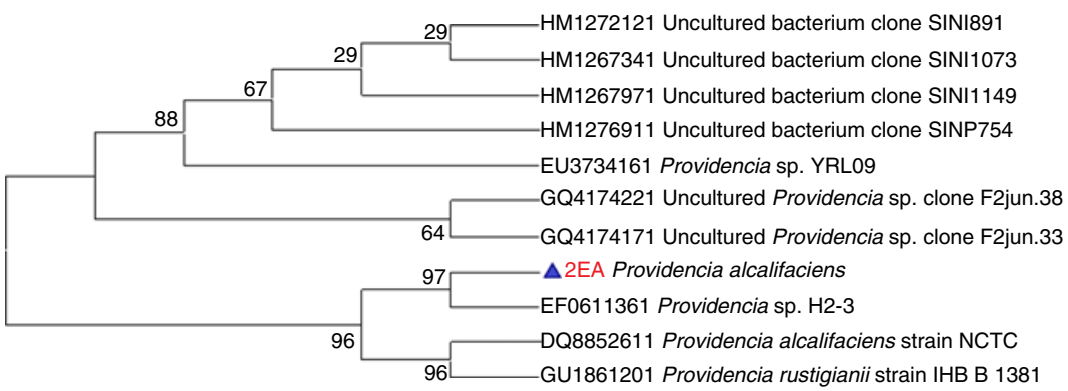

Figure 1 Phylogenetic tree showing the evolutionary relationship of the test bacterium with other closely related bacteria using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches.

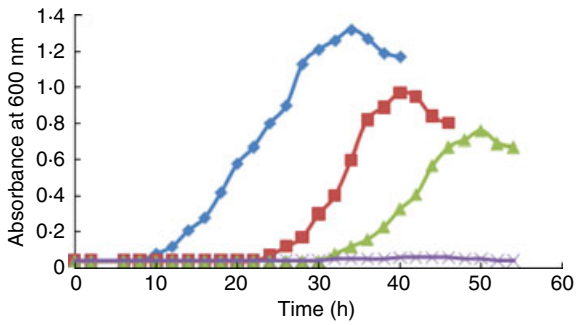

Figure 2 Growth behaviour of *Providencia alcalifaciens* strain 2EA in tris-minimal medium amended with different concentrations of lead nitrate. (—◆—) 0 mol l^{-1} (control); (—■—) 0·001 mol l^{-1}; (—▲—) 0·0014 mol l^{-1}; (—×—) 0·0015 mol l^{-1}.

(MIC values 0·0002 and 0·00003 mol l^{-1}, respectively). Antibiotic susceptibility test clearly revealed that this strain is resistant to several antibiotics viz. ampicillin (25 μg per disc), cephalexin (30 μg per disc), oleondamycin (15 μg per disc), ciphaloridine (30 μg per disc), erythromycin (15 μg per disc) and amikacin (10 μg per disc).

Providencia alcalifaciens strain 2EA produced insoluble brown precipitate in the growth medium when cells were grown in modified TMM amended with 0·0014 mol l^{-1} lead nitrate and 0·4% glucose as sole carbon source. X-ray diffraction pattern of the purified brown precipitate showed close resemblance with lead phosphate, that is, $Pb_9(PO4)_6$ by referring JCPDS card no. 33-0768 (Fig. 3) (Martinez *et al.* 2004; Mire *et al.* 2004). Scanning electron microscopy and EDX analysis of the brown precipitate further confirmed that the precipitate was a complex lead phosphate (Fig. 4a,b). On TPMG agar plates, *P. alcalifaciens* strain 2EA showed bluish green precipitation zone around deep green colony, which revealed involvement of phosphatase enzyme in lead immobilization (data not shown).

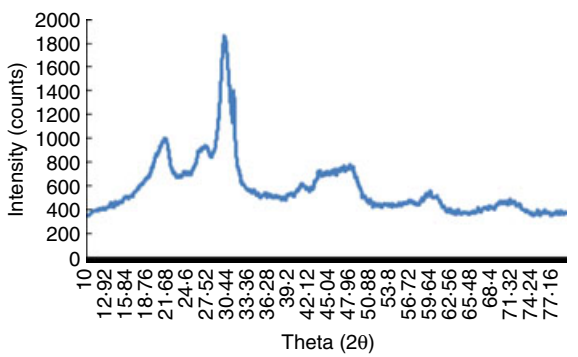

Figure 3 X-ray diffraction pattern of purified brown precipitate produced by *Providencia alcalifaciens* strain 2EA in the presence of 0·0014 mol l^{-1} lead nitrate in tris-minimal medium. (JCPDS pattern 33-0768).

