MOLECULAR SURVEY ON BACTERIAL DIVERSITY IN ARSENIC CONTAMINATED SUBSURFACE SEDIMENT IN WEST BENGAL, INDIA

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ABSTRACT

The present work investigated indigenous bacterial community composition within a highly contaminated aquifer zone of West Bengal, India. Culture independent method of Denaturing Gradient Gel Electrophoresis (DGGE) targeting 16S rRNA genes recovered from sediments collected from subsurface drilling was used to elucidate the bacterial community composition. DGGE based microbial community analysis of subsurface sediments was carried out for varying depths and hydrogeological settings. Presence of iron reducing Geobacter, gram positive high GC content Bacillus and C1 compound utilizing Methylophilaceae bacteria were detected along with the members of Firmicutes, Cyanobacteria and uncultured Bacteroidetes. The cluster analysis reveals most similar and possibly near similar type of microbial diversity in As-6 (grey fine sand) and As-1 (grey soft silt) samples. Also similar type community structure exists in As-7 (orange white coarse sand) and As-9 (orange sand) samples. Samples of As-5 (grey fine sand), As-2 (grey fine sand) and As-4 (grey fine sand) also share similar community structures. Detailed bacterial analysis of these subsurface samples would thus help in framing up a model for biogeochemical release of arsenic in specific microenvironments.

Key Words: Arsenic, Bacterial diversity, DGGE, Cyanobacteria, Gel-electrophoresis

INTRODUCTION

Elevated arsenic concentration in groundwater across several parts of the world including Bangladesh, India, China, Taiwan, Argentina, Mexico, USA, Thailand and Vietnam causes a severe problem in supplying drinking water from affected area.1,2 Exposure to arsenic can result in a variety of health problems in humans, including various forms of cancer (e.g. skin, lung and bladder), cardiovascular and peripheral vascular diseases and diabetes.3 The mechanism underlying the release of As from aquifer sediment within these affected regions has been a topic of intense scientific research.4-6 The alluvial aquifers of Bengal Delta Plain (BDP) located in West Bengal (India) and Bangladesh possess the most notorious As contamination. Mobilization of As in Pleistocene-Holocene aquifers of BDP is considered to be a complex interplay of geochemical and microbial reactions sensitive to site-specific hydrology and sediment composition.4,7 It has been proven beyond any doubt that throughout the world, microorganisms affect the geochemistry of arsenic release in sediments and groundwater that can lead to arsenic contamination of drinking water supplies and poisoning of epidemic proportions.8-10 Geo-microbiological activities within the subsurface sediments that effect release of arsenic have been a subject of intense scientific research in present time. Extensive hydrogeochemical studies have confirmed that source of As contamination in these regions is the sorbed As onto Fe-oxides/hydroxides4 and its release from these minerals is complex. Several investigations also revealed that along with geochemical processes indigenous microbes within the subsurface environment play important role in release of sediment bound As. This may occur as microbial reduction of Fe oxides/hydroxides...
under moderately reducing conditions thereby releasing sorbed As into the aqueous phase.\textsuperscript{7,11} Alternatively, competitive anion exchange with ionic groups of fertilizers i.e. $\text{PO}_4^{3-}, \text{NO}_3^-, \text{CO}_3^{2-}$ etc. mobilizes As.\textsuperscript{9} Additionally weathering of minerals for microbial nutrient acquisition may be effective mechanism in As release.\textsuperscript{12} In view of the complex interplay of various geo-hydro-microbiological processes occurring in contaminated aquifers, a thorough understanding of As biogeochemical activities catalyzed by indigenous microorganisms is imperative to develop strategies for sustainable drinking water supply to the affected areas.\textsuperscript{13}

Our efforts to explore the diversity of microorganisms has been transformed by molecular methods which allow information to be obtained about the species makeup of a microbial community, but are not dependent on the growth of organisms in the laboratory.\textsuperscript{14} The PCR (Polymerase Chain Reaction) is the most common basis for these methods because it can be used to amplify specific targeted DNA sequences. The gene encoding the small subunit rRNA (SSU or 16S rRNA in prokaryotes and 18S rRNA in eukaryotes) has been sequenced most often and is particularly useful for defining phylogenies at the genus and higher taxonomic levels.\textsuperscript{15} Apart from inferring phylogenetic relationships among microorganisms sequence variation in rRNA genes has been exploited for designing specific nucleotide probes for the detection of individual microbial taxa in natural habitats. These techniques have also been applied to determine the genetic diversity of microbial communities and to identify uncultured microorganisms. In the present work, we have employed culture independent 16S rRNA gene based molecular approach to compare the diversity of complex microbial populations that may exist within arsenic contaminated underground sediments. The particular technique used here is based on the separation of polymerase chain reaction-amplified fragments of genes coding for 16S rRNA, all with the same length, by Denaturing Gradient Gel Electrophoresis (DGGE). DGGE analysis of different microbial communities demonstrated the presence of many distinguishable bands in the separation pattern, which are most likely derived from as many different species constituting these populations, and thereby generated a DGGE profile of the populations.\textsuperscript{16}

