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Full Length Research Paper

Arsenic, boron and salt resistant *Bacillus safensis* MS11 isolated from Mongolia desert soil

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Arsenic (As) is a toxic metalloid, having both properties of a metal and a non-metal. Boron (B) is a non-metal, essential micronutrient for plants growth and development but its excess can be toxic to plants with various levels. In this study arsenic, boron and salt resistant bacteria were isolated from desert soil, Mongolia. The bacterial screening was carried out by serial dilution method. One hundred colonies were screened initially using of (2 mM) arsenic and (2 mM) boron containing LB agar medium. From this population, 10 bacterial isolates were selected based on arsenic and boron resistance, boron accumulation, salt tolerance and arsenic oxidizing capability. One of the potent strain, MS 11 from Mongolia desert soil, was tentatively identified as *Bacillus* sp. The phylogenetic and comparative analysis of 16S rRNA gene sequence with closely related validly published species available in the database showed that the isolate MS11 was closely related to *Bacillus safensis* FO-036b(T) with the highest sequence similarity (99.439%). The 16S rRNA gene sequence of the strain MS11 was submitted in the NCBI database with the accession number JF836885. *B. safensis* MS11 exhibited high level of resistance to arsenite (40 mM), arsenate (400 mM), boron (200 mM) and 15% salt tolerance in LB agar medium. *B. safensis* MS11 was also associated with resistance to multiple heavy metals such as Cd, Cr, Cu, Ni, Pb and Zn. Hence, this bacterium could be useful in the remediation of salt affected soils and biogeochemical cycles of arsenic pollution.

Key words: Desert soil, toxic metals, *Bacillus*, salt tolerant, bioremediation.

INTRODUCTION

Deserts are characterized by lack of moisture as a result of which biological activities are regulated by ephemeral water availability. However, total water availability is defined by the interaction of precipitation, temperature, evaporation and evapotranspiration. Based on the vegetative cover and total precipitation, arid ecosystems are called desert, semidesert, steppe, subdesert, semiarid or arid grasslands. Deserts are composed of sand that is larger and rounder than the sea beach sand (Bhatnagar and Bhatnagar, 2005). Mongolia is located in East and Central Asia and bordered by Russia to the north and China to the south, east, and west. Mongolia has a varied geography with regions such as the Gobi Desert in the south, and the cold and mountainous regions in the north and west (Okutani et al., 2011).

Microbial activity in desert soils is highly dependent on characteristics such as temperature, moisture and the availability of organic carbon (Buyanovsky et al., 1982; Parker et al., 1983, 1984). Arsenic (As) is a widespread contaminant in the environment. Arsenic is widely used in pesticide and wood preservative manufacturing, and is present in mining residues. Arsenic could also be found in nature, due to volcanic activity, forest fires and other natural phenomena in soils, and surface and ground water (Donahoe et al., 2004; Mandal and Suzuki, 2002). It exists mainly in two inorganic forms, trivalent As (III) and pentavalent As(V) (Jackson et al., 2003). Its contamination around the world include the historical use of As containing insecticides, herbicides and fungicides, mining and smelting, and the use of copper chrome arsenate as a wood preservative (Smith et al., 2003; Rahman et al., 2006). Microorganisms are known to play an important role in the biochemical cycling of arsenic, through its

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conversion to species with different solubility, mobility, bioavailability and toxicity (Silver and Phung, 2005). Arsenic resistant bacteria have been reported from contaminated sediments. Some species of *Aeromonas*, *Bacillus* and *Pseudomonas* were found to be highly resistant to both As (III) and As (V) (Pepi et al., 2007; Yamamura et al., 2007). Arsenic oxidizing bacteria could play a significant role in both arsenic oxidation and mobilization, which could be potentially used in bioremediation of polluted soils and water.

