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CHARACTERIZATION OF ARSENIC RESISTANT BACTERIA ISOLATED FROM THE SOIL OF KHULNA SHIPYARD

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Abstract: Thirty four arsenic resistant bacterial strains were isolated from the soil samples of Khulna Shipyard. They were isolated by growing them on nutrient broth medium containing 5 mgl^{-1} of arsenic. From them, five strains were finally selected, and studied their morphological, biochemical and ecological characters in details. They were identified as *Bacillus lichenifomis*, *Bacillus polymyxa, Listeria murrayi*, *Moraxella urethralis* and *Planococcus citreus*. All of these strains were able to tolerate upto100 ppm (mgl^{-1}) of arsenic (III). It is possibly due to the presence of arsenic (III) resistance mechanism(s) in these bacterial strains. The optimum pH and temperature for the growth of these bacterial strains were 8.5 and 37 °C, respectively.

Key words: Bacteria, arsenic, resistant, heavy metal

Introduction

Arsenic is a ubiquitous element that ranks 20th in abundance in the earth's crust, 14th in the seawater, and 12th in the human body (Woolson, 1975), and is widely distributed throughout nature as a result of weathering, dissolution, fire, volcanic activity, and anthropogenic input (Cullen and Reimer, 1989). Arsenic is used in pesticides, herbicides, wood preservatives, and dye stuffs as well as production of arsenic-containing wastes during smelting and mining operations. In arsenic-enriched environments, a major concern is the potential for mobilization and transport of this toxic element to groundwater and drinking water supplies. The standard of arsenic concentration in drinking water varies in different countries of the world. A few permissible limits in are given in the Table 1.

Table 1 show that the permissible limit of arsenic in drinking water in Bangladesh is 50 μgl^{-1}. In Bangladesh, out of 64 districts, drinking water of 53 districts having arsenic concentration well above 50 μgl^{-1} (Ahmed, 2002); and an estimated 57 million people have been exposed to arsenic through contaminated wells (Anon, 2001). Tondel *et al.* (1999) reported that about 25 million people of 2000 villages in 178 arsenic-affected blocks

Table 1. Permissible limits of arsenic in drinking water in some countries of the world (Grantham and Jones, 1977).

Country/Organization	Recommended limit (μgl^{-1})
Bangladesh	50
Canada	25
European Union	10
WHO (World Health Organization)	10

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of Bangladesh are in risk and 3695 (20.6%) out of 17,896 people examined are suffering from arsenicosis. This incident serves as an unfortunate reminder of the toxic consequences of arsenic mobilization and underscores the need to find a suitable process to mitigate this problem.

A number of microorganisms had been isolated that could use arsenic in their metabolism, either using arsenate as a terminal electron acceptor in anaerobic respiration (Ahmann *et al*., 1994; Stolz and Oremland, 1999) or as a means of generating energy through chemoautotrophic arsenite oxidation (Santini *et al*., 2000). Bacteria might show resistance to arsenite through the activity of an arsenite oxidase (Muller *et al*., 2003). Most studies on arsenic-metabolizing and arsenic resistant bacteria were focused on organisms isolated from environments showing significant arsenic contamination or increased global arsenic production from activities such as mining and fossil fuel consumption, as well as industrial use, is leading to increased arsenic contamination of many systems. Increased arsenic levels will result in new selective pressures for arsenic resistance, and an increased importance of examining microbial arsenic resistance in natural environments. This research was conducted to isolate and characterize potential bacterial strains, which are resistant to arsenic. These arsenic resistant strains could be used for bioremediation of arsenic from contaminated sites, especially from drinking water.

Materials and Methods

Soil samples were collected from different arsenic contaminated sites of Khulna shipyard, Rupsha, Khulna. From these soil samples, bacterial strains were isolated by "soil dilution technique" using nutrient agar medium containing 5 mgl^{-1} of arsenic (III) as sodium (meta) arsenite (Fluko, Switzerland).

