Biodegradation (2012) 23:803–812
DOI 10.1007/s10532-012-9567-4

ORIGINAL PAPER

Arsenic bioremediation potential of a new arsenite-oxidizing bacterium *Stenotrophomonas* sp. MM-7 isolated from soil

Md. Mezbaul Bahar · Mallavarapu Megharaj · Ravi Naidu

Received: 10 April 2012 / Accepted: 15 June 2012 / Published online: 4 July 2012

Abstract A new arsenite-oxidizing bacterium was isolated from a low arsenic-containing (8.8 mg kg^{-1}) soil. Phylogenetic analysis based on 16S rRNA gene sequencing indicated that the strain was closely related to *Stenotrophomonas panacihumi*. Batch experiment results showed that the strain completely oxidized 500 μM of arsenite to arsenate within 12 h of incubation in a minimal salts medium. The optimum initial pH range for arsenite oxidation was 5–7. The strain was found to tolerate as high as 60 mM arsenite in culture media. The arsenite oxidase gene was amplified by PCR with degenerate primers. The deduced amino acid sequence showed the highest identity (69.1 %) with the molybdenum containing large subunit of arsenite oxidase derived from *Bosea* sp. Furthermore the amino acids involved in binding the substrate arsenite, were conserved with the arsenite oxidases of other arsenite oxidizing bacteria such as *Alcaligenes feacalis* and *Herminnimonas arsenicoxydans*. To our knowledge, this study constitutes the first report on arsenite oxidation using *Stenotrophomonas* sp. and the strain has great potential for application in arsenic remediation of contaminated water.

Keywords Arsenic · Arsenite oxidizing bacteria · Arsenite oxidase gene (*aox*) · Detoxification

Md. M. Bahar (✉) · M. Megharaj · R. Naidu
Centre for Environmental Risk Assessment and Remediation (CERAR), University of South Australia, Building X, Room X2-03, Mawson Lakes Boulevard, Mawson Lakes, SA 5095, Australia
e-mail: bahmm001@mymail.unisa.edu.au

Md. M. Bahar · M. Megharaj · R. Naidu
Cooperative Research Centre for Contamination Assessment and Remediation of Environment (CRC CARE), Mawson Lakes Boulevard, Mawson Lakes, SA 5095, Australia

Introduction

Arsenic is a semi-metallic element and widely distributed in nature with a crustal abundance of 0.0001 % (Nriagu 2002). It is primarily released into the environment through natural activities such as volcanic emissions, weathering of arsenic-bearing minerals, etc. and anthropogenic activities like mining, smelting and combustion of fossil fuels (Bhumbla and Keefer 1994). Arsenic concentrations in the numerous aquifers that exist worldwide range from less than 5 to 5,000 μg L^{-1} and its subsequent contamination of drinking water and food is now a major concern to human health globally (Smedley and Kinniburgh 2002; Mandal and Suzuki 2002). The World Health Organisation (WHO) established a guideline value for arsenic in drinking water of 10 μg L^{-1} (WHO 1993) but many developing countries including Bangladesh adopted 50 μg L^{-1} as the guideline value for economic reasons (Ng and Moore 2005). Arsenic exists in four oxidation states in nature:

arsine(−III), elemental arsenic(0), arsenite(+III) and arsenate(V). Arsenite [As(III)] and arsenate [As(V)] are the predominant inorganic forms found in environmental samples, and the former two occur rarely (Smedley and Kinniburgh 2002). Studies have shown that As(III) is much more toxic than As(V). Further, it is more difficult to remove from water due to its high magnitude solubility (Clifford 1990; Rhine et al. 2006).

The removal of arsenic from contaminated water is generally accomplished using conventional treatment methods including adsorption, adsorptive filtration, coagulation, membrane filtration, ion exchange and reverse osmosis (US EPA 2000). The conventional processes generally require an oxidation step to transform As(III) to As(V). However, oxidation via reaction with oxygen under atmospheric condition is extremely slow and hence, chemical oxidants such as chlorine, hydrogen peroxide and ozone are widely used as part of arsenic removal processes (Simeonova et al. 2005). These chemicals result in high costs and may produce many harmful by-products. The use of biological treatment, either in situ or ex situ, has gained significant attention during the last two decades due to its advantages over conventional treatment methods. Thus, microbial transformation of As(III) to As(V) could be an eco-friendly and cost effective alternative to conventional methods.