In the Bengal delta, arsenic concentration greater than 0.01mg/l in groundwater is found to be restricted to the shallow aquifers (20-100m). The high-arsenic values are found clustered in isolated pockets occupying small areas and surrounded by values of low arsenic content. The study area of Nadia district, West Bengal is one such hot spot of arsenic contamination.\textsuperscript{17} The detailed subsurface geology, sediment chemistry and groundwater chemistry of the Gotra village have been described by Pal et al.\textsuperscript{18} Release of arsenic from the sediments into the groundwater is assumed to be a factor of specific litho package that exist in subsurface environments.\textsuperscript{18} Microbiological association of the sediments from these arsenic affected areas have already been reported by Sarkar et al.\textsuperscript{13}

**AIMS AND OBJECTIVES**

A detailed work of culture independent DGGE analysis of microbial community in subsurface sediment samples collected from the Gotra village.

**MATERIAL AND METHODS**

**Sample collection and analysis of geochemical constituents**

Subsurface sediments have been collected by percussion method with piston sampling in PVC tubes.\textsuperscript{18} At three borehole points each of 60m depth, viz., A (23°01’12.6’’:88°35’11.4’’), B (23°01’09.00’’:88°35’07.05’’) and C (23°01’18.8’’:88°35’16.0’’) (Fig. 1), the PVC tubes containing samples of desired depth and lithology were immediately preserved and transported to the laboratory. Approximateley 0.3 g dry sediment was digested according to Sultana et al.\textsuperscript{5} The quantity of As and Fe were determined with a hot HCl extract using HG-AAS. TIC and DOC were analyzed with an Elementar LiquiTOC analyzer. Major anions (nitrate and sulfate) were determined with an ion chromatograph (Dionex, CA, USA).
Isolation and quantification of community DNA

Total microbial community DNA from sediment samples were extracted using Powersoil™ DNA kit (MO BIO laboratories, Inc.). Yield and quality of the extracted DNA samples were determined spectrophotometrically using standard methods.

Denaturing Gradient Gel Electrophoresis (DGGE)

The V3 variable region of the bacterial 16S rRNA genes was amplified by PCR (Polymerase Chain Reaction) with primer 341f (5’GCCGCCGCGGCCGCGCGGCCGCGGCACCACCAGGAGCCCACTAGGGAGGGCAGCAG-3’) and 518r (5’-ATTACCGCGGCTGCTGTAAG-3’). The forward primer had a GC clamp at its 5’end. The PCR mixture (final volume 25 µl) contain template DNA, 0.4 M primers, 0.2 mM dNTPs, 1.5 mM MgCl₂, 1x of MgCl₂ free buffer and Taq polymerase (New England BioLabs). Touchdown PCR was performed with an initial denaturation step of 95°C for 15 min. followed by 10 touchdown cycles of 94°C for 30 S, 61°C (0.5°C per cycle) for 30 S and 68°C for 5 S followed by 25 cycles of 94°C for 30 S, 56°C for 30 S and 68°C for 45 S and a final elongation step of 68°C for 7 min.

All PCR products were analyzed by electrophoresis on 2% (w/v) agarose gel containing ethidium bromide to check their size (~177bp).

Polyacrylamide gel (8% w/v) as prepared and run with 1x TAE buffer (1x TAE is 0.04 M Tris base, 0.02 M sodium acetate and 1.0 mM EDTA pH adjusted to 7.4). A D Code™ universal mutation detection system (BIO-RAD) gel electrophoresis unit was used with glass plates (16 by 18cm), 1.5mm spacers and 1cm wide loading wells. A 10 liter aquarium (BIO-RAD) served as the lower buffer chamber. DGGE gels contained a 35 to 65% gradient of Urea and Formamide (UF) solution increasing in the direction of electrophoresis. A 100% UF solution is defined as 40% (v/v) formamide plus 7.0 M urea. DGGE was conducted at a constant voltage of 70 V at 60°C for 12 h, after which the band positions had stabilized. After electrophoresis, the gels were incubated for 15 min in Milli-Q water containing ethidium bromide (0.5 mg/ litre), rinsed for 10 min with Milli-Q water and photographed with UV transillumination. Representatives of 7 distinguished bands in DGGE profile were carefully excised, re-amplified with primers 341F-518R.
DNA sequencing and phylogenetic analysis
Partial sequences (nearly 200 bp) of 16S rRNA genes were determined commercially using an automatic 3100 DNA sequence. Sequence data were compared with 16S rRNA gene sequences deposited in public databases by using both the BLAST (NCBI) program followed by an initial classification using classifier program in Ribosomal Database Project (RDP). 16S rRNA gene sequences of various bacteria including those closely related to the unknown sequences, as indicated by BLAST search obtained from the GeneBank database were aligned with the new sequences by using ClustalW. Resulting alignments were used to construct the distance matrix followed by phylogenetic tree construction by neighbour joining method using MEGA 4.0 software package.

