Identification of Arsenic Resistance Genes from Marine Sediment Metagenome

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Abstract A metagenomic library of sea sediment metagenome containing 245,000 recombinant clones representing ~ 2.45 Gb of sea sediment microbial DNA was constructed. Two unique arsenic resistance clones, A7 and A12, were identified by selection on sodium arsenite containing medium. Clone A7 showed a six-fold higher resistance to arsenate [As(V)], a three-fold higher resistance to arsenite [As(III)] and significantly increased resistance to antimony [Sb(III)], while clone A12 showed increased resistance only to sodium arsenite and not to the other two metalloids. The clones harbored inserts of 8.848 Kb and 6.771 Kb, respectively. Both the clones possess A + T rich nucleotide sequence with similarity to sequences from marine psychrophilic bacteria. Sequence and transposon-mutagenesis based analysis revealed the presence of a putative arsenate reductase (ArsC), a putative arsenite efflux pump (ArsB/ACR) and a putative NADPH-dependent FMN reductase (ArsH) in both the clones and also a putative transcriptional regulatory protein (ArsR) in pA7. The increased resistance of clone A7 to As(V), As(III) and Sb(III) indicates functional expression of ArsC

and ArsB proteins from pA7. The absence of increased As(V) resistance in clone A12 may be due to the expression of a possible inactive ArsC, as conserved Arg60 residue in this protein was replaced by Glu60, while the absence of Sb(III) resistance may be due to the presence of an ACR3p-type arsenite pump, which is known to lack antimony transport ability.

Keywords Metagenomic · Sea sediment · Arsenic · Metagenome · Arsenite efflux pump · Arsenate reductase

Introduction

The marine ecosystem covers more than three-quarters of the earth surface and represents huge diversity of organisms. Metagenomic studies regarding exploration and exploitation of microbial diversity of sea revealed various microbial physiological pathways like light energy fixation by microorganisms, nutrient recycling etc. in conjunction with in-depth change in diversity with the gradient of temperature, pressure etc.[1–4]. The sea sediment is one of the complex ecosystems possessing burrowing and gliding organisms surviving at the oxic and anoxic interface utilizing the deposited organic, inorganic load and detoxifying pollutants like pesticides, metals, metalloids, detergents, etc.

Arsenic is the 20th most common element in the earth's crust but its pollution and exposure to living forms increases due to either natural (like withering rocks of arsenic ores) or anthropogenic processes (smelting, pesticides, roxarsone etc.). In most environments, majority of the arsenic is found in bound form as metal complexes, but in water, it is present as oxyanions with two major oxidation states; pentavalent arsenate [As(V)] and trivalent arsenite [As(III)]. In both forms, it induces toxicity by

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blocking general cell metabolism [5]. Microorganisms evolved mechanisms for detoxification of this toxic metalloid. *ars* operon is one of the well-characterized systems in *Escherichia coli* for arsenic detoxification. It comprises of genes encoding for arsenate reductase (ArsC), arsenite efflux pump (ArsB), a transcriptional regulatory protein (ArsR) along with an arsenite chaperon (ArsD) and an ATPase (ArsA) [5, 6]. Similar detoxification mechanism was also discovered in other bacterial [5, 7, 8] and fungal species [9]. With the advent of genome sequencing, new genes have been identified and demonstrated for resistance to arsenic like methyltransferases (*arsM*) from *Rhodospseudomonas palustris* [10] and aquaglyceroporin proteins (*aqpS*) from *Sinorhizobium meliloti* [11].

Marine organisms accumulate arsenic from the environment and convert it to various organic forms known as organo-arsenicals [5, 12]. Such dead organisms get deposited at sea bottom and increase the concentration of arsenic in the sea sediments. Microorganisms surviving and recycling sediment debris are expected to possess arsenic detoxification mechanisms and release its soluble form in the surroundings.

