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Biological responses of symbiotic *Rhizobium radiobacter* strain VBCK1062 to the arsenic contaminated rhizosphere soils of mung bean

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ABSTRACT

The rationale could be that mung bean is cultivated in areas of arsenic contamination and therefore it is worth investigating how *Rhizobium* is impacted by arsenic exposure. The objective(s) of the study deals with relationship between *Rhizobium* metal tolerance and its adaptations to metal stressed environment. The selected strain was recovered from root nodules of *Vigna radiata*, based on viscous EPS production and arsenic tolerant capacity, identified as *R. radiobacter by* 16 S rDNA sequencing. Batch studies were performed to evaluate toxic effects of heavy metal ions in decreasing order of MIC As(V) (10 mM), Cu (1.5 mM), Pb(0.18 mM), Cr(0.1 mM), Ni(0.08 mM) and Cd(0.04 mM). Scanning electron microscopy analysis of Arsenic resistant strain revealed evident changes in cell morphology. SDS-PAGE results showed altered expression of proteins in response to arsenate. One unique protein of approximately 21 kDa was highly expressed in 5 mM arsenate, but same protein was down regulated in 10 mM arsenate. The exopolysaccharide components such as total carbohydrates, proteins and uronic acids were significantly enhanced by 41%, 25% and 33% (P Value < 0.05) and also produced EPS under Arsenic stressed conditions. Fourier transformed spectroscopy analysis demonstrated arsenic metal ion-EPS interactions. The results obtained from SEM–EDS analysis clearly revealed mucous nature of Rhizobial-EPS surrounding bacterial cells and confirmed the role of EPS in arsenate sequestration (10% as weight). Interestingly total arsenate uptake by strain VBCK1062 in whole-cell pellet and EPS were 0.045 mg and 0.068 mg g^{-1} of biomass respectively. Thus these results significantly contribute to better understanding of plant-metal-microbe interactions, cellular-metabolic changes and As-enhanced EPSs, hence can serve as potential bioremediation agent for As-contaminated agrogeoecosystems.

1. Introduction

Heavy metals discharges from industrial operations are currently widespread in various ecological systems and cause a substantial threat to varied agrogeoecosystems (Cheung and Gu, 2007). Agriculture soil may become contaminated with heavy metals from a variety of anthropogenic sources such as smelters, mining, power station industries, application of metal containing fertilizers and sewage sludge (Robinson et al., 2001). Other sources of As contamination include the use of As containing insecticides, herbicides, fungicides and wood preservatives (Smith et al., 2003). Arsenic (As) contamination is considered as a global environmental problem. Arsenic contaminated ground water has been reported in many countries including Bangladesh, India, China and USA (Rahman et al., 2006). This metalloid is known to be carcinogenic in some forms, and is mostly found in the environment as arsenate including As (V) as $H_2AsO_4^-$, $HAsO_4^{2-}$, etc. and arsenite As (III) is found as H_3AsO_3 (Oremland and Stolz, 2005). Arsenic is bioaccumulated in human beings from drinking water as well as from agricultural crops and enhances human risk of arsenic poisoning through the food chain. (Das et al., 2004). Therefore it is desirable to remove arsenic from groundwater and contaminated agriculture soils. Several chemical methods have been established for decontamination of arsenic from ground water (Mayo et al., 2007) including biological treatments (Mandal et al., 2008). Interestingly the sequestration of arsenic by using biological methods is receiving a great deal of attention in a biotechnological perspective employing microorganisms (Satish Kumar et al., 2004).

Leguminous plants were identified as prominent species grown on arsenic contaminated sites (Reichman, 2007) and free-living rhizobia are generally colonized in soils with elevated arsenic content (Carrasco et al., 2005). Nevertheless, the root nodules of

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inoculated legumes grown in arsenic contaminated soil are generally much reduced or absent (Mench et al., 2006). Arsenate is known to interrupt various biological functions in microorganisms such as oxidative phosphorylation and ATP synthesis, also induces osmotic and oxidative stress by generating reactive oxygen species (ROS) (Carbonell et al., 1998). However, bacteria show a number of responses to metal ions that include metal biosorption, metal precipitation and enzymatic metal transformation, that permit their use for environmental restoration (Valls and de Lorenzo, 2002). Besides interacting with plant roots, *Rhizobium* also play an important role in arsenic biogeochemistry i.e. reduction and oxidation, methylation and demethylation, sorption and desorption in soils. As a result, bacteria have developed different detoxification strategies to withstand the growth restriction under arsenic stress. Apparently, *Rhizobium* bacteria tolerate high As concentrations by maintaining low internal As concentrations, in the form of As(V) is reduced to As(III) and then actively effluxed into the surrounding medium (Yang et al., 2005). Symbiotically effective *Rhizobium* strains tolerant to arsenic have been previously reported (Carrasco et al., 2005; Pajuelo et al., 2008; Mandal et al., 2008).

