CHAPTER 5

CHARACTERIZATION OF ARSENITE REDOX TRANSFORMING BACTERIA

5.1 Introduction

Arsenic (As) is a major contaminant of many agricultural ecosystems in Thailand and is responsible for serious public health issues. Agricultural and industrial activities have led to the release and prevalence of As in many regions and ecosystems of the world. However, various groups of microorganisms such as bacteria has evolved certain strategies that enable them to tolerate arsenic and to survive in arsenic-rich environments. Arsenic-resistant bacteria have been isolated from old mining areas and chemical wastes contaminated areas such as soils contaminated with chromated copper arsenate (CCA) and soils around a metallurgical factory (Turpeinen *et al*., 2004; Krumova *et al*., 2008; Jareonmit *et al*., 2010). However, recent studies revealed large proportions of arsenic-resistant bacteria (7–50% of the total isolates) in arsenic free soils (Jackson *et al*., 2005) and in cultivated soils (90% of the total isolates) (Shutsrirung *et al*., 2013). These suggest a variety of arsenic-resistant system and wide distribution of arsenic-resistant bacteria in the environment. Arsenic transformation of bacteria involves oxidation, reduction and/or methylation to overcome the toxic effects and survive in arsenic-rich environment. Various genera of bacteria are capable of using either arsenate (oxidized form of As) or arsenite (reduced form of As) in their metabolism, and many of them are capable of resisting arsenic toxicity though the ars genetic system (Jackson *et al*., 2003; Oremland and Stolz, 2003). Through a variety of detoxification and respiratory mechanisms, microorganisms significantly influence arsenic species, toxicity and mobility in the environment and thus play a significant role in the arsenic biogeochemical cycle. (Liu *et al*., 2012). Characterization of arsenic resistant bacteria could lead to a better understanding of the detoxification processes and the development of suitable, eco-friendly method for the treatment of arsenic contaminated soils. A diversity of bacterial

genera has been reported to tolerate high concentration of arsenic such as *Acidithiobacillus*, *Bacillus*, *Deinococcus*, *Desulfitobacterium* and *Pseudomonas* (Oremland *et al*., 2004; Suresh *et al*., 2004a; Pepi *et al*., 2007;Yamamura *et al*., 2007), *Pseudomonas fluorescens* (de Vicente *et al*., 1990; Prithivirajsingh *et al*., 2001) and *Bacillus subtilis* (Sato and Kobayashi, 1998). Novel arsenic resistant strains were also isolated; *Planococcus* (KRPC10YT) from bore-well of West Bengal, India (Chowdhury *et al.*, 2007) and *Bacillus arsenicus* from arsenic contaminated soils in Chakdah district of West Bengal, India (Shivaji *et al*., 2005). The extensive database for arsenic resistance genes in bacterial genomes indicates that arsenic resistance operon must have evolved due to the presence of arsenic in the natural environment. New arsenic resistance gene clusters continue to emerge, and varied mechanisms seem to occur in diverse biological systems. The increasing availability of genome data and proteome analysis will help researchers to explore metabolic diversity, which may possibly lead to the discovery of new pathways and regulatory elements.

In Thailand, arsenic contaminated soils are widely distributed as a consequence of several decades of high input of agro-chemical inputs in modern farming systems. However, the study of bacterial diversity is limited in the contaminated areas. Our knowledge about the arsenic-resistant bacteria in agricultural soils in Thailand and their possible role in arsenic transformation and mobilization is very limited. In the previous study (Chapter 2 and 3) four isolates (BAs 8, BAs 11, BAs 19, BAs 29) of arsenic-resistant bacteria with a high potential application in the biotransformation of arsenic compounds to less toxic ones were selected for further investigations. One arsenic sensitive isolate, BAs 7 was also selected to test along with the resistant isolates. The aim of the present work was (i) to evaluate the phenotypic traits of the selected isolates; (ii) to identify selected isolates by 16S rRNA sequencing (iii) to analyze genetic similarity between arsenic resistant isolates.