Boron is a member of the subgroup III of metalloids and has intermediate properties between metals and non-metals (Marschner, 1995). Boron exists in the environment mainly through the weathering of rocks, boric acid volatilization from seawater, and volcanic activity (WHO, 1998). Boron is also released from anthropogenic sources to a lesser extent. Anthropogenic sources include agricultural refuse and fuel wood burning, power generation using coal and oil, glass products manufacture, use of borates/perborates in the home and industry, borate mining/processing, leaching of treated wood, paper, and sewage sludge disposal. Many of these sources are difficult to quantify (WHO, 1998). Although boron plays important roles in many plant metabolic pathways (Lou et al., 2001) as an essential micronutrient (Marschner, 1995), however, high concentrations of boron are toxic for plants and other living cells. Boron exposure has been discussed as a potential cause of chronic kidney disease in Southeast Asia (Pahl et al., 2005). Recently boron resistant bacteria were characterrized from Turkey soil (Ahmed et al., 2007a, b, c) and the University of Tokyo experimental field soil (Miwa et al., 2008, 2009; Yoon et al., 2010). In this study, we identified and characterized the arsenic, boron and salt resistant bacterial strains isolated from desert soil of Mongolia.

MATERIALS AND METHODS

Sample collection and isolation of arsenic and boron resistant bacteria

The desert soil samples were collected from Mongolia. The characteristics of Mongolia soil was carried out by NITON XL3t XRF analyzer. For bacterial isolation, the samples were collected separately in sterile plastic sheets, stored at 4°C and used for further studies. For isolation and enumeration of arsenic and boron resistant bacteria, soil sample was serially diluted in sterile distilled water and plated on Luria–Bertani (LB) agar individually supplemented with 2 mM level of (As III) sodium arsenite ($NaAsO_2$) and 2 mM of boron (H_3BO_3). Plates were incubated at 30°C for 72 h. Resistant colonies differing in morphological characteristics were isolated in pure form and used for further studies.

Determination of arsenic resistance

Maximum resistance of selected 10 isolates against As (III) and As (V) was evaluated until the strains were unable to grow on LB agar medium. The initial concentration (4 mM) was added using 1 M stock solutions. The stock solutions of sodium arsenite ($NaAsO_2$) and sodium arsenate (Na_2HAsO_4) (Wako chemicals, Osaka, Japan) was prepared in sterilized deionized water. The growing colonies at a given concentration were subsequently transferred to the next higher concentration. Based on the evaluation minimum inhibitory concentration (MIC) of As (III) and As (V) was determined after three days incubation at 30°C.

Determination of boron resistance

Boron resistance of 10 isolates was carried out on LB agar medium supplemented with different concentration of boron. The concentration range of boron was selected from 4 to 250 mM. The working concentrations of boron were prepared from 1 M stock solution of boric acid (Wako chemicals, Osaka, Japan). The stock solution of boron was prepared in sterilized deionized water. Minimum inhibitory concentration (MIC) was evaluated until the selected isolates were unable to grow on boron containing LB agar medium. Based on this analysis, MIC was determined at 30°C in three days.

Arsenic transformation assay

The transforming ability of 10 isolates was carried out by using $AgNO_3$ method (Simeonova et al., 2004). Agar plates were flooded with a solution of 0.1 M $AgNO_3$. A brownish precipitate revealed the presence of arsenate in the medium (arsenite oxidizing bacteria), while the presence of arsenite was detected by a bright yellow precipitate (arsenate reducing bacteria) (Rahman et al., 2006). The transforming ability was also carried out in liquid medium. The bacterial suspensions were incubated at room temperature for 72 h. Subsequently, the bacterial cultures were centrifuged, and 100 µL of the liquid phase was mixed with 100 µL of a 0.1 M $AgNO_3$ solution. The resulting precipitates containing arsenic were from light yellow due to As (III) to light brown red due to As (V) (Liao et al., 2011).

Determination of other heavy metals resistance

The heavy metal resistance of 10 isolates was carried out in LB agar medium. The different working concentrations of heavy metals like cadmium as $CdCl_2$, chromium as $K_2Cr_2O_7$, copper as Cu SO_4, lead as $(CH_3COO)_2$ $Pb.3H_2O$, nickel as $NiCl_2$, and zinc as $ZnCl_2$, were prepared using 1 M stock solutions (Sigma-Aldrich, USA; Wako chemicals, Osaka, Japan). The initial concentration 1 mM was used for all the heavy metals. The culture growing on the last concen-tration was transferred to the higher concentration by streaking on the plates. Minimum inhibitory concentration (MIC) was noted when the isolates were failed to grow on the plates even after three days of incubation at 30°C.