Thirty four bacterial colonies were isolated from ten soil samples. Depending on cultural and morphological characteristics, 5 strains were finally selected. Selected strains were studied in details about their morphological, cultural, biochemical and ecological characteristics. Methods described by Collins and Lyne (1984) were followed for these purpose. With the help of Bergey's Manual (Krieg and Holt, 1984) identification was done.

Determination of optimal growth temperature and pH of the strains: For the determination of optimum growth temperature, strains were grown in Nutrient Broth (NB) medium. Tubes of NB were inoculated with fresh culture and incubated at different temperature viz. 4, 20, 37 and 60 °C. In case of pH test, tubes of NB at different pH viz. 4.5, 6.5 and 8.5 were prepared in duplicates and after inoculation incubated at 37 °C. In both cases, growth was measured by spectrophotometer reading at 600 nm after 24 h.

Growth responses at different concentrations of Arsenic (As^{3+}): The strains were tested for their growth response at different concentrations viz. 0, 1, 5, 10, 20, 50 and 100 ppm (mgl^{-1}) of As^{3+}. Tubes of nutrient broth containing different concentration of As^{3+} were inoculated with the fresh culture and incubated at 37 °C. After 24 h, growth was measured by spectrophotometer reading at 600 nm.

Results

Considering the cultural, morphological (Table 2), biochemical (Table 3) and ecological (Fig. 1 and Fig. 2) characters provisional identification was done. The identified five bacterial strains were *Bacillus lichenifomis*, *Bacillus polymyxa*, *Listeria murrayi*, *Moraxella urethralis* and *Planococcus citreus.*

Among the identified bacterial strains *B. lichenifomis*, *B. polymyxa, L. murrayi* and *P. citreus* were gram positive whereas *M. urethralis* was gram negative. All of the strains were rod shaped except *P. citreus,* which was spherical. Only two strains, *B. lichenifomis* and *B. polymyxa* were spore former while other strains were non-spore former. Except *B. lichenifomis* and *M. urethralis*, all other strains were motile.

In case of fermentation tests, it was observed that all of the five strains were able to ferment glucose, lactose, sucrose, mannitol and xylose. The optimum pH for the growth of all strains was 8.5 (Fig. 1). The optimum temperature for the growth of all the strains was 37 ^{o}C (Fig. 2).

Table 2. Morphological characters and staining properties of the selected bacterial strains

Strain No.	Vegetative cells	Spore	Gram reaction	Flagella
B. lichenifomis	Rods; occur in chain	+	+	-
B. polymyxa	Short rods; ends rounded; occur in chain	+	+	+
L. murrayi	Short rods; ends rounded; occur in chain	-	+	+
M. urethralis	Rods; ends rounded; occur in chain	-	-	-
P. citreus	Coccus; occurring in double	-	+	+

'+' sign indicates positive result, '-' sign indicates negative result.

Table 3. Major biochemical characters of the selected bacterial strains '+' sign indicates positive result, '-' sign indicates negative result.

Name of bacteria	DGA	H_2S	Cit.	Cat.	Cas.	α-A	Gel.	NR	Oxi.	MR	VP	Ind.
B. lichenifomis	FA	+	+	+	+	-	+	+	+	-	+	+
B. polymyxa	FA	-	+	+	+	+	+	+	+	-	-	+
L. murrayi	OA	+	+	+	-	+	+	+	+	-	-	+
M. urethralis	OA	+	+	+	-	+	+	+	+	-	+	+
P. citreus	OA	-	+	+	+	-	+	+	+	-	+	+

DGA = Deep Glucose Aagar; FA = Facultative anaerobe; OA = Obligate aerobe; Cit = *Citrate*; Cat = *Catalase*; Cas = *Caseiase*; *α-A = α- amylase*; Gel = *Gelatinase;* NR = *Nitrate Reduct-ase;* Oxi = *Oxidase*; Ind = *Indole*.