5.2 Materials and methods

Characterization was performed using five selected isolates i.e. BAs7 (arsenic sensitive isolates), BAs8, BAs11, BAs19 and BAs29.

5.2.1 Morphological and biochemical identification

A loopful of 7 days culture broth was smeared on a clear slide and stained according to Gram's staining method (Merchant and Packer 1969; Claus and Berkeley, 1986). The slide was observed under oil immersion objective lens of compound microscope (Olympus). Pure single colony of each isolate developed after streaking on nutrient agar (NA) was examined for morphological features (Bergey's manual of systematic bacteriology, 2001).

5.2.2 Molecular identification by 16S rDNA sequencing

5.2.2.1 Extraction of genomic DNA and PCR amplification products of 16S rDNA

Nucleic acid preparation: A Genomic DNA was extracted from each isolate of the arsenic resistant bacteria using the NucleoSpin® method (Nucleospin® Tissue, Germany) according to the manufacturer's instruction. All the selected isolates (BAs 7, BAs 8, BAs 11, BAs 19 and BAs 29) were grown in the nutrient broth at the ambient temperature with shaking condition (250 rpm). The cell population of seven-day-old bacterial cultures was adjusted to an OD between 0.5 to 1.0 at 620 nm and one mL of each isolate was centrifuge at 8,000 rpm for 5 min then the supernatant was removed to obtain the cell pellets. The pre-lysis of the cell pellets was performed by resuspending the pellets in 180 µl buffer T1. Proteinase K 25 µl was then added and vortexed vigorously and incubate at 56°C until compete lysis was obtained (at least 1-3 h), vortexed occasionally during incubation or use a shaking incubator. 25 µl of proteinase K, and 200 µl of Nucleospin lysis buffer B3 were added to each other and vortexed for 20 s. Then 200 µl of Nucleospin lysis buffer B3 were added to the complete lysis sample, vortexed vigorously and incubated at 70°C for 10 min, vortexed briefly. After the lysis steps, 210 µl ethanol (96-100%) was added to the sample, vortexed vigorously to adjust the DNA binding condition. To bind the DNA sample, each sample was separately placed in

NucleoSpin®Tissue column and put into a collection tube, centrifuge at 11,000 rpm for 1 min, the flow-through was then discarded and the column was placed back into the collection tube. Silica membrane was washed by adding 500 µl Buffer BW, centrifuged at 11,000 rpm for 1 min, again, the flow-through was then discarded and the column was placed back into the collection tube. Silica membrane was then dried by centrifuge at 11,000 rpm for 1 min. Highly pure extracted DNA was eluted by placing the NucleoSpin®Tissue column into a microcentrifuge tube and 100 µl prewarmed buffer BE (70°C) was added and the tube was incubated at room temperature for 1 min, then the tube was centrifuged at 11,000 rpm for 1 min and stored at -20°C for future analysis.

PCR amplification and purification: The extracted DNA of each isolate was used as the template for PCR amplifications. The 16S rDNA gene was amplified using PCR kit (Quick Taq HS DryMix, Japan) with primers (Pepi *et al.*, 2007) 27F 5'-AGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTAC GGCTACCTT GTTACGA-3'). The PCR protocol was as follows: initial denaturation at 95°C for 5 min melting at 95°C for 1 min, annealing at 55°C for 40 s and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. After 35 cycles, the amplification product was separated in a 1% agarose TAE gel by electrophoresis and visualized on a UV transilluminator (SYNGREN, Gene Genius Bio Imaging System).