Salt tolerance

The salt tolerance of selected MS isolates was determined in LB agar supplemented with NaCl. The growth was monitored after 72 h incubation at 30°C.

Estimation of boron accumulation

The bioaccumulation of boron was carried out using 10 isolates (resting cells) grown in 2 ml LB medium supplemented with 0.8 mM of boron and incubated at 30°C for seven days. After incubation, sample were collected and centrifuged at 13,000 rpm for 10 min. The pellet was air dried and used for boron analysis. The boron present in the dried pellets was determined by a HACH DR2800

Table 1. X-ray fluorescent analysis of Mongolia desert soil.

Component	ppm / % level
Ag	90.951
Ba	685.981
Ca	0.882
Cs	77.774
Cu	12.158
Fe	1.784
K	1.584
Mn	326.867
Ni	44.202
Pb	37.062
Rb	81.605
Sb	50.360
Sc	83.662
Sn	32.655
Sr	323.029
Te	153.144
Th	1.818
Ti	2155.726
Zn	22.446
Zr	102.815

spectrophotometer.

Selection of potent strains

Based on the growth performances in As (As III and V), boron and other heavy metals supplemented media, boron accumulation and salt tolerances, one of the potent resistant strain MS11 was selected for several biochemical and physiological characteristics. Colony morphology was observed on LB agar plates. The shape and colour of the colony was examined under the microscope after gram straining. The gram stain was determined using NEAT STAIN Gram stain kit (Polysciences, Inc. USA). Strain Ms11 was also differentiated (Gram negative or positive bacteria) by its growth on MacConkey agar (Wako Chemicals, Osaka). Ability to ferment glucose, lactose and sucrose and to produce H_2S was tested by triple sugar iron (TSI) agar (Wako Chemicals, Osaka). Strain Ms11 was biochemically analyzed for the activities of catalase, motility, indole production, starch and casein hydrolysis, nitrate reduction and citrate utilization. These tests were used to identify the isolate according to Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986). The strain was grown in LB medium, with varying pH values, 5, 6, 7, 8 and 9, and then incubated at 25, 30, 37 and 40°C. The optical density of the growing cultures in all the above-mentioned conditions was observed at 600 nm using a spectrophotometer to determine the optimum growth.

Polymerase chain reaction (PCR) amplification

Genomic DNA was isolated from strain Ms11 by QIAamp DNA mini kit (Qiagen, Hilden, Germany). Amplification of 16S rRNA was carried out by using the universal bacterial 16S rRNA primers, 27 F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1429 R 5'-GGT TACC TTG TTA CGA CTT-3' in thermal cycler under the following cyclic conditions as follows: 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. Polymerase chain reaction was performed in Gene AMP PCR Applied Biosystems. PCR product was analyzed in 1.0% agarose gel electrophoresis.

16S rRNA sequencing, alignment and phylogeny

The 16S rRNA gene sequencing of the strain MS11 was carried out in PRISM 3100 genetic analyzer and a dye-labelled terminator sequencing kit (Applied Biosystems, Foster, CA, USA) by direct sequencing of the PCR-amplified 16S rRNA gene. The sequences obtained were compiled and compared to the sequences in the Genbank databases using BLAST (Altschul et al., 1997) and megaBLAST (Zhang et al., 2000) programs against the database of type strains with validly published prokaryotic names (Chun et al., 2007). The 30 sequences with the highest scores were then selected for the calculation of pair-wise sequence similarity using global algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2007) and phylogenetic tree was created in MEGA5 (Tamura et al., 2004).

