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Mechanism of arsenic resistance prevalent in *Bacillus* species isolated from soil and ground water sources of India

Bhoomika Saluja*,

Abhishek Gupta, Reeta Goel

Department of Microbiology,
G. B. Pant University of Ag. & Tech.,
Pantnagar-263145, Uttarakhand, India

Arsenic is a naturally occurring element and its contamination of soil and groundwater is a major threat to the environment and human health. The ubiquity of arsenic in the environment has led to the evolution of the arsenic defence mechanism in certain microbes. The present study highlights identical mechanisms of arsenic resistance in two bacterial strains, namely AG24 and AGM13, which were taken from two different ecosources and states of India. The phylogenetic analysis of the strains using 16S rDNA sequence revealed both strains to be under the genus *'Bacillus'*. Furthermore, *arsC* gene was amplified from the strains, cloned and sequenced. The homology of the sequences of the two strains, when compared with available database using BLASTn search, showed that the 300bp amplicons obtained in both strains possess a partial *arsC* gene sequence which codes for arsenate reductase, an enzyme involved in the reduction of arsenate to arsenite, which is then effluxed out of the cell. Nevertheless, atomic absorption spectroscopy and scanning electron microscopy analysis of both strains also strengthen the presence of the efflux mechanism. Thus, the presence of a similar mechanism of resistance in both strains suggests the possible role of lateral gene transfer from soil to water system and vice versa which is an alarming situation for global concern.

Key words: arsenic, *arsC gene, Bacillus* species, efflux mechanism

INTRODUCTION

Arsenic (As) is a naturally occurring element that is widely distributed on the Earth's crust. It is classified chemically as a metalloid having both properties of a metal and a nonmetal. Inorganic As forms trivalent or pentavalent ions where trivalent arsenicals are considered 100 times more toxic than pentavalent derivatives (Al-Abed et al., 2007; Taerakul et al., 2007; Neff, 1997). Arsenic contamination of soil and groundwater is a major threat to the environment and human health (Escalante et al., 2009; Smedley, Kinniburgh, 2002). Its contamination of drinking water most often results from natural sources; however, it can result from human activities as well. Ever since industrialization, thousands tons of As have been poured out as waste from industrial processing, livestock farming, wool processing and from mining and metal industry (Jones et al., 2000; Oremland, Stolz, 2003; Jackson et al., 2003). Although As levels in natural waters are usually low (a few μg/l), there are several areas in the world where humans consume drinking water containing >100 μg As/l resulting in cancer, post neonatal mortality, ischemic heart disease (heart attack), diabetes mellitus, hypertension, emphysema, bronchitis, chronic airway obstruction, etc.

The ubiquity of As in the environment has forced the evolution of As defence mechanism in certain microbes (Gaballa, Helman, 2003;

* Corresponding author. E-mail: rg55@rediffmail.com

Rehman et al., 2007). These microbes have developed or acquired genes that permit the cell to neutralize the toxic effects of arsenic through its exclusion from the cells (Sa'-Pereira et al., 2007). Chromosomal and plasmid-based As-resistance genes are clustered in an operon, commonly known as the ars operon (Silver, Keach, 1982; Cervantes et al., 1982; Xu et al., 1998; Nies, 1999). This operon codes for (i) a regulatory protein (*ars*R), (ii) an arsenate permease (*ars*B), and (iii) a gene coding for an arsenate reductase (*arsC*) that converts arsenate to arsenite (Rosen, 2002). Arsenate (As V) is transported into the cell by the Pit system and is subsequently reduced to arsenite (As III) by a cytoplasmic arsenate reductase enzyme encoded by the *arsC* gene. This (As III) is then effluxed from cells through the chemiosmotic gradient by the As (III)-specific transmembrane protein *ars*B encoded by the *ars*B gene (Dey et al., 1994; Suzuki et al., 1994).