PCR products were purified by PCR clean-up Gel extraction (NucleoSpin®Gel and PCR Clean-up, Germany). The PCR products of each sample was loaded into a collection tube containing 50 µl PCR reaction, 100 µl Buffer NTI, a NucleoSpin®Gel and PCR Clean-up column, centrifuge at 11,000 rpm for 30 s. The flow-through was then discarded and the column was placed back into the collection tube. Silica membrane was then dried by adding 700 µl Buffer NT3 into the NucleoSpin®Gel and PCR Clean-up column, centrifuged at 11,000 rpm for 30 s. Again, the flow-through was then discarded and the column was placed back into the collection tube. The silica membrane was centrifuged again at 11,000 rpm for 1 min to remove Buffer NT3 completely. The NucleoSpin®Gel and PCR Clean-up column was placed into a new

microcentrifuge tube, then 30 µl Buffer NE was added and incubated at room temperature for 1 min, after that the tube was centrifuged 11,000 rpm for 1 min.

5.2.2.2 Nucleotide sequence accession numbers

The 16S rDNA gene sequences of the five selected strains were deposited in GenBank under the accession numbers KX897170 through KX897174

5.2.2.3 Sequence and phylogenetic analysis

The sequencing was detected by Bio Basic Inc., Canada. To analyze sequence similarity, the 16S rDNA gene sequence obtained were aligned using the BLAST program (http://blast.ncbi.nlm.nih.gov). The phylogenetic tree was constructed in MEGA 6.06 (The neighbor-joining method, sequence divergences between strains were quantified using the maximum composite likelihood) based on 1,000 bootstraps.

5.2.3 Evaluation of redox potential of arsenic resistant bacteria.

The ability of the five selected isolates to oxidize arsenite (As(III)) or reduce arsenate (As(V)) was evaluated by using silver nitrate ($AgNO_3$) method as described by Simeonova *et al*., 2004). Nutrient agar medium containing 1 mM $NaAsO_2$ was used for the determination of As (III) oxidation and 5 mM $Na_2HAsO_4 \cdot 7H_2O$ was used for the determination of As(V) reduction. The respective NA agar medium for each tested reaction was incubated at 30°C for five days. After incubation, the agar plates were flooded with 0.1 M $AgNO_3$ solution. On the agar plate, the reaction between $AgNO_3$ and As(III) or As(V) resulted in the formation of colored precipitate, a brownish precipitate revealed the presence of Ag_3AsNO_4 in the medium (colonies expressing arsenite oxidase) while a yellow precipitate confirmed the presence of Ag_3AsNO_3 colonies expressing arsenate reductase).

5.3 Results

5.3.1 Phenotypic identification of bacteria

All the colonies of the selected isolates were opaque with similar textures. The four isolates; BAs7, BAs8, BAs19, BAs29 showed similar colonies appearance with creamy-white or creamy-yellow in color, irregular in shape and undulate margin (Table 5.1, Fig. 5.1). Only isolate BAs11 exhibited obviously different colonies appearance from the rest of the isolates. Isolate BAs11 showed yellow-orange colored with entire margin. In Gram's staining technique, all the selected isolates were violet colored, Gram positive rod shaped or bacilli, small rod-shaped appearance, arranged in single or several cells attached together under the microscopic examination. In general, isolates from the As(V) enrichments displayed slower growth rates than those isolated from As(III) media (data not shown). However, this was most likely due to incubation conditions - anaerobic for As(V) versus aerobic conditions for As(III) - and not arsenic toxicity.

Table 5.1 Colony morphology and biochemical reaction of arsenic resistant isolates

Bacterial isolates	Colony color	Colony shape	Colony margin	Cell morphology	Gram reaction
BAs7	Creamy- yellow	Irregular	Undulate	rod	Gram +
BAs8	Creamy-white	Irregular	Undulate	rod	Gram +
BAs11	Yellow-orange	Circular	Entire	rod	Gram +
BAs19	Creamy-white	Irregular	Undulate	rod	Gram +
BAs29	Creamy-white	Irregular	Undulate	rod	Gram +