RESULTS AND DISCUSSION

The desert soil sample collected from Mongolia and physiochemical analysis was carried out by X-ray fluorescent method (Table 1). Initially hundred colonies were screened using 2 mM As (III) and 2 mM boron containing LB agar. The pure colonies were further subjected to screening for arsenic resistances ranging from arsenite (2 to 45 mM) and arsenate (10 to 400 mM). The concentration of arsenic resistance (As (III) and As (V)) tested in this study are higher compared to those used in the previous studies. Jackson et al. (2003) reported arsenic resistant levels of 400 and 10 mM of arsenate and arsenite, respectively. Macur et al. (2004) reported that arsenate resistance level of 0.25 mM was tested from estuarine samples. Anderson and Cook (2004) obtained one isolate able to grow at 100 mM arsenate, was isolated from arsenic contaminated gold mine tailings, but the majority of their isolates showed growth inhibition at lower concentrations. Therefore, based on high arsenic tolerance, 10 isolates were selected and designated as MS3, MS5, MS6, MS7, MS8, MS9, MS10, MS11, MS14 and MS15, which have been further studied. One of the isolates MS 11 exhibited high resistance to As III (40 mM) and As V (400 mM) compared to other MS isolates (Table 2). Resistance to As was defined as the ability to grow on agar containing either 7 mM As(III) or 20 mM As(V) at 25°C (Rokbani et al., 2007). The selected 10 isolates were arsenic oxidizing bacteria confirmed by silver nitrate test (Table 2). Three of the four arsenite resistant isolates were arsenic oxidizing bacteria under aerobic condition isolated from Atacama Desert, Northern Chile (Campos et al., 2011). Arsenite oxidation and arsenate reduction under aerobic conditions has been reported as detoxification mechanisms in several aerobic

Table 2. Arsenic resistance isolates from Mongolia desert soil.

Isolate /MI	As (III)	As (V)	As transforming ability
MS3	10	400	Oxidizing bacteria
MS 5	18	400	”
MS 6	18	400	”
MS 7	10	300	”
MS 8	10	400	”
MS 9	18	400	Oxidizing bacteria
MS 10	18	150	”
MS 11	40	400	”
MS 14	18	100	”
MS 15	18	100	Oxidizing bacteria

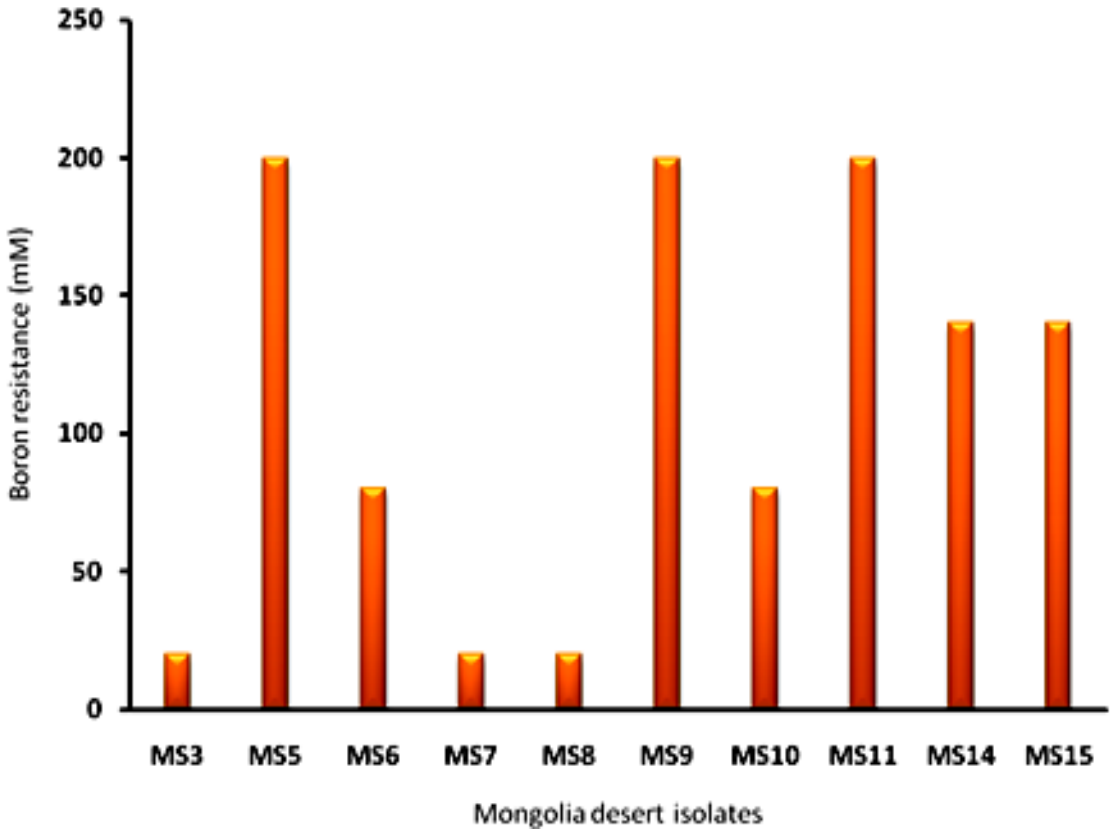

Figure 1. Boron resistance of Mongolia desert isolates determined in agar medium.