In view of above, the present study aims to describe a mechanism of As resistance prevalent in diversified *Bacillus* species strains AG24 and AGM13 isolated from different eco-sources and two different states of India. The *arsC* gene was amplified from both plasmid and genomic DNA of strains, cloned and sequenced. Further, to validate the mechanism, atomic absorption spectroscopy and scanning electron microscopy analysis was done. The study also throws light on the fact that the presence of a similar mechanism of resistance in the two strains isolated from two different sources may be due to horizontal gene transfer of the *arsC* gene from soil to water system and vice versa which is an alarming situation for global concern.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strain AG24 was isolated from soil sample of the Panki thermal power plant, Kanpur, Uttarpradesh, India, and strain AGM13 from groundwater wells of Hazrapara and Ghoshpara locality of Beldanga, Murshidabad (Distt.), West Bengal, India. The soil and water samples were collected in the months of September–October 2010 and the strains isolated thereafter. These were cultured in nutrient broth or nutrient agar plates (Himedia laboratories Pvt. Ltd., Mumbai, India) at 28 °C and were screened for their resistance to both forms of As. Further, the minimum inhibitory concentration (MIC) of these strains was calculated by growing them at 28 °C with varying concentrations of sodium arsenate and sodium arsenite, respectively.

16srDNA sequencing

The sequencing of 16s rDNA was done at the National Centre for Cell Sciences (NCCS), Pune, India. The phylogenetic relationship of organisms was then determined by comparison of individual 16srDNA sequence with other existing sequences in the public database [Genbank http:www.ncbi.nlm.nih.gov/]. Polymerase chain reaction (PCR) for the amplification of almost a full-length 16S rRNA gene was carried out with the bacterial primer set 16F27 (5'-CCAGAGTTTGATCMTGGCTCAG-3') and 16R1525XP (5'-TTCTGCAGTCTAGAAGGAGGTGWTCCAGGC-3'). PCR was performed in an automated gene amplification PCR system 9700 thermal cycler (Applied Biosystem, Foster City, U. S. A.), and the PCR product was sequenced using a BigDye terminator cycle sequencing kit (V3.1) in an ABI Prism 3730 Genetic Analyzer (Applied Biosystem, U.S.A.) using primer 16F27 to yield a 700-base 5' end sequence. The sequence was then analysed at the RD11 and NCBI database. The sequences of strains AG24 and AGM13 were deposited in the NCBI genebank database with accession numbers AY970346 and HQ738570, respectively.

Plasmid and genomic DNA isolation

The plasmid DNA was isolated using Anderson, McKay, 1983 protocol and genomic DNA by the Genomic DNA isolation Kit (Himedia laboratories Pvt. Ltd., Mumbai, India). The isolated DNA was run on 0.8% agarose gel and documented subsequently. Furthermore, the isolated plasmid DNA was eluted from gel using the Gel elution kit (Himedia, laboratories Pvt. Ltd., Mumbai, India) to prevent genomic DNA contamination in plasmid DNA.

PCR, cloning and sequencing of *arsC* gene

The *arsC* gene was amplified using degenerate primers *arsC*1'F' (TTT AYT TAT GYA CAG GHA ACT) and *arsC*1'R' (ATC RTC AAA TCC CCA RTG WWN) from both eluted plasmid DNA and genomic DNA of strains AG24 and AGM13, respectively. The polymerase chain reaction (50 µl) mixture contained 0.5 µM of each primer, 200 µM

dNTPS, 1.5U Taq DNA Polymerase, PCR buffer supplied with the enzyme and 1 µl (75 ng) of template DNA. The total volume of the reaction mixture was maintained with sterilized triple distilled water. PCR was performed in BioRad iQ5, thermal cycler, and was carried out as follows: a single denaturation step at 95 °C for 5 min followed by a 34 cycle programme which includes denaturation at 94 °C for 2 min, annealing at 54 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 10 minutes.

The purified product of *arsC* gene was ligated with pDrive vector for 2 hrs using the Qiagen cloning kit, Hilden, Germany, and was transformed into *E. coli* DH5α. DNA sequencing of a single strand of the *arsC* gene was done using primer T7. The sequences thus obtained were analysed using BLASTn search (http://www.ncbi.nlm.nih.gov/Blast) and were deposited in the gene bank database under the accession numbers HM191727 and HQ385922, respectively.

Atomic absorption spectroscopy and scanning electron microscopy

Atomic absorption spectroscopy (AAS) studies of both strains grown in the absence and presence of As were done according to the previously described protocol (Roane et al., 2001; Vasudevan et al., 2001) The actively grown cells were inoculated in medium containing 5 mM of arsenic and kept for shaking at 30 °C at 120 rpm. The cells were drawn at various time intervals (0, 5, 10, 15, 20 hrs) and arsenic estimation was done both in supernatant and pellet. Furthermore, samples were also drawn for scanning electron microscopy (SEM). For this, samples from both strains (AG24 and AGM13) grown in the presence and absence of 5 mM sodium arsenate were drawn during the late log phase and analysed to determine the changes in shape and size of the cell when grown in the presence of metal.