Statistical analysis
Relatedness of microbial communities was determined using similarity coefficients of bands common to any two samples. Our working definition was that two bands are common if they have migrated the same distance on the DGGE gel. First, the total number of different bands was determined for the samples being compared. Each sample was then scored for the presence or absence of each particular band within its profile when compared to the profile of each of the other samples. Jaccard’s index of similarity was used to make pair-wise calculations of band-sharing between samples. Cluster analysis and dendrograms were calculated using UPGMA (un weighted pair group method with arithmetic mean) by MVSP (Multi Variate Statistical Package) software.

RESULTS AND DISCUSSION
Chemical nature of the sediment samples
Sediments samples obtained from deep drilling within the subsurface environment of aquifer showed variations in terms of arsenic content as well as other important chemical constituents (Table 1). Iron concentration was ranged between 1835-2550 mg Kg⁻¹, nitrate and sulfate levels varied within the range of 100-365 mg Kg⁻¹. Total organic carbon was found low even in the presence of higher inorganic carbon.

Table 1 : Geochemical properties of the samples

<table>
<thead>
<tr>
<th>Properties</th>
<th>Sample ID (used in this study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth (m)</td>
<td>As-1</td>
</tr>
<tr>
<td>Lithology</td>
<td>Grey soft silt</td>
</tr>
<tr>
<td>Arsenic abundance in associated ground water</td>
<td>HA</td>
</tr>
<tr>
<td>Total iron (mg/kg)</td>
<td>ND</td>
</tr>
<tr>
<td>Nitrate (mg/kg)</td>
<td>ND</td>
</tr>
<tr>
<td>Sulphate (mg/kg)</td>
<td>ND</td>
</tr>
<tr>
<td>TOC (%)</td>
<td>ND</td>
</tr>
<tr>
<td>Inorganic carbon (%)</td>
<td>ND</td>
</tr>
</tbody>
</table>
PCR-DGGE and cluster analysis of bacterial communities

Bacterial community structures within the samples were elucidated using DGGE fingerprinting of PCR-amplified 16S rRNA genes (Fig. 2). The phylogenetic affiliations of partial 16S rRNA gene sequences were determined by comparing with sequences in the GenBank database and constructing phylogenetic tree by neighbour joining method. Even though a high number of bands appeared in DGGE gel, sequencing and identification were possible for a few most prominent bands only (Table 2). The prominent bands were marked and designated according to the sample number suffixed by a numerical value indicating the serial number of the band. Bacterial groups affiliated with Betaproteo, Deltaproteobacteria, Fermicutes, Cyanobacteria and Bacteroidetes were detected in the samples. The members of the bacterial communities retrieved in the present study were mostly reported earlier from several As contaminated aquifers including those of BDP (both ground water or sediments). Sequence of band As-5-e showed close similarity with uncultured Geobacter belonging to Deltaproteobacteria, previously reported as a predominant Fe(III) – reducing bacteria in many anoxic subsurface sediments. Their presence in bacterial communities had previously been correlated with As (III) release in BPD (Fig. 3). Band As9-g from sample As9 was affiliated to uncultured Methylphilaleae bacterium, C1, methanol as well as multicarbon compound utilizing facultative anaerobic bacteria were observed during this study as well as by previous investigators. Ability of these organisms to survive under low OC, high As environment, utilizing limited range of organic compounds seems in line with chemical nature of As contaminated aquifers of BDP that characteristically contain low OC and high As. Band As2-b affiliated to uncultured Bacillus (Firmicutes) had been previously identified as organisms with arsenic resistant and bioweathering activity. Weathering of minerals by bacteria may or may not be for energy, but certainly for supporting their growth and survival under nutrient limiting condition. Beside these bands, As1-a, As4-d and As7-f showed close similarity with uncultured Cyanobacteria and As3-c with uncultured Bacteroidetes, though they were previously noted in arsenic contaminated Bengal delta aquifer but their role in As biogeochemical cycle is still unclear.
Table 2: Taxonomic affiliation of the 16S rRNA gene clones retrieved from major DGGE bands