Microorganisms have developed high resistance to arsenic in the environment with some showing growth by driving energy from the oxidation of As(III) to As(V) (Santini et al. 2000; Garcia-Dominguez et al. 2009). In autotrophic As(III) oxidation, As(III) acts as the electron donor, whereas oxygen is used as the electron acceptor and carbon dioxide as the carbon source. For heterotrophic bacteria, the As(III) oxidation process is described as a detoxification mechanism catalysed by the enzyme- arsenite oxidase (Muller et al. 2003). Several bacterial strains have been isolated and identified as As(III) oxidizers from arsenic-rich environments and a very few of them are from low-stressed environments. This study reports on (i) isolation of a new As(III)-oxidizing bacterium from a low arsenic-containing soil, its phylogenetic analysis and evaluation of As(III) oxidation; and (ii) identification and characterization of arsenite oxidase gene and its potential for arsenic detoxification in the isolated strain.

Materials and methods

Isolation and culture condition of bacteria

A soil sample previously taken adjacent to a Lead Smelter Plant (Port Pirie, South Australia) was used as the inoculum for enrichment culture. The isolation and subsequent growth experiments were carried out with a modified Minimal Salts (MS) medium. Salts supplied per liter of medium were: 0.34 g KH_2PO_4, 0.06 g Na_2SO_4, 0.1 g KCl, 0.08 g $MgCl_2$, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.3 g KNO_3 and 0.2 g $(NH_4)_2SO_4$. Micronutrients and vitamins added from a concentrated stock, were (mg L^{-1}): *trace elements*—concentrated HCl (0.002 mL), $MnCl_2{\cdot}4H_2O$ (0.2), $CoCl_2{\cdot}6H_2O$ (0.24), $ZnCl_2$ (0.14), H_3BO_3 (0.12), $NiCl_2{\cdot}6H_2O$ (0.05), $CuCl_2{\cdot}2H_2O$ (0.03), $Na_2MoO_4{\cdot}2H_2O$ (0.025), $FeCl_2$ (1.9) and $Na_2SeO_3{\cdot}5H_2O$ (0.034); *vitamins* (mg L^{-1}): *p*-aminobenzoic acid (0.05), biotin (0.02), folic acid (0.02), nicotinic acid (0.05), thiamine HCl (0.05), pyridoxine HCl (B_6) (0.01), riboflavin (0.05), panthothenic acid (0.05) and cyanocobalamin (B_{12}) (0.001).

Enrichment cultures were established by adding 1 g of soil into 250 mL Erlenmeyer flask containing 100 mL fresh MS medium supplemented with 1 mM (equivalent to 75 mg L^{-1}) As(III) and glucose as the sole carbon source (0.5 %, w/v). The flasks were then incubated at 25 °C on a rotary shaker (130 rpm) for one week.

The enrichments were subcultured weekly into a fresh MS medium containing the same concentration of As(III). After the second subculture, the resulting enriched cultures were serially diluted and plated onto MS agar (1.5 %) medium containing 1 mM of As(III). Single colonies were picked and restreaked several times onto the same medium to obtain pure isolates. The obtained arsenite-resistant bacteria were then tested for their abilities to oxidize As(III) using a qualitative $KMnO_4$ screening method (Salmassi et al. 2002). Each isolated strain was inoculated into the MS medium containing 500 µM sodium arsenite and incubated on a rotary shaker at 25 °C for 48 h. For each isolate 500 µL culture was centrifuged at 10,000 rpm for 2 min. Nine micro liter of 10 mM $KMnO_4$ were added to the cell-free 300 µL culture medium. A pink coloured mixture indicated the presence of As(V) (which does not react with $KMnO_4$) and thus confirmed positive for As(III) oxidation. A clear or orange coloured solution indicated no As(III) oxidation. The sterile MS medium containing the same

amount of sodium arsenite served as an abiotic control. After confirmation of pure As(III)-oxidizing strains, they were preserved in 20 % glycerol medium at −80 °C and MS agar medium was used for routine maintenance.