RESULTS AND DISCUSSION

The present study takes into account two diversified *Bacillus* species strains, AG24 and AGM13, isolated from different eco-sources viz. soil and water, respectively and from two different states of India**.** The 16S rDNA sequencing revealed both strains to be under genus '*Bacillus*'. The strain AG24 was found to be *Bacillus cereus* and strain AGM13 was a novel *Bacillus* sp. strain whose identification using polyphaisc approach is underway at NCCS, Pune, India.

Gene location and gene characterization

Resistance to As is reported to be encoded by both plasmid and chromosomal resistance operons (Chen et al., 1986; Cai et al., 1998; Sinha et al., 2011). Therefore, to determine the location of resistant gene / genes; genomic composition in terms of presence or absence of plasmid DNA in strains AG24 and AGM13 was determined (Anderson, Mckay, 1983). The plasmid DNA profile of strain AG24 revealed the presence of plasmid DNA (5 kb approx) whereas AGM13 showed the presence of two small plasmids (≈ 2 kb approx) when the strains were grown in the presence of 5 mM sodium arsenate (Fig. 1).

The plasmid and genomic DNA isolated from the strains when subjected to the amplification of the *arsC* gene resulted in an amplicon of approximately 300 bp in size from the plasmid DNA of strain AG24 and from the genomic DNA

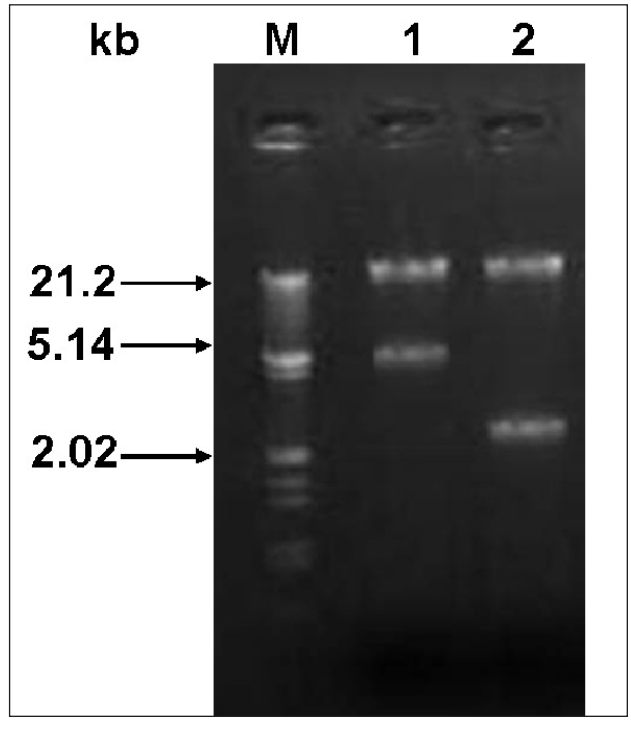

Fig. 1. Plasmid DNA profile of arsenic resistant strains: Lane M- lambda DNA/EcoRI/HindIII double digest, Lane1- Plasmid DNA profile of *Bacillus cereus* strain AG24, Lane 2- Plasmid DNA profile of *Bacillus* sp. strain AGM13

of strain AGM13. However, DH5α which served as a negative control showed no amplification. Furthermore, an attempt to amplify the *arsC* gene from genomic DNA of AGM13 and plasmid DNA of strain AG24 also gave negative results (Fig. 2). This showed that the gene was plasmid mediated in strain AG24 and was present on genomic DNA in strain AGM13.

The homology of the sequences of the selected clones, when compared with the available database using BLASTn search, showed that the amplicons contain a partial *arsC* gene sequence. The strain AG24 showed 98% similarity with *Bacillus* sp. CDB3 arsenical resistance gene cluster. Its homology comparison with other available database using BLASTn search showed 91% similarity to *Bacillus* sp. MB24 transposon TnARS1, complete sequence. The strain AGM13 showed 99% similarity with arsenate reductase gene of *B. licheniformis* ATCC 14580, complete genome and *B. licheniformis* strain DSM13. The presence of the *arsC* gene clearly suggests the presence of the efflux mechanism of resistance in both strains. Furthermore, to validate the mechanism, AAS and SEM analysis was done.

