8.1 Introduction

In the recent years, one major concern is release of arsenical compounds in particular arsenite and arsenate, into the environment. Their toxicity and/or carcinogenicity in some forms, arsenites in particular, to a variety of organisms and human beings is a major concern. Arsenate redox is mediated either enzymatically or through respiration (Silver and Phung, 2005; Chang et al., 2008; Yoon et al., 2008). Different resistance pathways for As species in Bacteria are reported by Mukhopadhyay et al. (2002) and Rosen (2002).

The *ars* operons encoded either chromosomally or by plasmids, three (*ars*RBC) or five (*ars*RDABC) genes (Silver and Phung, 1996) are well known and widespread. Ji and Silver (1992a); Ahmann et al. (1994); Cervantes et al. (1994); Santini et al. (2000); Jackson et al. (2001) and Lee et al. (2001) reported on the use of As and its species as either acceptors or donors of electrons, also on their possessing disparate As detoxification modes. Monitoring As pollution level is aided by detection and description of *ars* genes (Stocher et al., 2003; Ji and Silver 1992a, b; Cervantes et al., 1994). Many researchers have worked on different *ars* genotypes to relate them to As resistant phenotype (Saltikov and Olson, 2002; Anderson and Cook, 2004; Jackson et al., 2005; Sun et al., 2004). Molecular detection and genotyping *ars* genes is useful to discern the diversity of bacterial flora (Cervantes et al., 1994; Diorio et al., 1995; Oremland et al., 2004).

The ARB isolates from near coastal marine waters representing all the 27 genera/species identified through 16S rRNA gene sequencing were examined for a few chromosomally encoded *ars* genes in this study. This was done to check for the presence of *ars*, *aox* and *Acr*3 genes associated with As resistance in a set of ARB to describe the innate detoxification mode/s of As biotransformation.

8.2 Materials and Methods

8.2.1 PCR Amplification of *ars* (*ars*R, -B, -C, -A, -D, -AB and -H) Genes

As many as 27 representative isolates (Table 8.1) were examined for the presence of *ars* (*ars*R, -B, -C, -A, -D, -AB and -H), *aox* (*aoxB* and *aoxR*) and *Acr*3 genes. Genomic DNA extraction and sequencing of the isolates is described in Chapter 6.
PCR amplification of these seven, well known arsenic resistant (ars) genes was done using the PCR primers listed in Table 8.2. All these ars primers used have an annealing temperature of approximately 53°C to 60°C (Chang et al., 2008). PCR amplification carried out using 25 µl with 1 µl DNA of concentration within a range of 25 - 40 µg DNA µl⁻¹ and 10 pmol each of forward and reverse primers. PCR was conducted using a thermal cycler (Veriti, Applied Biosystes, USA). The gene/s amplification was done following a standard protocol with 5 min hot-start at 95°C initially, 1 min 94°C denaturation (35 cycles), annealing at 53-57°C for 1 min, finally 1 cycle of 2 min extension at 72°C. PCR was ended by a 7 min extension at 72°C for ars genes. PCR amplifications of all examined genes was detected through gel electrophoresis using 1% agarose gel. The PCR amplification of arsB (using different set of primer) and Acr3 was performed using primers as given in Table 8.2 and by following Achour et al. (2007). PCR primers of Achour et al. (2007) were used to confirm some of these results.

8.2.2 PCR Amplification of aoxB and aoxR Genes

The primer sets for aox gene PCR are presented in Table 8.2 and their amplification was done following Cai et al. (2009). The PCR of aoxR was carried out as described by Chang et al. (2010).PCR amplifications were examined through gel electrophoresis using 1% agarose gel.

8.2.3 Cloning of arsB Gene

Cloning kit (TOPO TA, Invitrogen, USA) was used for arsB gene cloning according to the standard protocol. The PCR amplicons were purified and ligated into TOPO and transformed in E.coli competent cells (one shot TOP10). The transformed cells were spread plated on Luria Bertani (LB) agar containing ampicillin and X-gal and grown for 16 h at 37°C following manufacturer’s protocol. Atleast 10 transformed (white) colonies picked up and sequenced following the standard sequencing procedure.

8.2.4 Sequencing of ars, aox and Acr3 Genes

Sequencing of PCR products was performed with 15-50 ng PCR products and one pmol of each forward and reverse primers. The sequencing was done using ABI
sequencing instrument following the dideoxy chain termination method. The samples were sequenced on a ABI 3130 Genetic Analyzer (ABI, USA) and all raw sequences were manually screened for low quality regions. After end trimming and removal of low quality/chimeric regions, sequencing results were compared with sequences from NCBI database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), for their matches using BLASTX programs for confirmation.