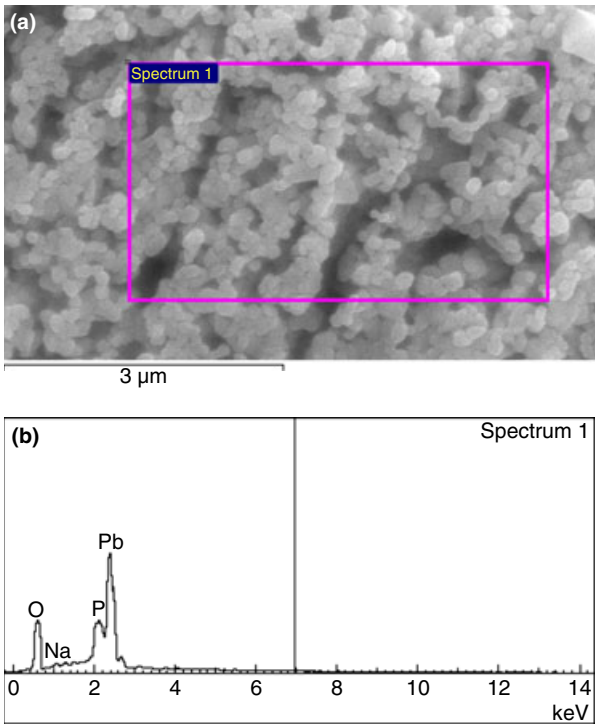

Figure 4 Scanning electron microscopy (a) and energy-dispersive X-ray analysis (b) of extracellular brown precipitate produced by *Providencia alcalifaciens* strain 2EA (Area marked with square is used for energy-dispersive X-ray analysis).

Concentration, bioavailability of heavy metals and composition of the medium are the important factors regulating the response of microbes to toxic heavy metals (Roane 1999; Pike *et al.* 2002; Park *et al.* 2011). Modified Tris-minimal medium (TMM) with 0·0005 mol l^{-1} β-glycerol phosphate was used instead of inorganic phosphate in lead resistance study to minimize lead precipitation as lead phosphate (Naik and Dubey 2011). Precipitating heavy metals into nonbioavailable form is one of the resistant mechanisms conferred by microorganisms against toxic heavy metals and radionuclides (Aickin *et al.* 1979; Levinson and Mahler 1998; Mire *et al.* 2004; Nilgiriwala *et al.* 2008; Park *et al.* 2011). Although there are several reports on microbial metal precipitation, exact chemical composition of precipitate has been elucidated in very few cases. It is interesting to mention that we have isolated highly lead-resistant *P. alcalifaciens* strain 2EA, which produced an insoluble extracellular brown precipitate when grown in TMM amended with 0·0014 mol l^{-1} lead nitrate. XRD and SEM-EDX analysis confirmed the brown precipitate as $Pb_9(PO_4)_6$. To survive toxic levels of lead, *P. alcalifaciens* has developed this resistance mechanism of converting soluble toxic lead into insoluble lead phosphate, that is, $Pb_9(PO_4)_6$. Phosphatase

enzyme produced by *P. alcalifaciens* acts on β-glycerol phosphate (organic phosphate) to release inorganic (ionic) phosphate, which complexes with lead ions (Pb^{+2}) to precipitate lead as lead phosphate. The hypothesis of this research is that precipitation of lead as lead phosphate was catalysed by enzyme phosphatase produced by *P. alcalifaciens* strain 2EA.

It is well known that environmental bioavailability of the toxicants to natural biota regulates their toxicity threshold. Lead was not available freely as Pb^{+2} ions to the *P. alcalifaciens* strain 2EA after lead was precipitated as unusual insoluble $Pb_9(PO_4)_6$ in TMM. Therefore, we can infer that precipitation of soluble lead as lead phosphate catalysed by phosphatase enzyme served as a resistance mechanism in *P. alcalifaciens* strain 2EA exposed to toxic lead. Lead precipitation as lead phosphate, that is $Pb_9(PO_4)_6$, by naturally occurring *P. alcalifaciens* strain 2EA isolated from soil contaminated with car battery waste containing high levels of lead was observed in this study. *Providencia alcalifaciens* strain 2EA can potentially be employed for bioremediation of lead-contaminated sites, as it can efficiently precipitate lead to lead phosphate to mitigate Pb^{+2} bioavailability.

Materials and methods

Determination of lead in the soil sample

Lead content of soil sample contaminated with car battery waste was determined by atomic absorption spectroscopy (AAS, Varian AA240 FS, Australia) as per the procedure of Khan *et al.* (2010). Dried and powdered soil sample (1 g) was taken into a Teflon beaker, and 15 ml of aqua regia was added. It was kept at room temperature overnight and followed by heating on the hot plate until no brown fumes were produced. Concentrated $HClO_4$ (5 ml) was added and heated again on low heat until the solution was evaporated near to dryness.