Bacterial As(III) oxidation experiment

A batch test was executed to determine the As(III) oxidation ability of strain MM-7. The cells were grown in MS medium supplemented with 0.5 % glucose and 500 μM of As(III). Once the cells reached the exponential phase, they were harvested by centrifugation at 4,000 rpm for 20 min at 4 °C and the cell pellets were washed three times with 0.85 % NaCl and resuspended in the required volume of MS medium to obtain the optical density (OD_{600}) of 2.0. One percent of the concentrated inoculum ($OD_{600} = 2.0$) was inoculated into the MS medium containing 500 μM of As(III) and the cultures were incubated on a rotary shaker (130 rpm) at 25 °C in a walk-in incubator room. The initial cell density in the test medium was 1.5×10^7 cells mL^{-1} and the pH value of the medium was adjusted to 7.2 with 1 mM NaOH and 1 mM HCl. Controls without inoculation were also incubated under the same conditions. Samples were taken at different time intervals to determine the As(III) and As(V) concentration as well as to determine the bacterial growth in terms of optical density (600 nm). All experiments were done in triplicate, including controls and the mean values were taken into account.

Effect of pH and carbon source on As(III) oxidation

The effect of pH on bacterial oxidation of As(III) was assessed following the same procedure used in the batch oxidation experiment. For the effect of pH, the pH values of the culture medium were adjusted to 4, 5, 6, 7, 8, 9 and 10 with predetermined amounts of filter-sterilized NaOH and HCl. Samples were taken at different time intervals to determine the As(III) and As(V) concentrations as well as to determine the bacterial growth in terms of optical density (600 nm). The As(III) oxidation by strain MM-7 was also investigated with other carbon sources such as dextrose, fructose, maltose, sucrose and mannitol in the MS medium. The initial pH was adjusted to 7.0 ± 0.2.

Effect of As(III) on cell growth

The effect of As(III) on the growth of bacterium was evaluated to determine the strain's tolerance to arsenic using 48 h growth-inhibition bioassays. The 50 % effective concentration (EC_{50}) was defined as the initial As(III) concentration resulting in a 50 % decrease in growth when compared with no-As(III) control culture. It was determined by applying a non-linear regression model with a Four Parameter Logistic Curve, using the software SigmaPlot (version 10.0). Minimum inhibitory concentration (MIC) represented the lowest concentration, which completely inhibited the growth. Minimal salts medium amended with arsenic in concentrations 0.1–80 mM for As(III) was inoculated with cell suspensions to a final density of 1.5×10^7 cells mL^{-1}. A non-amended MS medium with arsenic served as the control.

Chemicals and analytical methods

All chemicals used in this study were of analytical grade, obtained from Sigma-Aldrich. All solutions were prepared with double deionized water (18 Ω cm^{-1}, Milli-Q, ELGA Labwater, UK) and were sterilized using either filtration or autoclaving. The As(III) stock solution was prepared from sodium arsenite ($NaAsO_2$) and stored at 4 °C in the dark.

Arsenic species were separated by high-performance liquid chromatography (Agilent 1100, Japan) equipped with a guard column and separation column (Hamilton PRP-X100) and quantified by inductively coupled plasma-mass spectrometry (Agilent 7500C, Japan). Prior to arsenic speciation analysis samples from the experimental culture medium were centrifuged at 10,000 rpm for 8 min using a microcentrifuge (Sigma, Germany) to remove cells. Supernatants were frozen until samples were analysed. Cell density was measured as optical density at 600 nm (OD_{600}) using a Microplate Reader (Synergy™ HT, Bio-Tek).