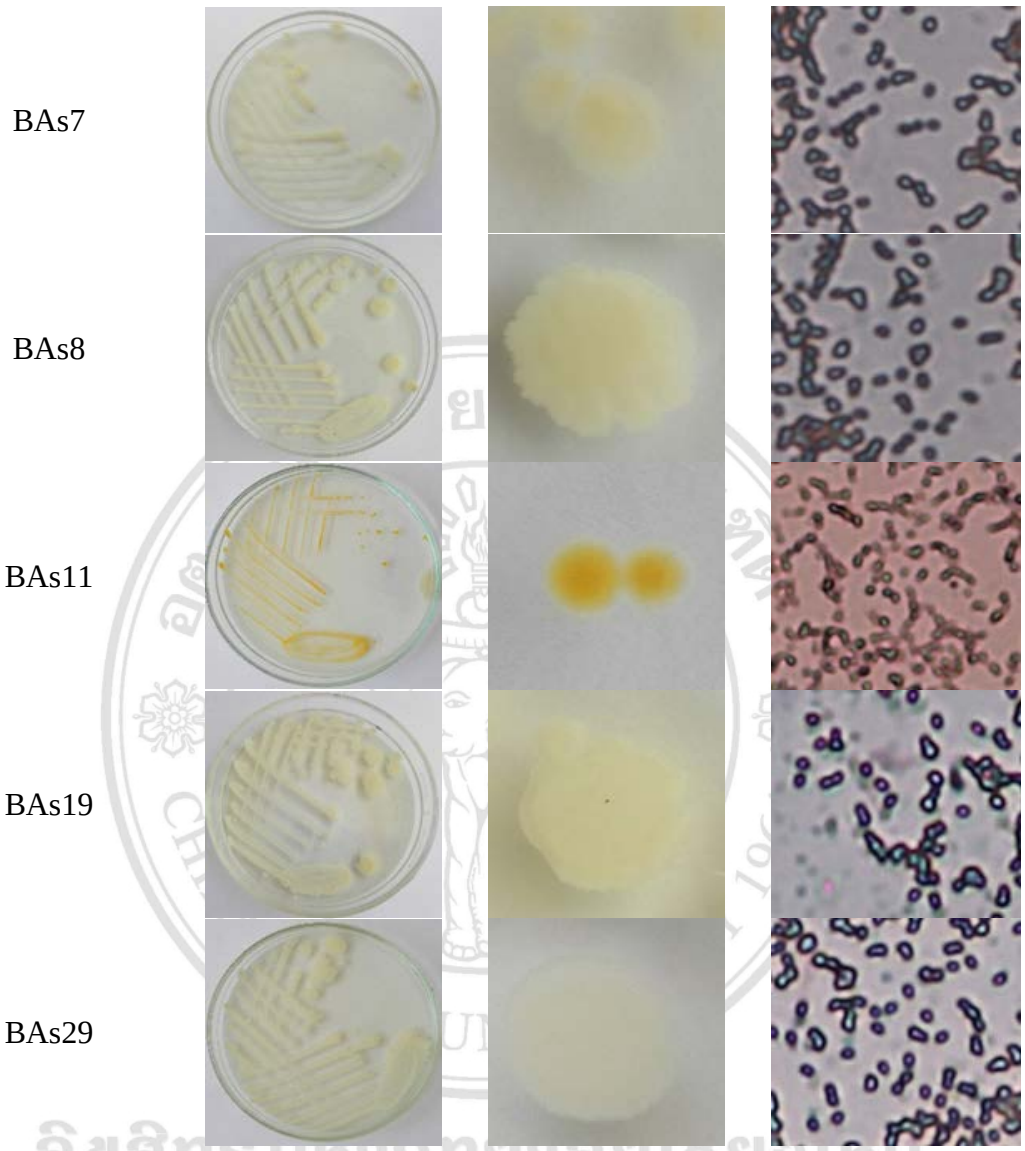

Figure 5.1 Colony morphology and gram strain of arsenic resistant bacteria on NA medium

5.3.2 Sequence and phylogenetic analysis.

The five selected isolates (BAs7, BAs8, BAs11, BAs19, BAs29) were extracted, yielding quantifiable amounts of DNA for all isolates (around 1,500 bp) (Figure 5.2).