Majority of the microorganisms remain uncultured and represent a large proportion of microbial community in any environment [13, 14]. This huge uncultured microbial diversity comprises of a massive untapped gene pool and novel physiological processes. Few attempts have been made to use metagenomics to understand arsenic resistance genes in the environment [15–17], these metagenome sequencing-based studies revealed the presence of arsenic resistance genes and their abundances in the contaminated environments [18–21]. The metagenome sequence-analysis studies merely reveal the presence of the genes and fail to indicate the functionality of the genes in the studied environment. Another disadvantage of the sequence-based metagenomic studies is that, due to homology-based analysis, it is not possible to identify novel genes involved in the particular function. On the other hand, functional metagenomics allows isolation of functional clones and many times leads to the identification of previously uncharacterized genes. There are few functional metagenomics studies to uncover arsenic detoxification mechanisms of unculturable bacteria. A novel arsenic resistance gene was identified from an effluent treatment plant sludge metagenome [22] and many new genes resulting in arsenic resistance were identified from acid mine drainage metagenome [23]. The goal of this functional metagenomics study was to understand potential adaptive strategies involved in arsenic detoxification from marine sea sediments, which is known to be the major sink for many pollutants including arsenic.

In this work, a sea sediment metagenomic library was prepared and clones were selected for arsenite tolerance.

Two unique arsenite resistance clones, A7 and A12, were selected. The sequence analysis and transposon mutagenesis of the clones helped in identification of the arsenic resistance genes. The genes similar to arsenate reductases and arsenite efflux pumps were identified from both the clones. Increased resistance to As(V), As(III) and Sb(III) of A7 indicated functional expression of ArsC and ArsB from pA7. The absence of significant increase in As(V) and Sb(III) resistance could be due to possible inactive ArsC of pA12 and presence of ACR3p-type arsenite pump, respectively. This study not only successfully demonstrated the application of functional metagenomics to characterize arsenic resistance genes but also opened new frontiers to identify arsenic detoxification mechanisms prevalent in marine ecosystems.

Materials and Methods

Sample, Strain and Plasmids

Sea sediment sample was collected in January 2004 from the coastal areas of Goa (India). The sample was collected in a sterile container and transferred to the lab in aseptic conditions and stored at 4 °C. The sample was dark grey in color having a mixture of silt and sand particles with a slight smell. *E. coli* DH10B was a gift from Dr. H. Shizuya, Caltech. *E. coli* cultures were grown on Luria–Bertani (LB) agar medium at 37 °C for 16 h. Plasmid pHB201 was a gift from Dr. Daniel R. Zeigler, Bacillus Genetic Stock Center. Plasmid culture was grown in LB broth or on LB agar plates at 37 °C supplemented with chloramphenicol to a final concentration of 5.0 µg/ml.

DNA Manipulation Techniques

The standard procedure for plasmid isolation, restriction enzyme digestion, ligation, and competent cell preparation were used as described by Sambrook and Russell [24].

Metagenomic DNA Extraction from Sea Sediment

Sea sediment sample was centrifuged at 8000 g for 2 min and excess water was removed. Metagenomic DNA was isolated using 25 g sea sediment sample. The sea sediment sample was suspended in 72.5 ml of extraction buffer (EDTA, 100 mM; sodium phosphate, 100 mM; sodium chloride, 1.5 M; cetyl trimethyl ammonium bromide, 1% w/v, Tris–Cl pH 8.0, 100 mM). Proteinase K was added to a final concentration of 1 mg/g of pellet and tubes were incubated at 37 °C for 1 h with gentle shaking. SDS was added to final concentration of 2% and tubes were incubated again at 65 °C for 2 h with occasional shaking. The

lysate was centrifuged at 8000 g for 10 min after addition of equal volume chloroform and isoamyl alcohol (24:1). The DNA was precipitated by 0.6 V of isopropyl alcohol and kept undisturbed for 1 h. The DNA was obtained by centrifugation at 10,000 g for 20 min and washed with 20 ml of 70% ethanol. The pellet was air dried and dissolved in 1 ml of TE, pH 8.0. DNA was further purified on 0.7% low melting agarose and high molecular weight DNA was collected from the gel for library construction.