PCR amplification of 16S rRNA and *aoxB* gene

Genomic DNA was extracted using a genomic DNA prep kit (Bioline) following the manufacturer's instructions. The extracted DNA was used in PCR amplification as a DNA template. The polymerase chain reaction (PCR) amplification of both 16S rRNA and arsenite oxidase gene were carried out in a 50 μL

reaction mixture containing 100 ng DNA template, 1 μL of each forward and reverse primer (100 μM), 1 μL of $MgCl_2$ (50 mM) and 25 μL PCR mixture containing PCR buffer, dNTPS and *Taq* polymerase. The total volume was adjusted with highly purified sterile water. Universal eubacterial primers (E8F: 5′-AG AGTTTGATCCTGGCTCAG-3′; 1541R: 5′-AAGGA GGTGATCCANCCRCA-3′) (Lane 1991) were used for 16S rRNA amplification, whereas for the arsenite oxidase gene, primers were degenerate (69F: 5′-TG YATYGTNGGNTGYGGNTAYMA-3′; 1374R: 5′-TA NCCYTCYTGRTGNCCNCC-3′) (Rhine et al. 2007). The forward primer of arsenite oxidase gene, 69F, corresponds to nucleotide positions 69–86 of the large subunit of arsenite oxidase (*aoxB)* gene derived from the arsenite oxidizing bacterium *Agrobacterium tumefaciens* (Accession No. DQ151549). In contrast the reverse primer, 1374R corresponds to the nucleotides 1,356–1,374 of the same gene. All PCR amplifications were performed in the MyCycler thermal cycler (Bio-Rad). The PCR for 16S rRNA consisted of initial denaturation step at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extending to 72 °C for 1 min, with a final jump to 72 °C for 10 min. Contrasting this, for *aoxB* gene amplification the protocol was an initial denaturation step at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extending to 72 °C for 2 min, with a final jump to 72 °C for 10 min. The yields of genomic DNA and all PCR products were checked via gel electrophoresis using 1 % agarose gel with ethidium bromide at 80 V for 35 min and the specific bands were visualized under UV trans-illuminator (Bio-Rad, USA). The PCR products were purified using an UltraClean PCR clean-up kit (Mo-Bio Laboratories Inc, CA, USA).

Following this the purified PCR product of *aoxB* gene was ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) and the ligation products were transformed into *E. coli* DH5α competent cells. Then the transformants were grown on LB agar containing ampicillin, X-Gal and IPTG at 37 °C for 16 h according to the manufacturer's recommendations and the plasmid DNA was extracted using an ultraclean standard plasmid mini prep kit (Mo-Bio Laboratories Inc, CA, USA). Finally the clones were sequenced using the T7 and SP6 primers from the vector.

Sequencing and phylogenetic analysis

DNA sequencing analysis was performed using ABI 3130 Sequencer (Applied Biosystems, USA) at Southpath and Flinders Sequencing Facility, Flinders Medical Centre, Adelaide. The initial sequence analysis was done using BlastN (for 16S rRNA) and BlastX (for *aoxB*). Phylogenetic analyses were carried out with MEGA 5.0 using a neighbour-joining method (Kumar et al. 2004).

GenBank accession numbers

The nucleotide sequences of 16S rRNA and *aoxB* gene of isolate MM-7 have been deposited in GenBank under the accession numbers JN009768 and JQ739209, respectively.

Results

Phylogenetic identification of MM-7

Several hundred bacterial colonies were able to grow on MS agar medium containing As(III). Of these, three strains capable of As(III) oxidation were isolated and their oxidizing ability was assessed in additional experiments. All the isolated strains were characterized as aerobic and gram-negative. The BLASTN search result of the near full length 16S rRNA gene sequence (1,428 bp) of strain MM-7 showed that it belonged to the genus *Stenotrophomonas* and possessed 99.8 % similarity with *Stenotrophomonas panacihumi* (Fig. 1). The phylogenetic relationships among the As(III)-oxidizing bacteria (genus level) isolated so far are shown in Fig. 2. The As(III)-oxidizing bacteria are phylogenetically diverse and grouped into α-, β- and γ- *Proteobacteria* except *Thermus* sp. The isolate MM-7 was grouped with *Pseudomonas* sp. and both of them belonged to the γ-*Proteobacteria.*

Arsenic oxidation by MM-7 and the effect of pH and carbon sources

Arsenite oxidizing ability of the strain MM-7 was evaluated in batch tests employing an initial As(III) concentration of 500 μM. The sampling was conducted at different time intervals until complete