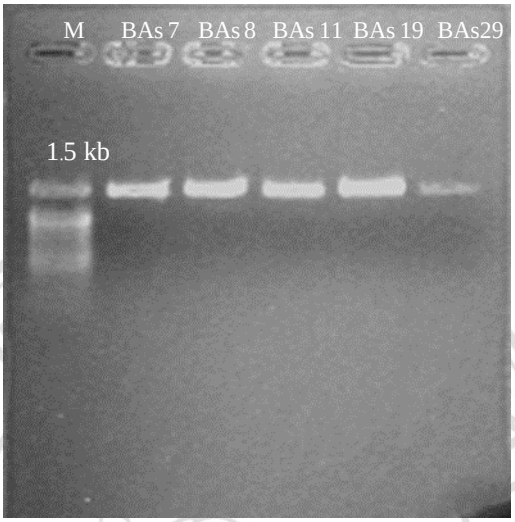

Fig 5.2 Representative gel of PCR amplicons of 16S rRNA gene of BAs7, BAs8, BAs11, BAs19 and BAs29. M is the molecular weight marker, with a DNA band of approximately 1,500 bp.

5.3.3 Nucleotide sequence accession numbers

Molecular identification was conducted based on amplification and sequence analysis of the 16S rDNA ribosomal gene of the five strains (BAs7, BAs8, BAs11, BAs19 and BAs29) of arsenic-tolerant isolates. The 16S rDNA gene sequences of the five strains were deposited in GenBank under accession numbers KX897170 to KX897174 (Table 5.2).

Table 5.2 The 16S rDNA sequence homologous identity of the seltected strains with the most relevant species retrieved from the GenBank by blast

Strain	Isolate Accession number	Closest neighbor/ accession number	Percentage similarity
BAs 7	KX897170	*Bacillus stratosphericus*	99
BAs 8	KX897171	*Bacillus pumilus*	98
BAs 11	KX897172	*Mycobacterium neoauurum*	99
BAs 19	KX897173	*Bacillus stratosphericus*	99
BAs 29	KX897174	*Bacillus altitudinis*	98

The 16S rRNA sequence analysis and phylogenetic analysis grouped the arsenic-tolerant isolates into two distinct genera; *Bacillus* spp. (BAs7, BAs8, BAs19 and BAs29) and *Mycobacterium* sp. (BAs11) (Fig. 5.3; 5.4). *Bacillus* spp. BAs7 and BAs19 showed 99% similarity to *Bacillus stratosphericus* (Accession No. AJ831841). *Bacillus* spp. BAs8 and BAs29 were closed to *Bacillus pumilus* and *Bacillus altitudinis* with 99 and 98% similarity (Accession No. AB098578 and AJ831842), respectively. Only one isolate, BAs11 belong to genus *Mycobacterium* with 99% homology to *Mycobacterium neoauurum* (Accession No. FJ172309). The analysis of 16S rRNA sequences indicated that isolates BAs7, BAs8, BAs19 and BAs29 were identified as *Bacillus* spp. and isolate BAs11 as *Mycobacterium*.