Metagenomic Library Construction

Four microgram of metagenomic DNA was partially digested with Sau3A1 and DNA fragments in the range of 5–23 kb were gel eluted using DNA isolation kit (Biological Industries, Israel). Two hundred nanogram of partially digested DNA was ligated to BamHI digested and dephosphorylated pHB201 vector. The ligated product was electroporated at 200 Ω , 25 μ f, and 12.5 kV/cm in *E. coli* DH10B using Micropulser II (Bio-Rad, USA). Transformants were obtained on LB agar plates containing chloramphenicol.

Selection of Arsenic Resistant Clones

Arsenicals resistant clones were selected by plating sea sediment metagenomic library on LB chloramphenicol plates containing 6.0 mM of sodium arsenite. The arsenic resistant clones were selected based on their ability to form colony after 24 h incubation at 37 °C. All arsenic resistant clones obtained after preliminary selection were patched on LB chloramphenicol plates containing 6 mM sodium arsenite. Randomly selected fifty arsenic resistant clones were checked for their restriction digestion patterns analysis and clones with unique digestion pattern were selected for further study.

Metalloid Resistance Assay

Unique clones were further studied for their resistance toward sodium arsenite (2, 4, 6, 8, 10, 12, 14 and 16 mM), sodium arsenate (2.5, 5, 10, 20, 30, 40 and 50 mM) and antimony potassium tartrate (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mM). Assay to determine resistance towards sodium arsenite and antimony potassium tartrate was carried out in LB broth. Overnight grown cultures were diluted to an optical density (O.D.) of 1.0 at 600 nm and 25 μ l of diluted cultures was inoculated in 5 ml fresh LB supplemented with chloramphenicol and concentrations gradient of metalloids. Cultures were incubated at 37 °C at 200 r.p.m. for 24 h and O.D. was measured at 600 nm. Arsenate resistance assay was carried out in low phosphate medium according to the standard procedure [25].

Sequencing and Phylogenetic Analysis

In-vitro transposon mutagenesis reaction was carried out to identify the gene conferring arsenic resistance. The plasmids of the arsenic resistant clones were mutated using genome priming system (GPS) kit following the manufacturer's instruction (New England Biolab, USA). The mutated plasmid was electroporated in *E. coli* DH10B and selected on LB agar plates containing chloramphenicol (5 μ g/ml) and kanamycin (20 μ g/ml). These colonies were then checked for arsenic resistance on LB agar plate with 6.0 mM sodium arsenite supplemented with kanamycin and chloramphenicol to screen the negative and positive mutants (the negative mutants were expected to have transposon insertion in the gene conferring arsenic resistance and fail to grow on growth medium supplemented with 6.0 mM arsenite). The mutated plasmids from the clones were isolated and sequenced using primers N and S (supplied with kit) using ABI prism big dye terminator protocol. Ends were sequenced using M13 forward primer and M13 reverse primer (MBI, Fermentas, Lithuania). Transposon insertion map was constructed using information about the position of transposon insertion in positive and negative transposon insertion mutants in the gene sequence of arsenic resistant clones. Primer walking was done to fill the gaps and to obtain the double strand sequence. Open reading frames in the assembled sequence for each clone were identified by ORF Finder at National Center for Biotechnology Information (NCBI) website and the amino acid sequence of each identified ORF was used to find the closest match by BLAST 2.0 program. Multiple sequence alignments were carried out by T-COFFEE software at EBI (<http://www.ebi.ac.uk/t-coffee/>). Conserved domains and patterns were analyzed using conserved protein domain database at NCBI and transmembrane helices were predicted using server HMMTOP [26]. The phylogenetic tree was constructed for putative arsenate reductases and arsenite efflux pump proteins along their homologous protein in a database using the maximum-likelihood method with MEGA, version 7.0 software [27]. The bootstrapping value was used to estimate the reliability of phylogenetic construction (1000 replicates).