bacteria isolated from different arsenic contaminated sites (Jones et al., 2000; Macur et al., 2001), suggesting that arsenic (arsenite/arsenate) resistance plays an important role in the biogeochemical cycling of this element in nature (Inskeep et al., 2007). The isolates MS5, MS9 and MS11 exhibited 200 mM boron resistance, while in contrast, MS3, MS7 and MS8 showed 20 mM boron resistance (Figure 1).

Various levels (up to 450 mM) of boron tolerances have been reported previously for *Bacillus boroniphilus*, *Gracilibacillus boraciitolerans*, *Chimaerecicella boritolerans*, *Lysinibacillus boronitolerans*, and *Lysinibacillus parviboronicapiens* (Ahmed et al., 2007 a, b, c, d; Miwa et al., 2009). In this study, boron accumulation was carried out using MS isolates grown in 2 ml LB medium supplemented with 0.8 mM of boron. The MS isolates accumulated boron concentration ranging from 0.3 to 2.6 mg/L from dry weight (Figure 2). The highest boron accumulation was detected by MS6, MS9 and MS11 when compared to other isolates. The variation of

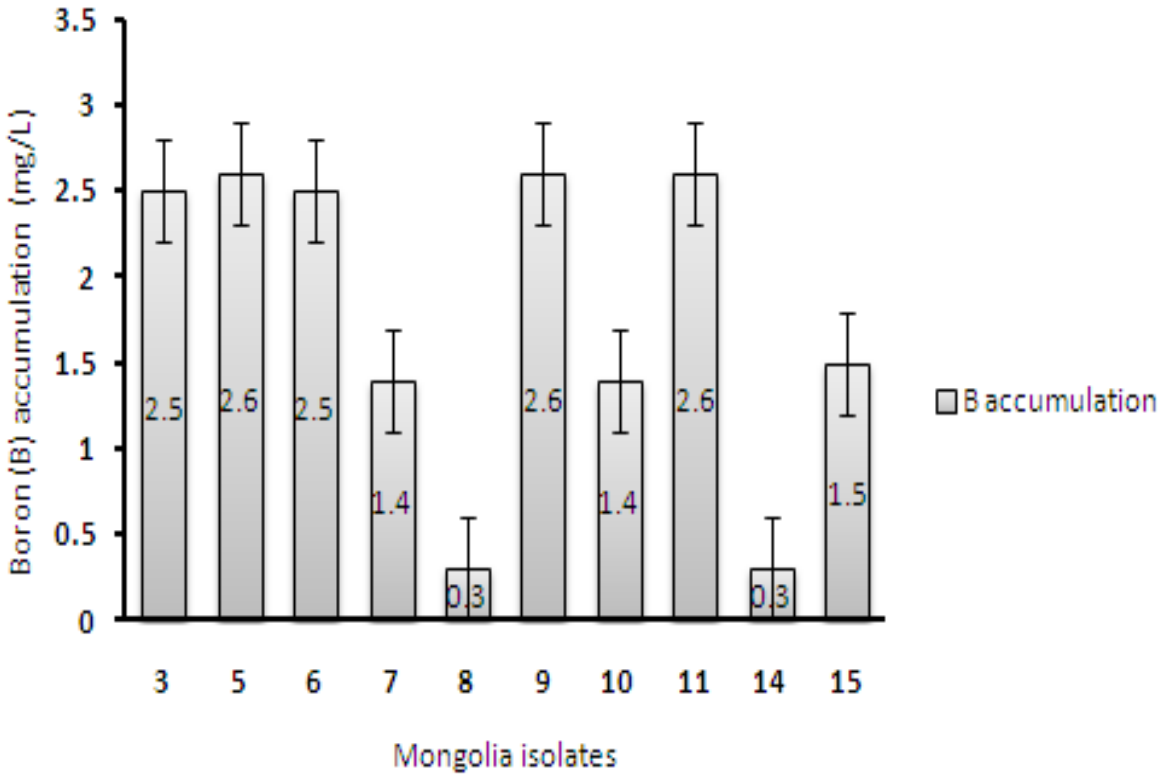

Figure 2. Boron accumulation of Mongolia desert isolates estimated by Carmine powder pillow method.