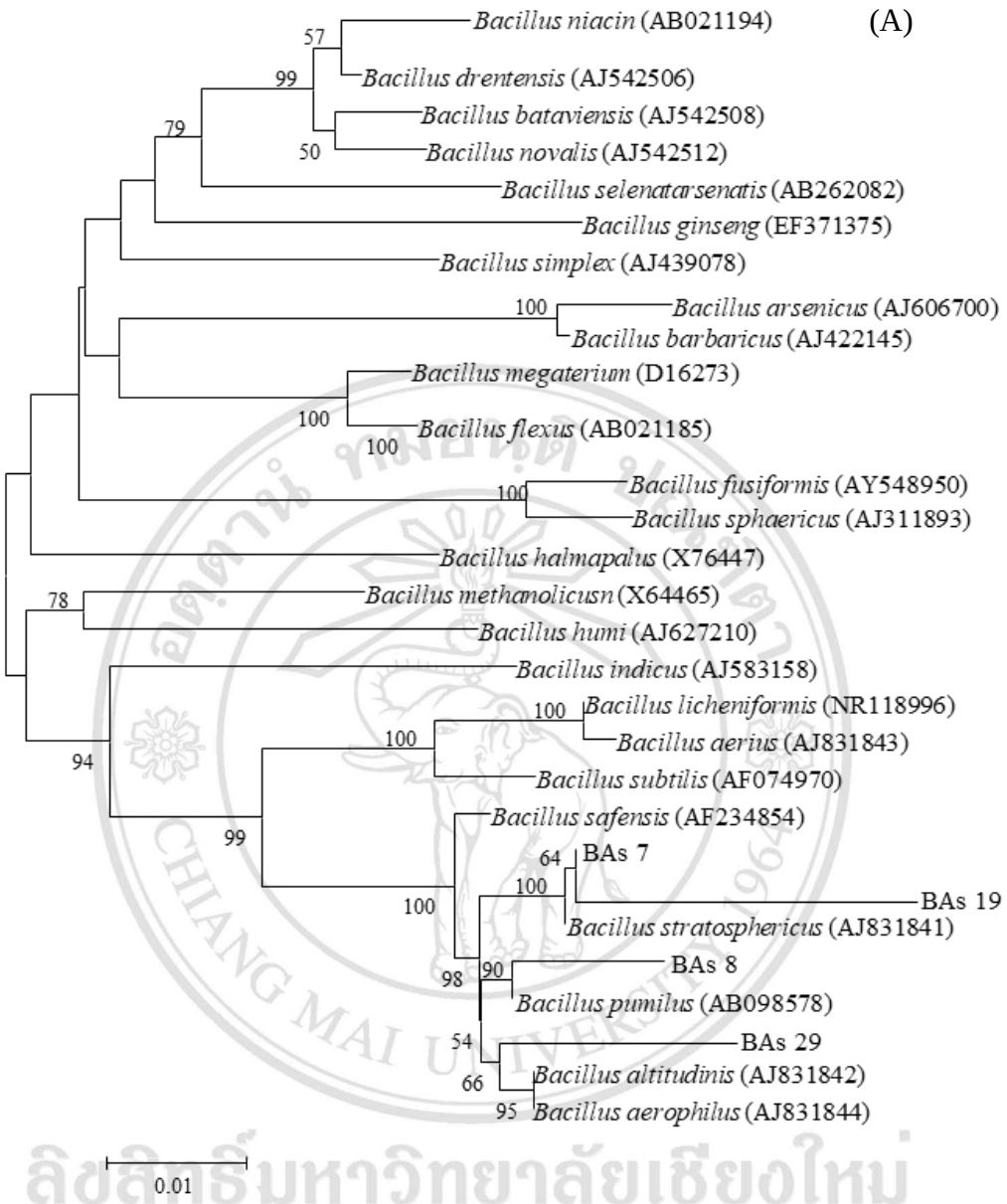

Figure 5.3 Phylogenetic tree based on 16S rDNA sequence showing the position of BAs 7, BAs 8, BAs 19, BAs 29 (A). The tree was constructed from a matrix of pair-wise genetic distances using the neighbor-joining tree method. Bootstrap values over 50% are shown.