Accession No

The nucleotide sequences of the clones A7 and A12 were deposited in GenBank under accession numbers EU315066 and EU315067, respectively. The amino acid sequences of the predicted proteins from the two clones were deposited under accession numbers ACA14307-ACA14316 and ACA14317-ACA14323.

Results and Discussion

The metagenomic DNA was isolated from the sea sediment, which represented gene pool of the microbial communities of the habitat. Approximately 7 µg of DNA was extracted from each gram of the sea sediment sample. The estimated size of sea sediment DNA molecules was in the range of 40–50 Kb. Crude metagenomic DNA of the sea sediment sample was pale yellow in color, which might be due to the presence of humic acid and other impurities. Gel extraction resulted in the removal of most of the impurities from the metagenomic DNA, which was later used for metagenomic library construction. Gel extraction from low melting agarose gel followed by the release of DNA with agarase has been used successfully to obtain cloning-ready metagenomic DNA from various environmental samples. Our results indicate that this method is also suitable for purification of high quality and higher molecular weight sea sediment metagenome. The sea sediment plasmid library contained about 245,000 recombinant clones. Average insert size of the library was calculated to be ~10.13 Kb with inserts ranging between 4.6 and 23 Kb. Sea sediment metagenomic library represented 2.45 Gb of microbial community DNA, which is equivalent to ~600 *E. coli* genomes indicating good representation of sea sediment microbial community in the metagenomic library.

About 100 clones were obtained after selection of the sea sediment library on 6 mM arsenite containing medium. Two unique digestion patterns were observed in total fifty randomly selected clones after digestion with EcoRI and HindIII. One clone from each pattern was selected for further characterization and the clones were designated as A7 and A12. Retransformed *E. coli* DH10B strains with pA7 or pA12 demonstrated similar arsenite resistance as the parent clones confirming plasmid-borne nature of

arsenite resistance. The plasmids pA7 and pA12 contained inserts of ~8.8 and ~6.7 Kb, respectively.

Clone A7 showed a six-fold higher resistance to sodium arsenate, a three-fold higher resistance to sodium arsenite and significantly increased resistance to potassium antimony tartrate, while clone A12 showed increased resistance only to sodium arsenite and not to the other metalloids (Fig. 1).

Clone pA7 and pA12 were sequenced to full length and found to contain inserts of 8848 and 6771 base pairs, respectively. DNA sequence of clones pA7 and pA12 was found to be A + T rich and contained 52.41 and 60.61% of A + T bases in the sequences, respectively. DNA sequence of pA7 and pA12 show similarity with A + T rich genome sequences of psychrophilic bacteria *Psychrobacter* sp. P2G3 with 91% identity and 97% query coverage and *Pseudoalteromonas* sp. SM9913 with 83% identity and 10% query coverage, respectively. DNA sequence of pA7 and pA12 was analyzed for protein coding regions by ORF Finder. BLAST analysis of these coding regions revealed homology to putative arsenic resistance proteins and other proteins involved in general microbial metabolism (Table S1 and Table S2). All the encoded proteins from pA7 had the best match with proteins from *Psychrobacter* spp. with an amino acid identity ranging from 87–99%, while the encoded proteins from pA12 had the best match with proteins from *Pseudoalteromonas* spp. with amino acid identity ranging between 84 and 100%. The genus *Psychrobacter* and *Pseudoalteromonas* belong to *Gammaproteobacteria* and have been mainly isolated from cold marine environments including deep sea sediments and most of them have been shown to be adapted to the deep-sea life style [28, 29]. High similarity of the DNA and encoded proteins from pA7 and pA12 may indicate the origin of the metagenomic DNA in these clones from