All 5 strains were able to tolerate >100 ppm (mgl^{-1}) of arsenite (Fig. 3). Among the tested strains growth of *B. licheniformis* was highest whereas, *B. polymyxa* had lowest at 100 mgl^{-1} of arsenite. Reportedly, *B. licheniformis* also resistant to various heavy metals Such as Zn^{2+}, Cu^{2+}, Co^{2+}, Ag^{+}, Cr^{3+} etc.

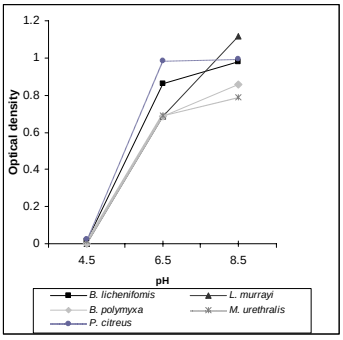

Fig. 1. Growth responses of the tested strains at different pH (Optical density after 24 h).

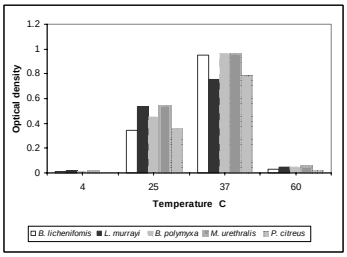

Fig. 2. Growth responses of the tested strains at different temperature (Optical density after 24 h).

Discussion

Anderson and Cook (2004) reported 17 morphologically distinct arsenic-resistant heterotrophic bacteria to be members of the genera *Exiguobacterium*, *Aeromonas*, *Bacillus*, *Pseudomonas*, *Escherichia* and *Acinetobacter*. Macur *et al.* (2001) also found that members of the *Caulobacter*, *Sphingomonas* and *Rhizobium* may be responsible for the reduction and mobilization of arsenic. Hoeft *et al*. (2002) found that *Sulfurospirillium* and *Desulfovibrio* use arsenate as an electron acceptor for their growth. This study reveals that the three genera such as *Listeria*, *Moraxella* and *Planococcus* are also resistant to arsenic. The diversity of arsenic resistance genes is probably much greater and more complex than is apparent from studies on known arsenic resistant isolates (Jackson and Dugas, 2003). Heavy metal toxicities and binding are pH-dependent (Wood, 1983) and it appears that the strains require environmentally relevant pH for growth.

Bouchard *et al*. (1996), Christiansen and Ahring (1996) and Niggemyer *et al.* (2001) reported optimum pH 7.5 for arsenic resistant strains *Desulfitobacterium frappieri*, *D. hafniense* and *Desulfitobacterium* strain GBFH, respectively.

Temperature is another important environmental factor, which affects bacterial growth (Herbert and Bhakoo, 1979).

The optimum temperature (37 ^{o}C) for the growth of all the strains in the present study support the results observed by Bouchard *et al*. (1996); Christiansen *et al*. (1996) and Niggemyer *et al.* (2001).

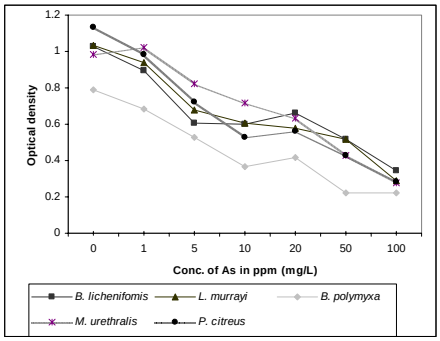

Fig. 3. Growth responses of the tested strains at different concentration of arsenite (Optical density after 24 h).