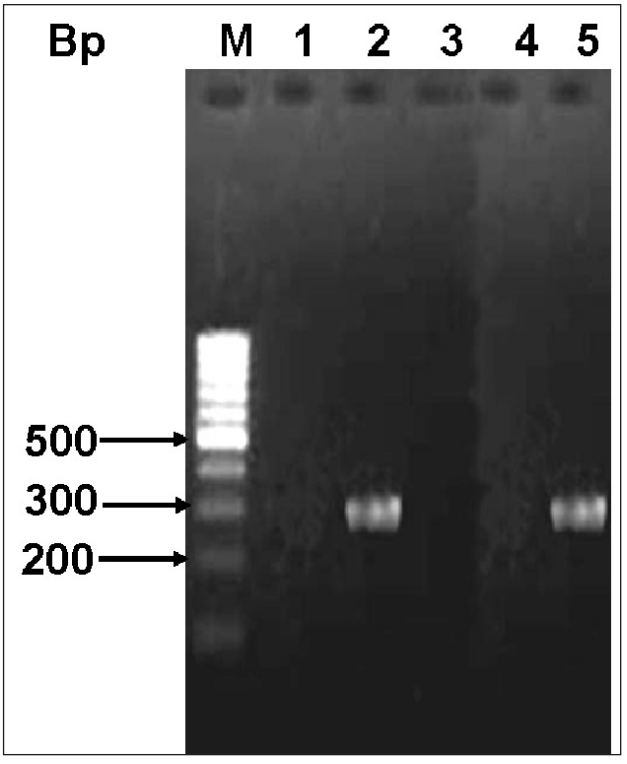

Fig. 2. The *ars*C gene amplification profile. Lane M-100 bp DNA ladder, Lane1- control DH5α, 2, 3- amplicon from plasmid DNA, 4, 5- amplicon from genomic DNA of strains AG24 and AGM13, respectively

Atomic absorption spectroscopy and scanning electron microscopy

The AAS results showed that the concentration of arsenic in strains increased in the pellet with the start of the log phase and then decreased with the end of the log phase whereas in the supernatant the concentration of arsenic followed the reverse trend, i. e. the concentration of metal decreased in the early log phase and then increased depicting the efflux of metal out of the cell in the late log phase (Fig. 3). Moreover, the data of AAS was substantiated by

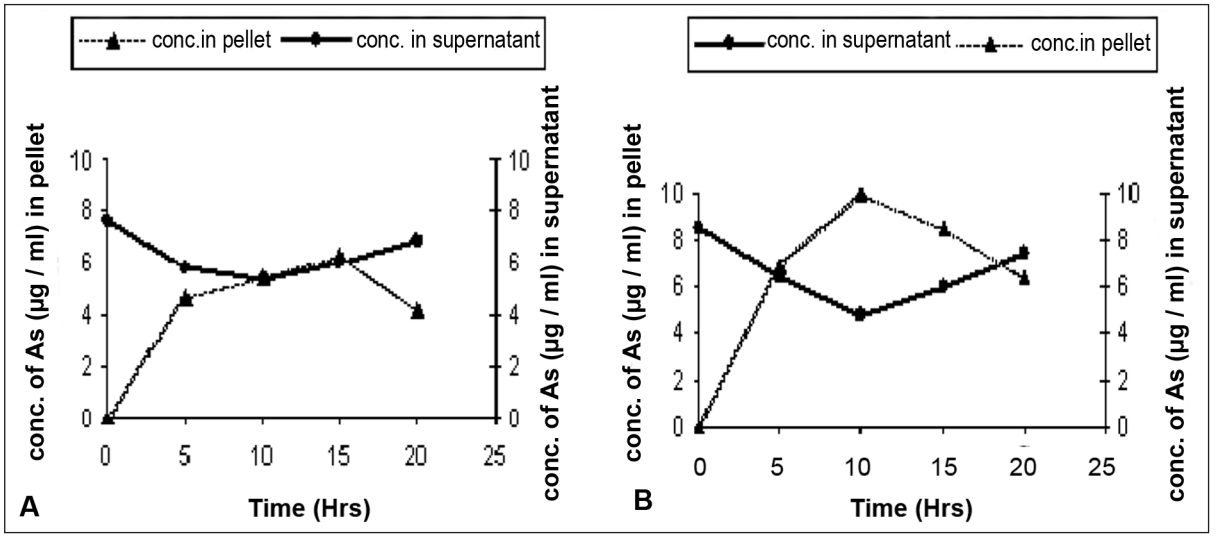

Fig. 3. Arsenic concentration in pellet and supernatant fraction of (A) strain AG24 and (B) strain AGM13, respectively, at different time intervals of growth