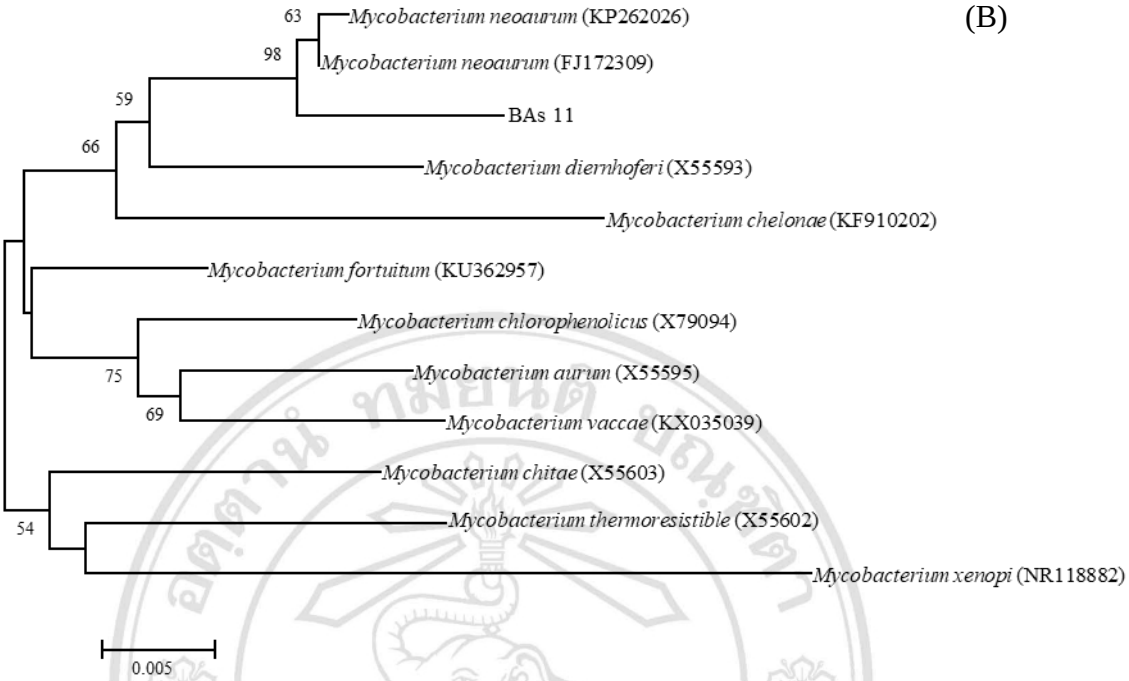

Figure 5.4 Phylogenetic tree based on 16S rDNA sequence showing the position of BAs 11 (B). The tree was constructed from a matrix of pair-wise genetic distances using the neighbor-joining tree method. Bootstrap values over 50% are shown

5.3.4 Screening of the arsenite oxidation and arsenate reduction bacteria

A Qualitative silver nitrate ($AgNO_3$) screening technique was used to detect the oxidation of arsenite to arsenate or the reduction of arsenate to arsenite. The interaction of $AgNO_3$ generates bright yellow precipitate and brown precipitate with arsenite and arsenate, respectively. The $AgNO_3$ test of five isolates revealed that, isolates BAs11 and BAs29 exhibited both oxidizing and reducing abilities. Isolate BAs8 and BAs19 showed only the ability to oxidize arsenite. In contrast to the tolerant isolates (BAs8, BAs11, BAs19 and BAs 29) one sensitive isolate, BAs7 could not oxidized arsenite and showed only the ability to reduce arsenate (Table 5.3).

Table 5.3 Evaluation of the arsenite oxidation and arsenate reduction by selected isolates

Bacterial isolates	Arsenite oxidation	Arsenate reduction
BAs7	-	+
BAs8	+	-
BAs11	+	+
BAs19	+	-
BAs29	+	+

5.4 Discussion

According to differences in morphological characteristics i.e. visible physical appearance and growth of the isolated colonies, the arsenic-resistant bacterial isolates in this study could be grouped in 2 different morphotypes. The four isolates, BAs7, BAs8, BAs19, and BAs29 belong to the same phenotype and only one isolate, BAs11 was in separated morphotype. The morphotype results are in consistent with 16S rRNA gene analysis. The same morphotype results in similar 16S rRNA gene sequences. Based on phylogenetic analysis of 16S rDNA sequence, the arsenic-resistant bacterial isolated were grouped into two distinct genera, *Bacillus* (BAs7, BAs8, BAs19, and BAs29) and *Mycobacterium* (BAs11)., Based on the phylogenetic analysis, various genera of the arsenic-resistant bacteria e.g. *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Enterobacter* and *Comamonas* (Suresh *et al*., 2004; Shivaji *et al*., 2005; Krumova *et al*., 2008; Chitpirom *et al*., 2009; Jareonmit *et al*., 2012) could be obtained from arsenic contaminated soils.