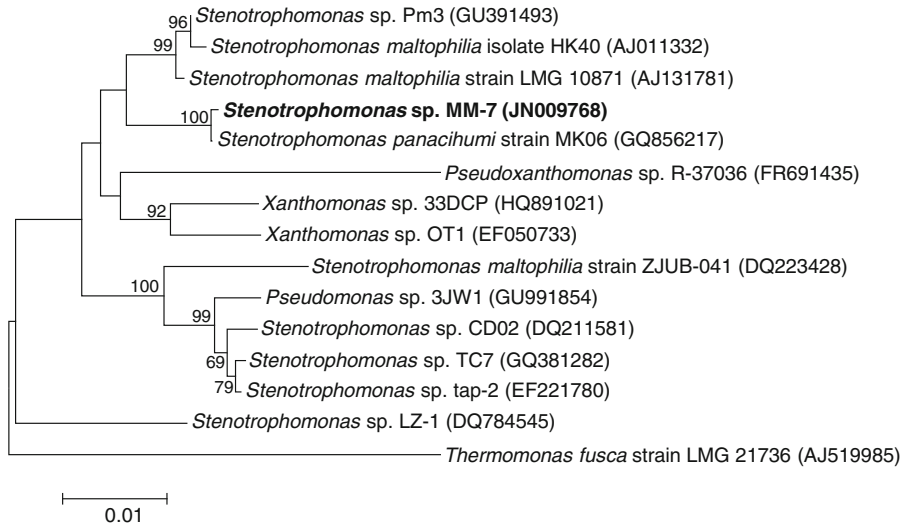

Fig. 1 Neighbour-joining tree showing the phylogenetic relationship of the isolated strain MM-7 compared with species belonging to the *γ-Proteobacteria*. The analysis included data from 1,428 unambiguous nucleotide positions. The bootstrap values (expressed as percentages of 1,000 replicates) above 60 % are shown at the branch points of the trees. The *scale bar* represents 1 nucleotide substitution per 100 nucleotides of 16S rRNA sequence

oxidation of As(III) in the culture medium was complete. The strain with initial cell density of 1.5×10^7 cells mL^{-1} was able to completely oxidize 500 μM As(III) (equivalent to 37.5 mg L^{-1}) within 12 h (Fig. 3). No growth was observed when the strain was cultured with As(III) as a sole energy source, indicating that it is a heterotrophic oxidizer.

The strain MM-7 was found to oxidize As(III) over a wide range of pH. Thus, complete oxidation of As(III) occurred within 12 h of incubation at pH ranging from 5 to 7 while it took 24 h for pH between 8 and 10 (Table 1). No growth as well as no oxidation of As(III) were observed at pH 4 even after 48 and 72 h of incubation (data not shown). The As(III) oxidation was fast when the strain was grown with glucose and maltose followed by fructose, mannitol, sucrose and dextrose (Table 1).

Effect of As(III) on cell growth

The effect of As(III) on the growth of MM-7 was evaluated prior to the As(III) oxidation experiments. The strain MM-7 was exposed to different concentrations of As(III) in MS medium and the growth in terms of cell density (OD_{600}) was measured at different time intervals. A decrease in growth was observed upon increasing the As(III) concentration at any given time interval compared to the control without As(III) amendment (Fig. 4). Thus there was nearly 31, 46 and 64 % decrease over control in the growth of the strain at 48 h incubation with 20, 30 and 50 mM As(III), respectively. At 60 mM As(III) although the growth declined initially but resumed after 48 h of incubation. This could be due to the very high toxicity at a higher concentration requiring an adaptive phase in the initial hours and subsequently entering a growth phase after 48 h. No growth was observed at 70 mM concentration of As(III) so the MIC would lie between 60 and 70 mM. The isolate MM-7 was found to be highly tolerant to As(III) with 48 h EC_{50} value of 34.51 mM (95 % confidence limits, 30.42–39.04 mM).