SEM. It had been reported earlier that SEM analysis of *Pseudomonas aeruginosa* strain MCCB 102 showed an increase in cell size due to Cd and lead accumulation in the cell wall and along the external cell surfaces (Zolgharnein et al., 2010). The effect of metal on cell morphology was also demonstrated by transmission electron microscopy analysis of *P. putida* strain 62BN which showed an increase in size of the cells grown in the presence of Cd and also showed intracellular and periplasmic accumulation of metal in the cells (Rani et al., 2009). However, the scanning electron micrographs of the strains (AG24 and AGM13) depicted no prominent change in cell morphology and size when the strains were grown in the presence of As (Fig. 4). This is in agreement with the results of AAS as the metal which entered the cells during the early log phase got extruded out of the cell in the late log phase, so no change in cell size and morphology was observed in scanning electron micrographs of cells grown in the presence of As as compared to cells grown in its absence, clearly indicating the presence of the efflux mechanism of resistance in the strains.

Lateral gene transfer (LGT) plays a vital role in increasing the genetic diversity of microorganisms and promoting the spread of fitness-enhancing phenotypes throughout microbial communities. To date, LGT has been investigated in surface soils, natural waters, and biofilm communities (Coombs, Barkay, 2004). The possibility that LGT is an important force in shaping the structure and