8.3 Results

PCR detection of ars genes in the chromosomal DNA of 27 representative bacterial isolates was done in this study. Notably, the isolates differed in harbouring As modulating genes. For example, the arsB of ~700 bp (Figure 8.1a) encoding efflux pump was observed in six of 27 representative ARB strains in as many different genera identified by 16S rRNA gene sequencing. These are GN34, Acinetobacter sp. (KJ719350); GN1, Psychrobacter sp. (KJ719317); GN35, Pseudomonas sp. (KJ719351); GN10, Staphyloccus arlettae (KJ719326); GN2, Bacillus subtilis (KJ719318) and GN31, Pseudomonas putida (KJ719347) encoding ArsB efflux pump (KM673296), arsenic efflux pump protein (KM820844), ArsB efflux pump (KM820845), arsenical pump membrane protein (KM820846), arsenical pump membrane protein (KM820847) and arsenical pump membrane family protein (KM820848) respectively.

The isolates differed with respect to the arsB gene they carried from two differently used primers. For example, with the primers of Achour et al. (2007), the arsB gene was observed in six ARB strains. These are GN84, Bacillus aquimaris (KJ719399); GN1, Psychrobacter sp. (KJ719317); GN2, Bacillus subtilis (KJ719318); GN25, Bacillus cereus (KJ719341); GN10, Staphyloccus arlettae (KJ719326) and GN96, Bacillus sp. (KJ719411) encoding arylsulfatase (KP161070 to KP161073) and arsenite-antimonite efflux pump (KP161074 to KP161075) respectively (Figure 8.1b). With both sets of PCR primers isolates Psychrobacter sp., Bacillus subtilis and Staphyloccus arlettae were positive for arsB gene.

Presence of the aox (aoxB) genes within the chromosome was detected in only one isolate GN96, Bacillus sp. (KJ719411) encoding NADH oxidase (KP100806). The fragment length of aoxB is ~350 bp (Figure 8.2). The arsD gene is encoded by
GN25, *Bacillus cereus* (KJ719341) showing the presence of chemotaxis protein CheY (KP100804) at ~350 bp (Figure 8.2). *Acr3* gene fragment ~350 bp is found to be encoded (Figure 8.2) by GN25, *Bacillus cereus* (KJ71934) asABC transporter (KP100805).

Phylogenetic tree was constructed for *arsB* gene detected in the strains (Figure 8.3). It is useful to recognize that the *arsB* fragments from GN1, *Acinetobacter* sp. (KM673296), GN2, *Psychrobacter* sp. (KM820844), GN3, *Pseudomonas* sp. (KM820845) and GN6, *Pseudomonas putida* (KM820848) were found to cluster with sequences to *Staphylococcus epidermidis* (WP_031272610.1), *Shigella flexneri* (WP_032322508.1) and *Psychrobacter* sp. JCM 18902 (GAF59143.1). All these clustered in the same clade. The sequences from GN1, *Acinetobacter* sp. and GN3, *Pseudomonas* sp. clustered in the same node. The fragments from GN5, *Bacillus subtilis* (KM820847) and GN4, *Staphylococcus arlettae* (KM820846) were found to cluster with sequences of *Escherichia coli* (WP_032249039.1) and *Escherichia coli* (WP_032298753.1). A very high homology between GN4, GN5 and *E. coli* clustering in the same node is useful to note the identical evolutionary process of this gene.

Phylogenetic tree was constructed for *arsB* gene detected in the strains (Figure 8.4). It is seen that no much homology is found between fragments from *arsB* genes. The GN11, *Bacillus aquimaris* (KP161070); GN12, *Psychrobacter* sp. (KP161071); GN13, *Bacillus subtilis* (KP161072); GN14, *Bacillus cereus* (KP161073); GN15, *Staphylococcus arlettae* (KP161074) and GN16, *Bacillus sp.* (KP161075) were found to cluster with sequences to Uncultured bacterium (ACA14310.1) in the same node.