Sequencing and characterization of arsenite oxidase gene *aoxB*

Using degenerate oligonucleotide primers, a fragment of the expected size (about 1,200 bp) of the large subunit of arsenite oxidase was amplified from the genomic DNA of *Stenotrophomonas* sp. MM-7. The PCR product was then cloned and several clones were sequenced. The nucleotide sequence of the *aoxB* obtained from the isolate was translated and the deduced amino acid sequences (404 aa) showed clear homology to the *aoxB* proteins from different organisms. Alignment with different proteins showed the

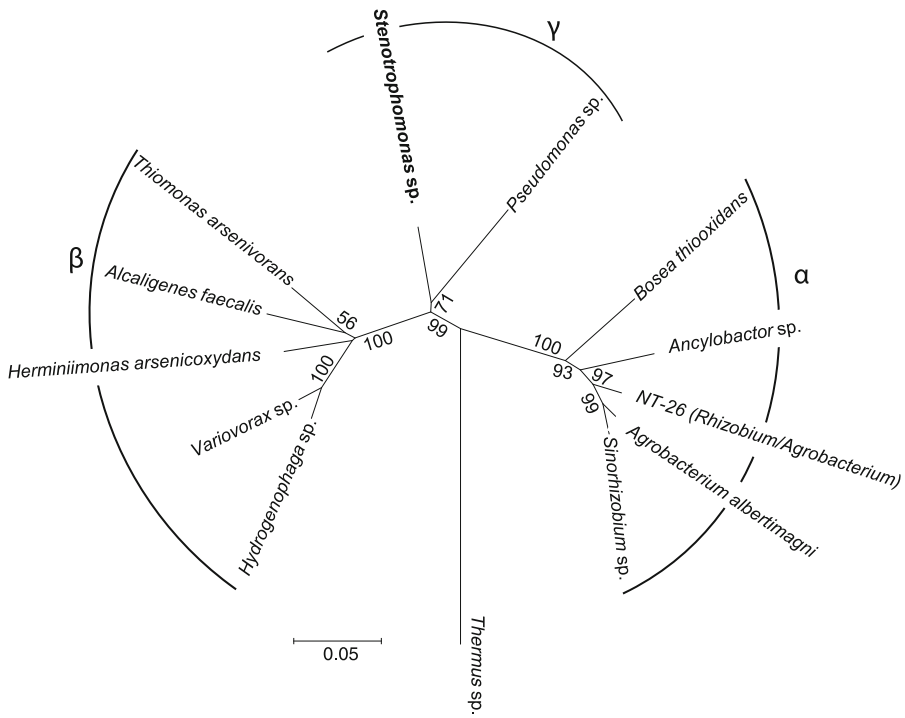

Fig. 2 Phylogenetic relationships among As(III)-oxidizing bacteria. The strain in each branch represents the respective genera. The bootstrap values (expressed as percentages of 1,000 replicates) are shown at the branch points of the trees. The *scale bar* represents 5 nucleotide substitutions per 100 nucleotides of 16S rRNA sequence. *α, β, γ* indicate the respective proteobacterial cluster. Strain and accession numbers of the species shown in this tree: *Hydrogenophaga* sp. (DQ986320), *Variovorax* sp. 34 (AF388028), *H. arsenicoxydans* ULPAs1 (NR043147), *A. faecalis* (HLE) (AY027506), *T. arsenivorans* b6 (AY950676), *Stenotrophomonas* sp. MM-7 (JN009768), *Pseudomonas* sp. VC-1 (FN645213), *B. thiooxidans* BI-42 (AF508803), *Ancylobactor* sp. OL1 (DQ986318), NT-26 (AF159453), *A. albertimagni* AOL15 (AF316615), *Sinorhizobium* sp. DAO10 (DQ336178), *Thermus* sp. HR13 (AF384168)

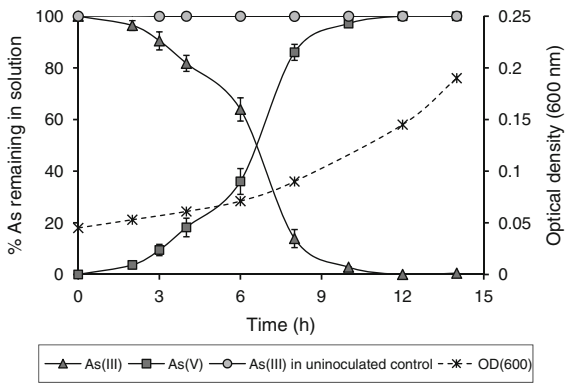

Fig. 3 Growth (expressed as OD_{600}) and As(III) oxidation by strain MM-7 in MS medium containing 500 μM As(III). *Data points* represent the averages of triplicate incubations. *Vertical bars* represent standard deviation