Fig. 1 Metalloid resistance conferred to *Escherichia coli* DH10B by metagenomic clone pA7 and pA12. Resistance to **a** Sodium arsenite, **b** Sodium arsenate and **c** Potassium antimony tartrate was examined with *E. coli* DH10B + pHB201(control) (filled circle), *E. coli*

DH10B + pA7 (filled triangle) and *E. coli* DH10B + pA12 (filled square). Experiments performed in triplicates, values are averages and standard deviations are shown as error bars

Fig. 2 Genetic map of clones pA7 **a** and pA12 **b** showing location and orientation of protein coding regions in respect to orientation of *lac* promoter of pHB201, Vertical arrows in figure indicates position

of transposon insertion in arsenic sensitive mutant's clones (arrow heads with triangles) and circle indicates arsenic resistant mutant's (arrows heads with ovals)

similar marine bacteria. A genetic map was constructed describing protein coding regions along with the position of transposon insertion in mutants with or without arsenite resistance ability (Fig. 2).

In pA7, transposon insertions were identified in the genes encoding for arsenite-antimonite efflux pump (ArsB), NADPH-dependent FMN reductase (ArsH), arsenate reductase (ArsC) and transcriptional regulator ArsR family protein (ArsR) in the mutants which lost the arsenite resistance ability, indicating these genes played role in arsenite resistance of the clones. While transposon insertions in genes encoding for two component heavy metal transcriptional regulator (SilR), periplasmic sensor signal transduction histidine kinase (H₂ATPase) and hypothetical proteins had not resulted in any change in arsenite resistance indicating these genes have no role in arsenic resistance of pA7. Putative arsenite-antimonite efflux pumps (ArsB) of clone pA7 have conserved domains of arsenical pump membrane protein and *arsB/NhaD* permease and an anion permease. This protein was membrane bound having 12 transmembrane helices and it shows 59% similarity to arsenite-antimonite efflux pump (ArsB) of *E. coli* and no similarity to ARR3p related proteins (Fig. 3a). It helps in the efflux of arsenite and provides resistance to arsenite. A putative arsenate reductase with similarity to putative arsenate reductase from *Psychrobacter cryohalolentis* K5 was identified in pA7. This protein possessed conserved domain for glutaredoxin-dependent ArsC family. It shows 56% similarity to well characterized arsenate reductase (ArsC) of *E. coli* [25, 30] and no significant homology to other arsenate reductase proteins from gram positive system or fungal origin, ARR2p [9] (Fig. 3b). Sequence alignment of this protein with *E. coli* arsenate reductase indicated the presence of essential residue for arsenate reduction such as His8, Cys12, Ser15, Arg60 and Arg94 [30] (Fig. 4). A putative protein of 151 amino acids was homologous to the putative transcriptional regulator ArsR.

ArsR is known to regulate expression of arsenic resistance operon in presence of arsenite and maintains stringent regulation of gene expression [31].

In pA12, transposon insertions in arsenical pump membrane protein (ARR3p) and NADPH-dependent FMN reductase (ArsH) resulted in the loss of arsenite resistance property of the plasmid indicating the role of these genes in arsenite resistance. A putative arsenate reductase was identified downstream of these genes but transposon mutation in this genes did not show any affect on the arsenite resistance of the clone. Transposon insertions in putative mannose-sensitive agglutinin biogenesis protein (MshI), D-cysteine desulfhydrase (DcyD), EAL domain containing protein and single strand binding protein (SSB) also did not result in loss of arsenite resistance property of the pA12 indicating these genes have no role in arsenite resistance of the clone. The putative arsenite pump of pA12 posses eight transmembrane helices with amino-terminal inside the cell. This protein shows no similarity to *E. coli* arsenite efflux pump (ArsB) and low similarity to ARR3p of *Saccharomyces cerevisiae* (24%) [9] and arsenite efflux pump of *Bacillus subtilis* skin element (31%) [8]. This protein showed affiliation to clade 2 containing proteins related to ARR3p in the phylogenetic tree (Fig. 3a). The putative arsenate reductase from pA12 had very low similarity (39%) to arsenate reductase of *E. coli*. This encoded protein belongs to glutaredoxin-dependent arsenate reductases (Fig. 3b) that are a peculiar characteristic of gram negative bacterial ArsC [25, 30]. Along with that, it possesses conserved residue for arsenate reduction like His8, Cys12, Ser15 and Arg94 except for Arg60 which was replaced by glutamic acid (Fig. 4). An Earlier study has shown Arg60 and Arg94 function as a reciprocating engine by temporarily exchanging places in the presence of arsenate to enhance hydrogen-bonding of the intermediate and these interactions are not possible in the ArsC Arg60 mutants. The