Table 3. Determination of heavy metal resistances by Mongolia desert isolates.

Isolate	Cd	Cr	Cu	Pb	Zn	Ni
MS3	-	-	-	4	2	-
MS 5	2	4	2	4	2	-
MS 6	-	4	-	4	2	-
MS 7	-	4	-	4	2	-
MS 8	-	4	-	6	5	-
MS 9	2	2	2	4	2	-
MS 10	2	2	2	6	2	-
MS 11	**2**	**6**	**4**	**9**	**10**	**3**
MS 14	2	4	2	8	5	-
MS 15	2	4	2	8	5	-

High resistance represented in bold and underline.

boron accumulation by MS isolates may reflect the utilization of boric acid, membrane composition, transport mechanisms and protein and organic compounds that can bind to boric acid (Miwa and Fujiwara, 2009). The order of heavy metals resistance by MS isolates may be found to be Zn>Pb>Cr> Cu>Cd, respectively (Table 3).

All MS isolates were sensitive to Ni except MS11. The maximum resistance to Zn and Pb was determined by MS11. The MS isolates were extremely resistance to As III (40 mM), As V (400 mM) and B (200 mM), respectively. Previously, mercury, arsenic and boron resistance bacteria were screened from geothermal area in Italy (Baldi et al., 1995). The MS isolates showed that salt tolerances ranging from 4 to 15% was determined in LB agar medium (Figure 3). Halophilic or halotolerant spore-forming gram-positive rod-shaped bacilli are a diverse phylogenetic group of bacteria which have adapted to life at the lower range of salinities and with the possibilities of rapid adjustment to changes in the extreme salt concentration (Ventosa et al., 1998). One of the potential strains showing high degree of resistance to arsenite (40 mM), arsenate (400 mM), boron (200 mM), boron accumulation

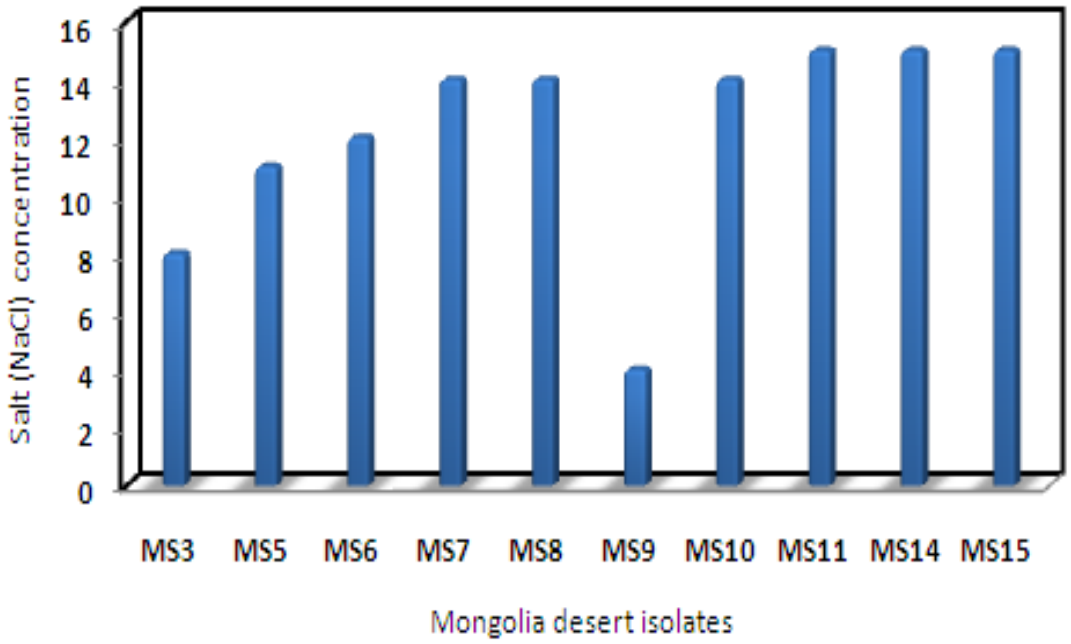

Figure 3. Salt tolerance of Mongolia desert isolates determined in LB agar medium.