Isolation of lead-resistant bacteria

Lead-resistant bacteria were isolated from soil sample contaminated with battery waste containing lead as contaminant by dilution plating of soil samples on PYT80 agar plates supplemented with 0·0001 mol l^{-1} lead nitrate (Naik and Dubey 2011). Filter-sterilized 1 mol l^{-1} lead nitrate solution was used as stock and was added to PYT80 medium after sterilization. Discrete colonies appeared were further inoculated on PYT80 agar plate amended with gradually increasing concentration of lead nitrate, and bacteria, which can grow on agar plate containing highest lead concentration, were chosen for further characterization.

Identification of bacterial strain

Identification of lead-resistant bacterial strain was carried out based on morphological, biochemical characteristics, fatty acid methyl ester profile (Sherlock version 6·0B) and 16S rDNA sequence analysis. 16S rDNA was PCR amplified using following eubacterial primers: 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′ACGG-CTACCTTGTTACGACTT-3′). Sequencing was performed at Xcelris Laboratories, Ahmedabad, Gujarat, India, and compared against GenBank database using NCBI Blast search (Altchul *et al.* 1997). Phylogenetic analysis of the bacterial isolate was also performed by constructing phylogenetic tree using Mega4 software (Saitou and Nei 1987; Tamura *et al.* 2007).

Growth and lead resistance studies

Modified Tris-minimal medium (TMM) supplemented with different concentrations of lead nitrate was used to study growth behaviour and lead resistance limit of the lead-resistant bacterial strain 2EA (Mergeay *et al.* 1985; Naik and Dubey 2011). Growth study was carried out at 30°C, pH 7·2, with constant shaking at 150 rev min^{-1} using 0·4% glucose as sole carbon source. Absorbance of culture broth was recorded at 600 nm using UV–vis spectrophotometer (UV-2450; Shimadzu, Tokyo, Japan) at regular time intervals, and graph was plotted between absorbance and time interval. Cross-tolerance to cadmium and mercury was also studied in TMM.

Antibiotic susceptibility testing

Antibiotic susceptibility of this lead-resistant strain was also explored following Kirby–Bauer disc diffusion technique on Muller–Hinton agar using antibiotic discs (Himedia, India) (Bauer *et al.* 1966).

Purification of extracellular precipitate

Tris-minimal medium broth (1 l) supplemented with 0·0014 mol l^{-1} lead nitrate was inoculated with overnight-grown culture of lead-resistant bacterial strain 2EA. After incubation for 48 h, culture broth was harvested at 1500 rev min^{-1} at room temperature to separate extracellular precipitate from bacterial cells. Because the brown precipitate was insoluble, it was located in the pellet fraction; no significant amount of brown solid was present in the supernatant. At this point, the precipitate was washed with distilled water and was centrifuged again to remove bacterial cells. Similarly, centrifuge and wash the resultant precipitate six times to completely remove bacterial cells. After the final centrifugation step, the supernatant was

decanted, and the pellets were gently washed off the walls of the centrifuge tubes with deionized water and decanted into two clean, empty plastic Petri dishes. Precipitate was dried in desiccator for 48 h. The entire procedure yielded a light brown powder.

XRD analysis of the precipitate

To perform X-ray diffraction analysis of the brown precipitate, it was grounded and smeared on glass slide precoated with ultrathin layer of petroleum jelly. Scintag X3 diffractometer coupled with Cu–K radiation (λ = 1·5418 nm) and Li-drifted germanium detector was used. Data were collected at a speed of 2 degrees per min and were analysed using XRD library supplied by Joint Commission on Powder Diffraction Software (JCPDS).

SEM-EDX analysis of the precipitate

Scanning electron microscopy coupled with energy-dispersive X-ray spectrometry (SEM-EDX, JEOL JSM-5800LV; Tokyo, Japan) was performed to examine the chemical nature and particle size of precipitate produced by the lead-resistant bacterial strain. The precipitate was placed on the metal stub followed by coating with gold and analysed using SEM-EDX (Naik and Dubey 2011).

Detection of phosphatase enzyme

The bacterial strain was plated on tryptose phosphate methyl green agar (TPMG) plates containing 0·001 g ml^{-1} phenolphthalein diphosphate (PDP) and 0·00005 g ml^{-1} methyl green (MG) to detect phosphatase activity as described earlier (Martinez *et al.* 2007; Nilgiriwala *et al.* 2008).

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