Jackson *et al.* (2005) isolated numbers of culturable arsenate (V) resistant bacteria from which some were capable to tolerate very high (100 mM) level of arsenate, although arsenite resistance was generally much lower. In addition, Zelibor *et al.* (1987) isolated As $(V)^{r}$ bacteria in well water samples. These isolates tolerated up to 2,000 µg of As (V) per ml. However, they did not test for As (III) resistance. Honschopp *et al*. (1996) isolated an arsenic resistant and arsenic methylating bacterium belonging to the *Flavobacterium-Cytophaga* group, which was able to tolerate 200 ppm concentration of As in the culture media. Plasmids also have been detected in some bacteria exhibiting high levels of resistance to arsenate, arsenite, and antimonate (Cervantes *et al*., 1994; Dabbs and Sole, 1988; Mobley *et al*., 1983).

The bioremediation of arsenic from contaminated sites involves reduction and oxidation of arsenic with the use of arsenic resistant microorganisms. The successful exploitation of these bacterial strains with proper biotechnology for bioremediation of arsenic will be beneficial. Therefore, more advance research is required for a deeper understanding about these bacterial strains to improve arsenic bioremediation process.

Conclusion

The World Health Organization (WHO) describes the arsenic contamination of ground water as "the largest mass poisoning of a population in history" (Anon, 2001). The development partners and Government of The People's Republic of Bangladesh have pumped millions of dollar to mitigate this problem. But a suitable process for mitigation of arsenic from soil and ground water is yet to be established. The identified arsenic resistant bacterial strains could be used to the mitigation of arsenic from contaminated sites, especially from drinking water to save millions of lives from arsenicosis.

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References

Ahmann, D.; Roberts, A.L.; Krumholz, L.R. and Morel, F.M.M. 1994. Microbe grows by reducing arsenic. *Nature,* 371: 750.

Ahmed, M.F. 2002. Water supply situation analysis. *In:* Ahmed, M.F. and WSP. ITN Center (eds.), *Alternative Water Supply Option for Arsenic Affected Areas of Bangladesh*. BUET, Dhaka, pp: 5-18.

Anderson, C.R. and Cook, G.M. 2004. Isolation and characterization of arsenate-reducing bacteria from arsenic-contaminated sites in New Zealand. *Current Microbiology*, 48:341-347.

Anon. 2001. Phase two groundwater studies of arsenic contamination in Bangladesh. British Geological Survey, Nottingham, United Kingdom.

Bouchard, B.; Beaudet, R.; Villemur, R.; McSween, G.; Lepine, F. and Bisaillon, J.G. 1996. Isolation and characterization of *Desulfitobacterium frappieri* sp. nov., an anaerobic bacterium which reductively dechlorinates pentachlorophenol to 3-chloropHenol. *International Journal of Systematic Bacteriology,* 46: 1010-1015.

Cervantes, C.G.Jr.; Ramairez, J.L. and Silver, S. 1994. Resistance to arsenic compounds in microorganisms. *FEMS Microbiology Reviews,* 15: 355–367.

Christiansen, N. and Ahring, B.K. 1996. *Desulfitobacterium hafniense* sp. nov., an anaerobic, reductively dechlorinating bacterium. *International Journal of Systematic Bacteriology*, 46: 442-448.

Collins C.H. and Lyne P.M. 1984. *Microbiological Methods*. Butterworth and Co. Ltd., New York, pp. 1-435.

Cullen, R.W. and Reimer, J.K. 1989. Arsenic speciation in the environment. *Chemical Reviews,* 89: 713-764.

Dabbs, E.R. and Sole, G.J. 1988. Plasmid-borne resistance to arsenate, arsenite, cadmium and chloramphenicol in a *Rhodococcus* species. *Molecular & General Genetics*, 211: 148–154.

Grantham, D.A. and Jones, J.F. 1977. Arsenic contamination of water wells in Nova Scotia. *Journal of American Water Works Association,* 69: 653-657.

Herbert, R.A. and Bhakoo, M. 1979. Microbial growth at low temperatures. *In:* Rusell, A.D. and Fuller, R. (eds.), *Cold Tolerant Microbes in Spoilage and the Environment,* Academic Press, London, pp. 1-16.