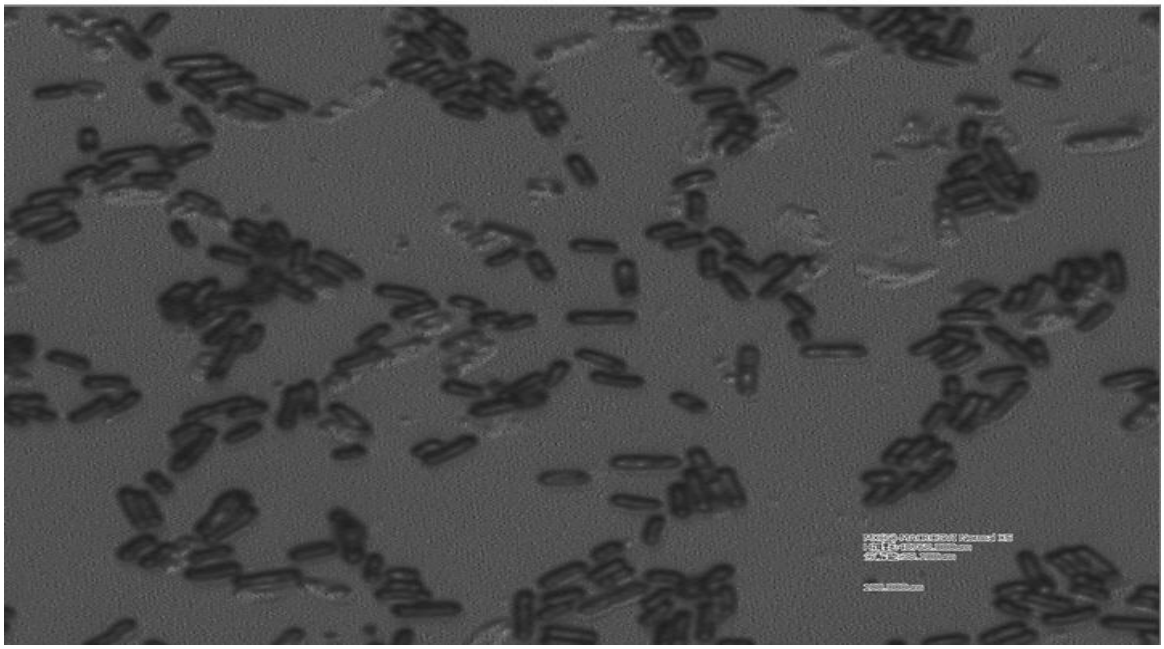

Figure 4. Microscopic view of strain MS11.

potential (2.6 mg/L) and salt tolerance (15%) was selected for further studies. The strain MS11 was a gram positive, rod shaped motile bacterium forming white colonies (Figure 4). The strain positive for catalase, utilize citrate and showed optimum growth at 30°C and pH 7. The morphological, physiological and biochemical characteristics of strain MS11 is shown in Table 4. The phylogenetic analysis based on 16S rRNA gene sequence clearly indicated that strain MS11 belongs to the genus *Bacillus.* Comparative analysis of 16S rRNA gene sequence with closely related validly published species available in the database showed that the isolate MS11 was closely related to *Bacillus safensis* FO-036b (T) (AF234854) with the highest sequence similarity

Table 4. The morphological and biochemical characteristics of strain MS11.

Characteristic	Strain MS11
Colony colour	White
Colony morphology	Rod
Motility	Motile
Gram reaction	Positive
Catalase	+
Indole production	_
Starch hydrolysis	_
Casein hydrolysis	_
Citrate Utilization	+
NaCl tolerance (%)	15
Optimum growth temperature	30℃
pH	7
TSI agar	
Glucose fermentation	_
Lactose	_
Sucrose	_
H2S production	_
Nitrate reduction	_
MacConkey agar	_
Pseudomonas agar	_

+, Growth; -, no growth.