Phylogenetic tree was constructed for *arsD* gene detected in the strain *Bacillus cereus* (Figure 8.5). It is useful to recognize that the *arsD* fragments from GN7, *Bacillus cereus* (KJ719341) encoding *arsD* gene, chemotaxis protein CheY (KP100804) were found to cluster with sequencers to *Pseudomonas mosselii* (WP_028869077.1), *Pseudomonas mosselii* (AIN61540.1), *Pseudomonas putida* (WP_012316605.1) and *Pseudomonas* sp. (WP_027917512.1). All these clustered in the same clade. The sequences from GN7 and *Pseudomonas* sp. (WP_027917512.1) clustered in the same node.

Phylogenetic tree for *Acr3* gene was constructed (Figure 8.6). It is evident to recognize that the *Acr3* fragments from GN8, *Bacillus cereus*, asABC
transporter(KP100805) were found not to cluster with any sequences. It is useful to recognize from phylogenetic tree that aoxB gene detected in the strain GN9, *Bacillus* sp.(Figure 8.7) encoding NADP oxidase (KP100806) were found to cluster with sequences to *Paenibacillus lactis* (WP_007128835.1), *Bacillus* sp. (WP_028404116.1), *Paenibacillus* sp. (ETT41226.1), *Clostridium* sp. (KGG85949.1) and *Paenibacillus* sp. (GAK41357.1) All these clustered in the same clade. A very high homology between GN9, *Bacillus* sp. and *Bacillus aquimarlis* (WP_032087021.1) clustering in the same node is useful to note the identical evolutionary process of this gene.

Eight of the 10 randomly picked out transformants/clones were positive for the presence of arsB gene. All these positive arsB clones had homology with arylsulfatase. The accessions numbers for each of positive clones are as follows. Clone 1 (KP090137), Clone 2 to clone 8 (KP100797 to KP100803).
8.4 Discussion

The As mobilization across soil and groundwater is facilitated by arsenate reduction/methylation by in situ microflora (Oremland and Stolz, 2003). Arsenic resistant *ars* genes, arsenite oxidising *aox* genes and arsenic transported *Acrs3* genes examined from 27 different arsenic resistance bacterial strains suggest strains specific responses. For instance, the function of *arsR*, which encoding for regulator seem to be very specific. so also are the functions of *arsB*, *aoxB* and *aoxR*. The last two performing oxidation of arsenite to arsenate and *Acrs3* the arsenic transport. Some of the bacterial isolates possessed *ars* genes which encode various functions. High tolerance/resistance of As in some isolates I examined could be ascribed to an existance of many *ars* operon types on their chromosomes (*sensu* Ordenez et al., 2005) or transposon (Tuffin et al., 2006). Restoration of As-laced habitats by applying such microbes is known (Carbonell et al., 1998; Dedysh et al., 1998; Ibrahim et al., 2006; Weber et al., 2006; Chang et al., 2007).

In this study, the *arsD* gene encoded by *Bacillus cereus* (KJ719341) showing the presence of chemotaxis protein (CheY) is useful to suggest the abilty of many ARB to sense the toxic moiety. Similar to this study, Muller et al. (2007) isolated an heterotrophic bacterium, *Herminiimonas arsenicoxydans* from a wastewater treatment unit and is the first fully characterized arsenic-metabolizing microorganism known to possess unsuspected mechanisms for coping with arsenic. Apart from arsenic oxidation/reduction, oxidative stress resistance and As(III) extrusion. It is therefore of key significance in the bioremediation of contaminated environments, leading to the sequestration of this toxic metalloid As.

Detection of *aoxB* in the chromosome of *Bacillus* sp. (KJ719411; NADH oxidase; KP100806), is pertinent to suggest as Lynn et al. (2000) report that activation of NADH oxidase is brought about by arsenite leading to the formation of superoxide. This superoxide is detrimental as it damages the DNA. It is quite likely that such mechanisms do operate in certain bacteria. The *Acr3* gene is encoded by *Bacillus cereus*, asABC transporter(KP100805). These ABC transporters are involved in resistance (Tamas and Wysocke, 2001) to arsenic and antimony-containing compounds. Rosen (1999) found that extrusion of arsenite in yeasts aided by Acr3p.
As Yang et al. (2012) point out, such efflux/extrusion processes are governed by protein families that also confer drug resistance in some microbes.

The *ars* genes are normally located as clusters either on plasmids or chromosomes. However as exceptions they may occur as single units (Diorio et al., 1995; Cai et al., 1998; Sato and Kobayashi, 1998; Suzuki et al., 1998; Butcher et al., 2000; Butcher and Rawlings, 2002; Maury et al., 2003). Bacterial strains possessing *ars* operons control As mobility or, consequently its detoxification through anyone of the possible array of speciation. Thus, any ARB bearing *ars* genes are vital and prospective candidates As bioremediation.