Biosynthesis of exopolysaccharides (EPS) by rhizobia is essential for the formation of nitrogen fixing nodules on legumes, for example, *Leucaena, Medicago, Pisum, Vicia, Trifolium* and *Vigna* (Wilbert et al., 1998). EPS produced by *Rhizobium* species are macromolecular complexes crucial for the establishment of symbiotic relationship between *Rhizobium* and leguminous plants. Rhizobial exopolysaccharides have been studied extensively for their role in plant host specificity (Reichman, 2007) but only recently their metal sorption capacity has been investigated (Wu et al., 2010). However a number of microorganisms have been shown to produce polysaccharides and other biopolymers that exhibit metal-binding properties (Gutnick and Bach, 2000). Bacterial EPSs are chemically diverse and are mostly acidic heteropolysaccharides with ionizable functional groups such as hydroxyl, carboxyl, amide, sulfate and phosphoryl which interestingly exhibit very high affinity to heavy metals (Bramhachari et al., 2007). Metal immobilization strategies applied by microbes to counteract toxic effect of heavy metals include precipitation, intracellular accumulation and extracellular exclusion in biopolymers. These polymers bind metals by electrostatic interaction resulting in metal immobilization within exopolymeric matrix. However few enzymatic reactions in bacterial EPS assist degradation of recalcitrants and transformation of heavy metals together with their subsequent precipitation in the exopolymeric mass (Pal and Paul, 2008). Moreover, the EPSs are usually composed of a variety of biodegradable organic substances, such as carbohydrates, proteins, uronic acids and nucleic acids, etc. (Sponz, 2002), and are therefore recommended as promising biosorbents for removal of heavy metals due to their extensive capacities (Salehizadeh and Shojaosadati, 2003).

The objectives of the present study deals with characterization of mucous EPS producing arsenic resistant *Rhizobium* bacteria associated with leguminous plant *Vigna radiata* in the metal contaminated agriculture zones and their adaptations to this stressed environment. Further the characterization of arsenate resistance limit, adaptive changes in cell morphology, altered protein expression, enhanced EPS production under arsenic stressed conditions and biosorption properties of the strain were also presented in this study.

2. Materials and methods

2.1. Study area and sampling sites

The current study involved 65 rhizobial isolates were obtained from nodules of the leguminous plant *Vigna radiata* (mung bean) harvested from soil samples contaminated with effluents from the chemical and fertilizer industries of Kakinada. This town is situated between the latitude 16°57′ North and longitude 82°15′ East. The study area consisted vast number of industries related to arsenate pollution includes the discharges from chemical, petrochemical, pharmaceutical, pesticide, fertilizer, electroplating and metal processing plants. This area is predominantly affected by the release of liquid effluents from these industries. We selected soil samples from three different environmentally significant locations 1. Atcham Peta, 2. Nagarjuna Nagar and 3. Valasapakala at Kakinada Industrial area, where heavy metals, metalloids and other pollutants are emitted as industrial byproducts on a regular basis. These industries discharge their treated effluents into unlined canals through drains, besides most of all agricultural lands are dependent on these water sources. We have also selected pristine controls (unpolluted) soil samples for each sampling site. The nodule occupancy test was analyzed by comparing mean values in each experimental site using Manne Whitney statistical test. The mean difference in the treatments was considered to be significant at the level of $P=0.05$.

2.2. Soil chemical analyses

The experimental soil samples, from (20 cm) depth of the nodule collection sites, were collected in polythene bags and sieved through 2 mm mesh. The soil was classified as sandy loam Alfisol type as per USDA soil taxonomy (Bhattacharyya et al., 2009). The soil pH and temperature were determined in the field using a CONTECH pH meter model-102 and the redox potential was determined by CONTECH Eh meter model EORP-01. The soil organic matter content was determined by loss on ignition at 600 °C using a muffle furnace. The experimental soil samples were put in clean petri-dishes and dried in oven at 50 °C for 12 h. Approximately 1 g soil sample was acid digested with aqua-regia on a hotplate at 120 °C. The digested soil samples were diluted up to 100 mL by adding ultrapure water and filtered. A sample of non contaminated soil was also processed in the same way and used as pristine control. Toxic elements were determined by inducted coupled plasma optical spectrophotometry (ICP-OES, Perkin Elmer, USA) after aqua-regia treatment in a microwave system according to Murillo et al. (1999).

2.3. Isolation of arsenate resistant Rhizobium sp.

Isolation was performed from surface sterilized nodules recovered directly from *Vigna radiata* plants found in the sampling area. Briefly, nodules were surface sterilized with ethanol 95% (v/v) for 30 s and $HgCl_2$ 0.1% (v/v) for 45 s and washed with sterilized distilled water. Nodules were then cut, ground and a loopful of tissue containing bacteria was transferred to modified yeast extract mannitol (YEM) medium. Randomly chosen nodules from *Vigna radiata* plants of selected area were examined for existence of arsenate resistant bacteria. Isolates were routinely cultivated at 28 °C, maintained and preserved in YEM broth containing 20% (v/v) glycerol at −80 °C. After several serial transfers, the media with highest number of single colonies were inoculated to plates containing 4 different concentrations of arsenate (5, 10, 15, 20 mM). The survived colonies were enumerated after 2 days of growth. Single colonies from plates containing 10 mM of arsenate were sub-cultured thrice to obtain axenic cultures. The culture