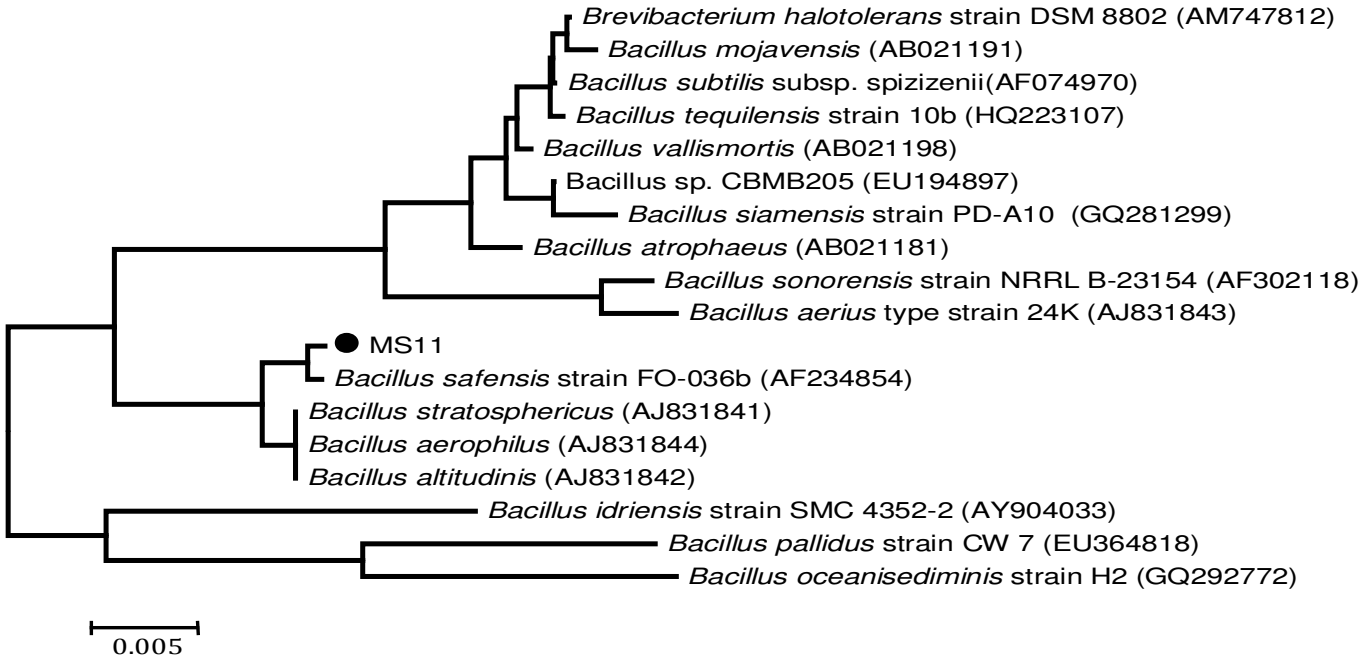

Figure 5. Phylogenetic tree of strain MS11 and related species from Genbank based on 16S rRNA sequences. The tree was generated using neighbour-joining method. The scale bar represents the unit length of numbers of nucleotide substitutions per site.

(99.439%) (Figure 5). The 16S rRNA gene sequence of the strain MS11 was submitted in the NCBI database with the accession number JF836885.

Conclusion

Arsenic (arsenite and arsenate) and boron resistant

bacteria were isolated from Mongolia desert soil. The MS isolates were arsenic oxidizing bacteria and their arsenic transforming ability was confirmed by silver nitrate test. One of the bacterium, *B. safensis* strain MS11, showed high resistance against arsenite (40 mM), arsenate (400 mM) and boron (200 mM). The strain MS11 also showed resistance against other heavy metals resistance such as Cd, Cr, Cu, Pb, Ni and Zn as determined on an agar medium. In addition, MS11 exhibited high boron accumulation potential (2.6 mg/L) and tolerance to NaCl salt (15%). Therefore, the arsenic resistance (arsenite and arsenate), oxidation characteristics, boron accumulation potential and salt tolerance of *B. safensis* MS11 could be useful in the bacterial remediation of salt affected soils and biogeochemical cycles of arsenic in the near future.

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