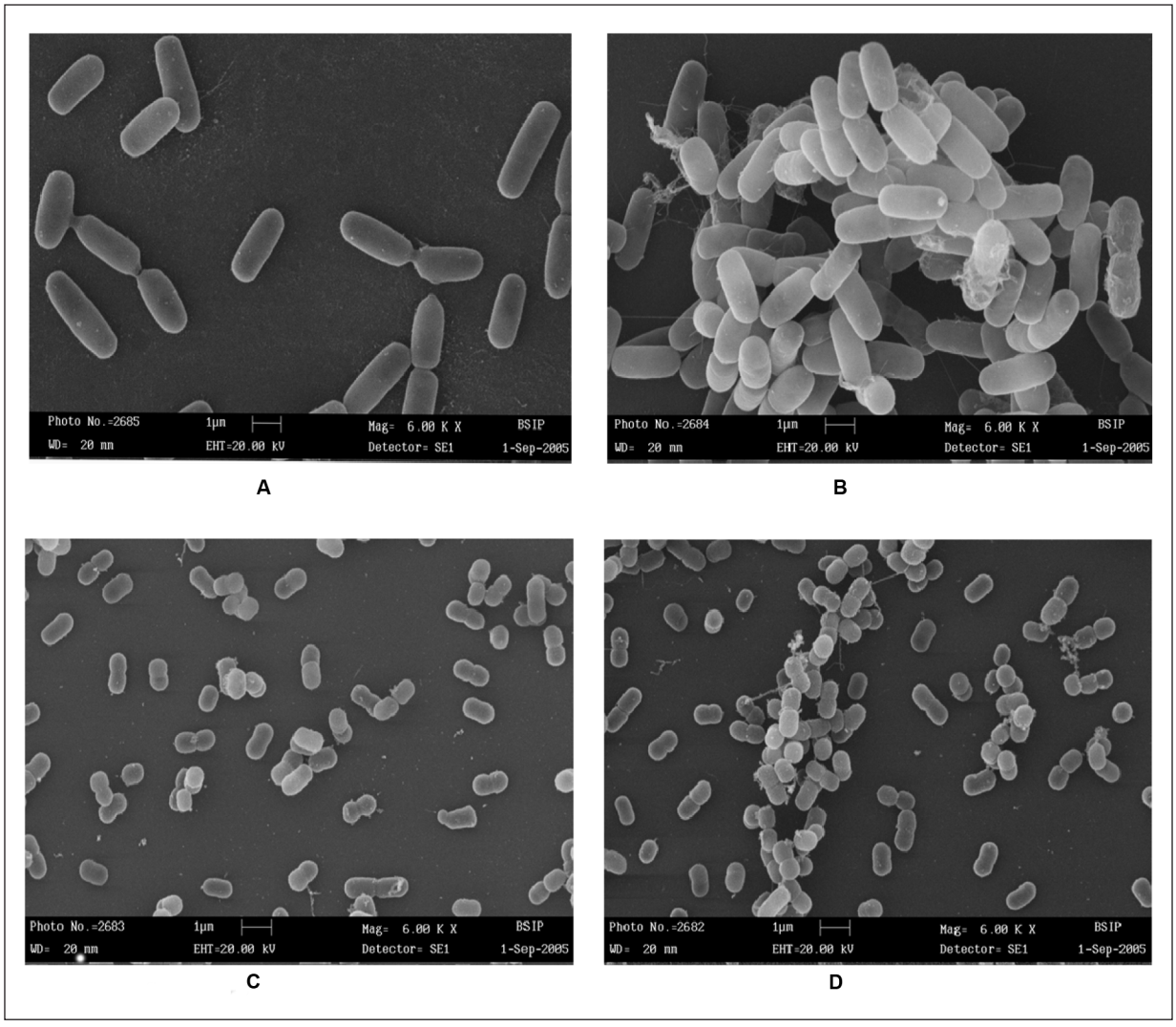

Fig. 4. Scanning electron micrographs of strains (A, B) AG24 and (C, D) AGM13 in the presence and absence at a magnification of 6000X

function of microbial communities in their natural habitats is suggested by the fact that abundant and diverse plasmids, insertion sequences (Smalla et al., 2000), integrons (Nield et al., 2001) etc. are common among microbes and occur in soils (Claverys et al., 2003) and natural water (Williams et al., 1996). Therefore, the presence of a similar resistance mechanism in two strains isolated from two different habitats viz. soil and water is perhaps an outcome of LGT from soil to water system and vice versa but this may be a threat to the environment, because the presence of the *arsC* gene protects the bacterial cell from hazardous effects of As(V) but in turn gives out more toxic As(III) into the surroundings. Therefore, the spread of the gene may contribute more to As pollution and a number of lethal diseases in humans.

Thus, from the above results it is clear that both strains possess a similar mechanism of As resistance, in spite of their origin from two different ecosources. These strains were found to possess a partial *arsC* gene sequence which was plasmid mediated in case of strain AG24 and was present on genomic DNA in strain AGM13. Further, the efflux mechanism was strengthened by AAS and SEM analysis. The study further indicates the possible role of LGT in the spread of the gene in bacteria living in different habitats which is a major environmental concern.

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ATSPARUMO ARSENUI MECHANIZMAS *BACILLUS* RŪŠYSE, IŠSKIRTOSE IŠ DIRVOŽEMIO IR POŽEMINIO VANDENS INDIJOJE

Santrauka

Dirvožemio ir požeminio vandens užterštumas arsenu ir jo junginiais kelia didelę grėsmę aplinkai ir žmogaus sveikatai. Dėl gana plataus paplitimo aplinkoje kai kurie mikroorganizmai tapo atsparūs arsenui. Šiame darbe aptarti identiški atsparumo arsenui mechanizmai, funkcionuojantys dviejose bakterijų padermėse – AG24 ir AGM13A, kurios buvo išskirtos iš dviejų pavyzdžių – dirvos bei vandens ekosistemų, paimtų skirtingose Indijos valstijose. Padermių filogenetinė analizė, naudojant 16S rDNR seką, atskleidė, kad abi šios padermės priklauso *Bacillus* genčiai. Be to, padermių *arsC* genas buvo padaugintas, klonuotas ir sekvenuotas. Palyginus dviejų padermių homologiją taikant BLASTn paiešką nustatyta, kad 300 bp PGR fragmentai abiejose padermėse turi dalinę *arsC* geno seką, koduojančią arsenato reduktazę – fermentą, redukuojantį arsenatą iki arsenito, kuris vėliau yra pašalinamas iš ląstelės. Be to, abiejų padermių analizė atominės absorbcijos spektroskopijos ir nuskaitančios elektroninės mikroskopijos metodais patvirtino arseno pašalinimo mechanizmą. Taigi, panašūs atsparumo mechanizmai abiejose padermėse leidžia teigti horizontalią genų pernašą pro dirvožemio vandens sistemą, kuri kelia susirūpinimą dėl tokio galimo plitimo nepaprastai plačiu mastu.

Raktažodžiai: arsenas, *arsC* genas, *Bacillus* rūšys, išmetimo mechanizmai