Fig. 3 Phylogenetic relationship of **a** arsenite transporters and **b** arsenate reductases of clone pA7 and pA12 along with their homologous protein present in the database. The Phylogenetic tree was constructed by the maximum-likelihood method with MEGA,

version 7.0 software. The number associated with branches refer to bootstrap value (confidence limit) representing the substitution frequency per amino acid

Fig. 4 Multiple sequence alignment of arsenate reductases (ArsC) from clone pA7, pA12 and *Escherichia coli* W3110 (BAE77791). Arginine 60 is shown in **bold** and with an arrow mark

<i>E.coli</i> W3110	MSNIIYHNPAAGTSRNTLEMRNSGTEPTIIHYLETPTTRDELVKLIADMGISVRALLR
pA7	—MKPLIFHNPNCGTSRNTLAIMKASGEQPEVVEYLKNPFSRDELVELLAKMNI SPRELIR
pA12	—MVVIHNPDCGTSRNVLQIIKAAGYNPVIIEYLKKA—EQNSIVSVICRSKLTNKNSE
	*.*** *****. * : : : * : * : *
<i>E.coli</i>	KNVEPYEELGLAEDKFTDDRLLIDFMLQHPILINRPVIVTPLGTRLCRPSEVVLEILPDAQ
pA7	SKETINDELGLDNPELSDQIIDAMI AHPILINRPVIVTDKGAALCRPSEVFELEN—P
pA12	SEKSPAKKGLLNENITDEAILEAMLAHPVLVNRPIVCTEKGVKLCRPSQVLTLLAQWP
	. : *
<i>E.coli</i>	KGAFSKEDGEKVDEAGKRL—K
pA7	VSSFTEKEDGEVL—HHGK—D
pA12	KGELYKEDGEMILNAQGEQPKN
	. : *

mutation of Arg60 to alanine, glutamic acid or lysine leads to 1 to 5% of wild-type activity of the enzymes [30]. The absence of this conserved residue in pA12 ArsC indicates that this protein may not be active in the native organism. Lower concentrations of As(V) are expected in sea sediment and As(III) is expected to be the major species present in the sea sediments due to anoxic conditions. ArsC, conversion of As(V) to As(III), may not be required by the marine bacteria from which the pA7 metagenomic insert has originated due to the absence of As(V) and this may be the reason why a natural ArsC mutant was observed in this clone. A putative NADPH-dependent FMN reductase protein (ArsH) was also identified in pA12. This protein is part of the arsenic resistance operon and provides arsenic resistance in *Yersinia* species [7].

Overall, these results indicate that the metagenomic library comprises of the gene pool of psychrophilic microbial community of the sea sediment and can be exploited for identification of cold-adapted novel biocatalysts and bioactive compounds. The metagenomic library can be used to identify and characterize genes involved in various physiological functions of microorganisms residing in sea sediment complex ecosystem. Isolation of functional arsenic resistant clones indicates that the genes from psychrophilic marine bacteria can express in *E. coli*. Arsenic resistance genes identified in this study can be used to study their function at lower temperatures and can be used to engineer strains required for bioremediation in high arsenic and low-temperature conditions.

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Compliances with Ethical Standard

Conflict of interest The authors declare that they have no conflict of interest.

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