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Max. identity</th>
<th>NCBI match</th>
<th>Putative taxon</th>
</tr>
</thead>
<tbody>
<tr>
<td>As1-a</td>
<td>100%</td>
<td>Uncultured bacterium (FJ499356.1)</td>
<td>Cyanobacteria</td>
</tr>
<tr>
<td>As2-b</td>
<td>100%</td>
<td>Uncultured <em>Firmicutes</em>. (HM659580.1)</td>
<td>Firmicutes</td>
</tr>
<tr>
<td>As3-c</td>
<td>95%</td>
<td>Uncultured <em>Bacteroidetes</em> (EF562555.1)</td>
<td>Bacteroidetes</td>
</tr>
<tr>
<td>As4-d</td>
<td>89%</td>
<td>Uncultured bacterium (FJ499356.1)</td>
<td>Cyanobacteria</td>
</tr>
<tr>
<td>As5-e</td>
<td>100%</td>
<td>Uncultured <em>Geobacter</em> sp. (EF414783.1)</td>
<td>Deltaproteobacteria</td>
</tr>
<tr>
<td>As7-f</td>
<td>80%</td>
<td>Uncultured bacterium (FJ499356.1)</td>
<td>Cyanobacteria</td>
</tr>
<tr>
<td>As9-g</td>
<td>82%</td>
<td>Uncultured <em>Methylophilaceae</em> bacterium (GU229825)</td>
<td>Betaproteobacteria</td>
</tr>
</tbody>
</table>

Note: All the sequence highlighted in bold were retrieved from this study.

Fig. 3: Neighbour-joining tree of 16S rRNA gene sequences showing the genetic distances among individual clones.

Bootstrap values of >60% based on 500 replicates are indicated by the numbers at the nodes. DGGE fingerprinting allowed comparison of total bacterial communities of the different samples through pattern analysis (Fig. 4). The results demonstrated that the complexity and diversity of microbial communities within the subsurface sediments...
can be adequately represented by this analysis. The DGGE based banding pattern among the samples indicated that sediment As-4, having low arsenic content had maximum number of bands followed by As-2 and As-9 (both low As samples). Samples As-7 (arsenic-free) and As-5 (low arsenic), both showed moderate number of bands while samples As-8 (moderate As sample), As-6 (low As sample), As-3 and As-1 (both high As samples) had the least number of bands. Cluster analysis (Fig. 4) showed that As-5 and As-1 had the similar distribution of bands. Bands’ distributions of As-4, As-3 were similar to that of A-2. Beside this it was also observed that As-9, As-7 and As-8, As-6 had similar banding pattern. Since each DGGE band obtained for a particular community metagenome represents a different 16S rRNA gene sequence and hence possibly a different microbial species, the observed increase in band numbers for total bacterial communities can possibly be correlated with an increase in total bacterial species. The highest number of bands, with strong band intensities, therefore indicates an elevated number of predominating species within the sample.

![Fig. 4: UPGMA dendogram constructed using Jaccard’s similarity index generated from the above DGGE profiles](image)

From this DGGE gel the maximum number of bands as observed in As-4 followed by As-2, As-9 and As-5 indicate maximum number of bacterial species likely to be present in these samples. Similarly, least number of bands as observed in samples As-6, As-8 and As-1 may indicate less number of bacterial species present in these samples. Beside this, moderate number of bands is found in samples As-7 and As-3 indicating that average number of bacterial species is present in these samples. Band intensity has also been compared among these samples and it was found that 3 bands are predominant in case of samples As-1, As-2, As-6 and As-8, which possibly denotes that 3 types of bacterial species are likely predominant among these samples. Similarly, 4 bands are predominant in case of As-4, As-7, As-9; 5 bands in case of As-3 and 6 bands in As-5, which indicates that 4 types of bacterial species are predominant in samples As-4, As-7, As-9, 5 types of bacterial species in the case of As-3 and 6 types of bacterial species are predominant in the case of sample As-5. Cluster analysis is a useful tool to compare and classify different systems. In this study, the cluster analysis illustrates that As-5 and As-1 have the highest similarity and possibly near similar type microbial diversity. At the same time, cluster analysis also showed that the As-9 and As-7 collected from below orange and orange sand respectively, give similar type of community structure. As evident from this analysis As-4, As-3 and As-2 share similar community structure.

CONCLUSION

The culture independent study revealed presence of iron reducing *Geobacter*, gram positive high GC *Bacillus* and C1 compound
utilizing *Methylphilaceae* bacteria within the sediment along with *Cyanobacteria* and uncultured *Bacteroidetes*. The DGGE banding pattern indicated an inverse relation between the number of bands and the As-content in samples. Sediments of different composition as well as those of similar composition at different depths may have different bacterial species, as the geochemical parameters vary in different microenvironments. Further study on depth-specific, undisturbed, sediment samples from the zones of contrasting groundwater-arsenic values would thus help in understanding the biological control on arsenic fixation/release in this part of the Bengal delta.

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