Isolate BAs11 was identified as *Mycobacterium* sp. (99% similarity to *Mycobacterium neoaurum*). *Mycobacterium neoaurum* are naturally found in various arsenic contaminated environments such as soil, fresh cow manure and compost (Hruska and Kaevska, 2012; Padya *et al*., 2015; Zhai *et al*., 2017). Although several species of *Mycobacterium* are known to be pathogenic to humans however *Mycobacterium neoaurum* infection has rarely been found in humans, and only a limited number of cases have been reported (Morimoto, 2007). Methylation is regarded as one of the main detoxification pathways for arsenic (As) and *Mycobacterium neoaurum* had been reported to demethylate several arsenic species (Lehr, 2003) suggesting its ability to tolerate arsenic in a specific environment.

Studies have shown that the genera of gram positive bacteria particularly the *Bacillus* genus, often exhibited high resistance to arsenic. Our results are in agreement with the previous studies that the gram-positive bacteria including most of the *Bacillus* strains appeared to tolerate high concentrations of arsenic (Suresh *et al*., 2004; Shivaji *et al*., 2005; Tantry, 2015; Alaniz-Andrade *et al*., 2017). In our study all the strains are gram positive and short rod shaped. The high percentage of Gram-positive bacteria may reflect the toxicity of arsenic, which may result in high selection pressure for Gram-positive organisms. Once inside a cell, As(III) disrupts protein folding and protein-DNA interactions. Gram-positive cells have a thicker cell wall compared to Gram-negative cells. The thicker cell wall might make it more difficult for As(III) to enter the cell (Norman 1998; Alaniz-Andrade *et al*., 2017)

Studies have reviewed that bacterial strains can resist the toxicity of arsenic using various cellular and metabolic mechanisms such as active cellular transportation of the toxic material out of the cellular environment and oxidation-reduction reaction (Mukhopadhyay *et al*., 2002; Silver and Phung, 2005). The arsenic resistant bacteria perform oxidation-reduction reaction using specific enzymes, arsenic oxidase and arsenic reductase to oxidize or reduce arsenic. (Banerjee *et al*., 2011). In the present study, we evaluated the ability of the resistant isolates to reduce and oxidize Na-As(III) using $AgNO_3$ solution. The $AgNO_3$ test of the five isolates revealed that the two highest arsenic-resistant (BAs11 and BAs29) exhibited both arsenic oxidizing and reducing abilities. The results implied that the two isolates could produce both arsenite oxidase and arsenate

reductase enzymes thus effectively control the toxicity levels of arsenite. Isolate BAs8 and BAs19 were able to produce only arsenite oxidase enzyme while sensitive isolate BAs7 produce only arsenate reductase enzymes.

5.5 Conclusion

Phylogenetic analysis based on 16S rRNA gene sequences indicated that the five selected isolates, representing two major bacterial genera; *Bacillus* and *Mycobacterium*. Isolates BAs7 and BAs19 showed 99% similarity to *Bacillus stratosphericus*, BAs8 showed 98% similarity to *Bacillus pumilus*, BAs 29 showed 98% similarity to *Bacillus altitudinis* and BAs11 showed 99% similarity to *Mycobacterium neoauurum*. A qualitative silver nitrate ($AgNO_3$) indicated that isolates BAs11 and BAs29 exhibited both arsenite oxidizing as well as arsenate reducing abilities suggesting their ability in producing both arsenite oxidase and arsenate reductase enzymes. BAs8 and BAs19 showed only the ability to oxidize arsenite. A sensitive isolate, BAs7 was not able to oxidize arsenite and could only reduce arsenate. The ability to reduce arsenate. The resistant isolates capable of producing arsenite oxidase and arsenate reductase could be applied as the promising microorganisms for bioremediation, as an alternative to existing physico-chemical methods of arsenic removal.

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