Hoeft, S.E.; Lucas, F.; Hollibaugh, J.T. and Oremland, R.S. 2002. Characterization of microbial reduction in the anoxic bottom waters of Mono Lake, California. *Geomicrobiology Journal*, 19: 23-40.

Honschopp, S.; Brunken, N.; Nehrhorn, A. and Breunig, H.J. 1996. Isolation and characterization of a new arsenic methylating bacterium from soil. *Microbiological Research,* 151(1): 37-41.

Jackson, C.R. and Dugas, S.L. 2003. Phylogenetic analysis of bacterial and archaeal arsC gene sequences suggests an ancient, common origin for arsenate reductase, *BMC Evolutionary Biology,* 3: 18.

Jackson, C.R.; Harrison, K.G. and Dugas, S.L. 2005. Enumeration and characterization of culturable arsenate resistant bacteria in a large estuary. *Systematic and Applied Microbiology*, 28(8): 727-734.

Krieg, N.R. and Holt, J.G. 1984. *Bargey's Manual of Systematic Bacteriology*. *Vol. 1, 9th edn.,* Williams and Wilkins Co., Baltimore, pp. 1-836.

Macur, R.E.; Jackson, C.R.; Botero, L.M.; McDermott, T.R. and Inskeep, W.P. 2004. Bacterial populations associated with the oxidation and reduction of arsenic in an unsaturated soil. *Environmental Science & Technology*, 38: 104–111.

Macur, R.E.; Wheeler, J.T. and McDermott, T.R. 2001. Microbial populations associated with the reduction and enhanced mobilization of arsenic in mine tailings. *Environmental Science & Technology***,** 35(18): 3676-3682.

Mobley, H.L.; Chen, C.M.; Silver, S. and Rosen, B.P. 1983. Cloning and expression of R-factor mediated arsenate resistance in *Escherichia coli*. *Molecular & General Genetics*, 191: 421–426.

Muller, D.; Lievremont, D.; Simeonova, D.D.; Hubert, J.C. and Lett, M.C. 2003. Arsenite oxidase aox genes from a metal-resistant b-*Proteobacterium*. *Journal of Bacteriology*, 185: 135–141.

Niggemyer, A.; Spring, S.; Stackebrandt, E. and Rosenzweig, R.F. 2001. Isolation and characterization of a novel As(V)-reducing bacterium: implications for arsenic mobilization and the genus *Desulfitobacterium*. *Applied Environmental Microbiology,* 67(12): 5568-5580.

Santini, J.M.; Sly, L.I.; Schnagl, R.D. and Macy, J.M. 2000. A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: phylogenetic, physiological, and preliminary biochemical studies. *Applied Environmental Microbiology,* 66: 92–97.

Stolz, J.F. and Oremland, R.S. 1999. Bacterial respiration of arsenic and selenium. *FEMS Microbiology Reviews,* 23: 615–627.

Tondel, M.; Rahman, M.; Magnuson, A.; Chowdhury, I.A.; Faruquee, M.H. and Ahmad, S.A. 1999. The relationship of arsenic levels in drinking water and prevalence rate of skin lesions in Bangladesh. *Environmental Health Perspectives,* 107: 727.

Wood, J.M. 1983. Selected biochemical reactions of environmental significance. *Chemical Scripta,* 21: 155-9.

Woolson, E.A. 1975. The persistence and chemical distribution of arsanilic acid in three soils. *Journal of Agricultural and Food Chemistry*, 23: 677-81.

Zelibor, J.L.Jr.; Doughten, M.W.; Grimes, D.J. and Colwell, R.R. 1987. Testing for bacterial resistance to arsenic in monitoring well water by the direct viable counting method. *Applied and Environmental Microbiology*, 53: 2929–2934.

Uddin, M.N.; Hossain, S.J. and Uddin, S.N. 2007. Characterization of arsenic resistant bacteria isolated from the soil of Khulna shipyard. *Khulna University Studies*, 8(1): 93-97.