Detection of *aox* genes in Mandovi-Zuari estuarine waters, is useful to suggest its utility in arsenite oxidation. Philips and Taylor (1976) demonstrated that arsenite oxidation of *Alcaligenes faecalis* is brought about by an enzyme and/or an electron acceptor which is formed when grown in media with As(III). Whereas Osborne and Ehrlich (1976) show that a strain of *Alcaligenes* sp. acquired its arsenite-oxidizing enzyme through growth-dependent induction. In this study, ability of arsenite oxidation (the trait enabled by *aoxB* gene) was identified in only one strain, *Bacillus* sp. (Figure 8.2). It is to be noted that in most ARB all genes essential for thwarting As toxicity were not present. This may be due to divergence in the *ars* operons as has also been noted earlier by some researchers (Jackson and Dugas, 2003 and Achour et al., 2007). Therefore, it is worthwhile to explore possible diversity of As resistance genes.

Migration of more toxic arsenite across sediment and aquifer is a serious concern. Microbial arsenic resistance is a common characteristic not only in contaminated areas but also in arsenic-free environments (Jackson et al., 2003). As an example Nagvenkar and Ramaiah, (2010) isolated bacterial strains from low As contaminated coastal region and reported many strains of ARB tolerating high concentrations (1000 ppm) of arsenite and also found their arsenite detoxifying potential to be very high.

Previously, Anderson and Cook (2004); Pepi et al. (2007); Fan et al. (2008) and Chang et al. (2010) reported arsenic resistant strains from the genera *Staphylococcus, Acinetobacter, Vibrio* and *Pseudomonas* from natural and arsenic...
contaminated sites. In this study, ARB isolates in the genera *Psychrobacter, Bacillus, Pantoea, Staphylococcus, Dietzia, Rhodococcus, Pseudomonas, Acinetobacter, Kocuria, Erwinia, Paenibacillus* and *Planococcus* were identified from estuarine waters of Mandovi and Zuari.

The results obtained in this study are certain evidences to the presence of numerous arsenic resistant genetic material in estuarine bacterial populations. Regardless of As concentration levels the resistance to As in many bacterial strains is formidable. Their ability to encode a few *ars* if not all the battery of genes brings forth the fact that such strains do perform As detoxification in the natural ecosystems. In any event, strains possessing a few to many *ars* operons seem to be conferred of arsenic resistance.
Studies on prevalence of flora capable of As tolerance are of pertinence for an understanding on As pollution and to realize their potential detoxification of As and other toxicants. It is evident from this study that sizable fraction of native bacteria growing in medium containing As is quite widespread. A broad range of bacterial types seem to carry out either reduction of arsenate or oxidization of arsenite. This aspect is clearly realized in this study.

As acknowledged variously, environmental effects of metal pollution are broad. Since various adverse effects due to a plethora of toxicants affect the coastal systems different experiments were conducted out to see whether marine ARB are adept enough in detoxifying arsenic and also to understand the ars gene constitution. The hypothesis putforth was whether native isolates capable of tolerating high As could detoxify it.

Water and sediments samples collected from coastal locations of River Mandovi reveal that bacterial fraction capable of growing in nutrient medium containing 15, 25 and 50 ppm arsenic is sizable. Thirteen randomly isolated environmental ARB strains capable of tolerating 1000 ppm arsenite were characterized biochemically. In addition, the effects of antibiotics were examined to check if certain alterations are due to As. Genomic DNA from a total of 500 isolates of ARB was extracted and, ARDRA other analyses were carried out. Twenty seven strains which represented all the genera of ARB isolated in this study were investigated for presence of ars, aox and Acr3 genes. Further, proteins expressed in Bacillus baekryungensis at different As concentrations were also analysed.

The following are the major features emanating from this study.

- The ARB have adapted to lower stretches of Mandovi-Zuari, exposed to low or moderate pollution. These systems do harbor ARB possessing mechanism(s) to deal with As toxicity. A total of 500 ARB (isolates tolerating \( \geq 100 \) ppm arsenite) from different locations (midstream: 187, estuarine: 218 and marine: 95) were isolated on NA medium amended with As. Thirteen of these isolates tolerating 1000 ppm were submitted to an extensive biochemical characterization. Two out of thirteen isolates were found to be Enterobacteriaceae, two Pseudomonas sp., one Corynebacterium sp., one
Xanthomonas sp., one Acinetobacter sp., one Flavimonas sp., one Micrococcus sp., one Bacillus sp., one Staphylococcus sp., one Rhodococcus sp. and one Planococcus sp.

- Some of these isolates bore resistance to many antibiotics suggesting metal and antibiotic resistance go hand in hand. This observation brings to fore the need to evaluate the ecological significance of native flora capable of tolerating such high concentrations of As and/or other toxic metals.

- All environmental strains tolerant to 1000 ppm arsenic were tested to realize their potential to detoxify arsenic. From the quantification of arsenic removal by 13 ARB strains, it was observed that amount of As removed was substantial (>85% of initial concentration) within 120 h by all of them. The highest removal rate was by Planococcus sp. (13) and Micrococcus sp. (9) followed by Enterobacteriaceae (3), Xanthomonas sp. (5), Rhodococcus sp. (12), Pseudomonas sp. (2 and 7), Staphylococcus sp. (11), Bacillus sp. (10), Corynebacterium sp. (4), Flavimonas sp. (8) and Acinetobacter sp. (6).

- The DNA from all 500 ARB isolates which tolerated arsenic concentration ≥ 100 ppm, was extracted and studied for the amplified ribosomal DNA restriction (ARDRA) of PCR-amplified 16S ribosomal RNA. My ARDRA analysis generated 123 different profiles from the 16S rRNA gene restriction digestion of 500 cultures. The sequencing was performed for a total of 123 amplified PCR products selected on their basis of unique ARDRA profiles.

- Analyses of 16S rRNA gene sequences represent 12 different genera and 27 different species with 99% similarity.

- Sequencing data of these isolates were used to construct phylogenetic trees to establish their evolutionary relationship to other bacterial clades. Based on 16S rRNA gene sequencing data, species were found to belong to three Phylum: Actinobacteria, Firmicutes and Proteobacteria.

- Whole cell lysate proteins from Bacillus baekryungensis through LCMS QToF analysis yielded respectively a total of 119, 101 and 16 proteins when grown
without As, with 50 ppm and 100 ppm As (as arsenite). By comparing control and As grown samples, it was found that only 5 proteins were similar.

- Analyses of *ars* genes from the 27 representative strains yielded interesting insights. Nine isolates namely *Acinetobacter* sp. (KJ719350), *Psychrobacter* sp. (KJ719317), *Pseudomonas* sp. (KJ719351), *Staphylococcus arletae* (KJ719326), *Bacillus subtilis* (KJ719318), *Pseudomonas putida* (KJ719347), *Bacillus aquimaris* (KJ719399), *Bacillus cereus* (KJ719341) and *Bacillus* sp.(KJ719411) possessed efflux pump system (Accession no. KM673296; KM820844 to KM820848; KP161070 to KP161075) encoded by the *arsB* (important in arsenite extrusion). The isolate *Bacillus* sp.(KJ719411) was found to encode *aoxB* gene (Accession no. KP100806). Detection of *aox* genes in the estuarine isolates, responsible for arsenite oxidation would be important in ecological functions pertaining arsenite oxidation. Further, *arsD* gene encoded by *Bacillus cereus*, achemotaxis protein CheY (KP100804) and *Acr3* gene encoded by *Bacillus cereus*, asABC transporter(KP100805) were also identified.

**Future Prospects**

In principle, higher the abundance of ARB, more likely could be the As metalloid contamination in that environment. Enumeration of ARB, if carried out on a regular basis might be a vital aspect in the up-keep of environmental health.

More detailed studies on ARB from marine environment would give rise to novel insight on, for instance, presence of non-*ars* ARB strains. Such studies are needed for acquiring more information on arsenic resistance and therefore the possibility of obtaining a set of hither to unknown, robust candidates of ARB useful for As bioremediation. Absence of *ars* (*arsR,-C, -A, -AB and -H*) and *aoxR* in the marine ARB strains of arsenic resistance/tolerance needs to be affirmed.

Importance of each proteins in the modulation of As are quite important. It is therefore worthwhile to check/look for enzymes or ionic processes conferring arsenic resistance.
Arsenic is a metalloid that causes harm to humans and environments. It is important to remove and reduce this pollutant from the environment through different methods such as physical, chemical, and biological. The use of bioremediation to remove arsenic from contaminated soils and aquifers could be an effective and economic way. This is because, a wide range of microorganisms have been found in this study to be successfully detoxifying As. For this, protocols for removing arsenic from the environment should meet basic technological criterion that include reliability, eco-safety, and safe from human-systems perspectives.