highest identity (69.1 %) with the molybdenum containing large subunit of arsenite oxidase (*aoxB*) from *Bosea* sp. WAO, involved in As(III) oxidation. It showed 52.2 % identity with the *aoxB* from *Alcaligenes faecalis*, in which the crystal structure of two subunits of arsenite oxidase enzyme was demonstrated by Ellis et al. (2001) along with complete sequence (*aoxA* 174 aa and *aoxB* 826 aa). The sequences were also 65 % identical to the arsenite oxidase (*AroA*) found in autotrophic As(III)-oxidizing bacteria NT-26. The large subunit of arsenite oxidase contains a molybdenum site, consisting of a molybdenum atom coordinated to two pterin cofactors, and a [3Fe−4S] cluster (Ellis et al. 2001). The motif, Cys-X2-Cys-X3-Cys-X70-Ser that binds the [3Fe−4S] cluster in the large subunit of *A. faecalis* arsenite oxidase is conserved in the *aoxB* of strain MM-7 (Fig. 5). Moreover, the four amino acids (His, Glu, Arg, and His) which were shown in *A. faecalis* to be involved in binding the substrate As(III), also appeared to be conserved in *aoxB* of strain MM-7 (His195, Glu203, Arg419, and His423).

Table 1 Amount of As(III) oxidation at different pH and carbon sources (mean ± SD)

	% As(III) oxidized	
	12 h	24 h
Initial pH		
4	0	0
5	100	100
6	100	100
7	100	100
8	46.9 ± 2.9	100
9	41 ± 3.3	100
10	24.5 ± 2.5	100
Carbon source (0.5 %)[a]		
Dextrose	24.8 ± 2.3	100
Fructose	46.9 ± 3.5	100
Glucose	100	100
Maltose	100	100
Sucrose	33.1 ± 2.9	100
Mannitol	40.7 ± 3.7	100

The initial As(III) concentration was 500 µM

[a] The initial pH was 7.0 ± 0.2

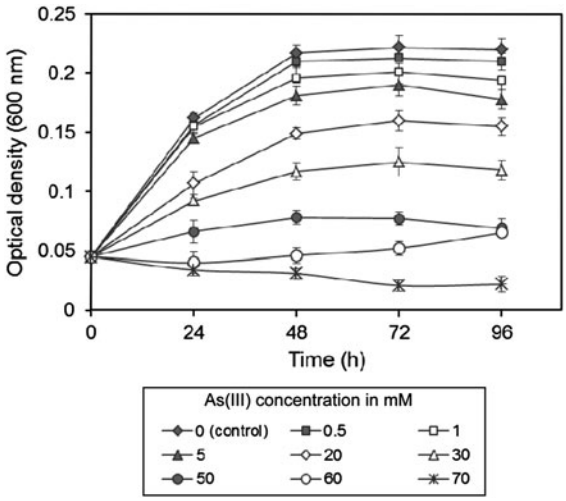

Fig. 4 Growth curves of strain MM-7 exposed to different concentrations of As(III) in MS medium. *Data points* represent mean ± SD

Discussion

Microbial oxidation of toxic As(III) has great potential as a preoxidation strategy in the treatment process of arsenic from contaminated water; it is emerging as superior to conventional chemical methods. Since the first report of the presence of As(III)-oxidizing bacteria in cattle dip soil (Green 1918), most of the bio-oxidizers have been isolated from highly arsenic contaminated environments such as gold and sulphur pyrite mine wastewater (Ilyaletdinov and Abdrashitova 1981), gold mines (Santini et al. 2000), aquatic environments (Weeger et al. 1999), hot creeks (Salmassi et al. 2002) and ground water (Liao et al. 2011). In this study, the arsenic oxidizing strain was isolated from a heavy metal contaminated soil but having very low levels of arsenic (8.8 mg kg^{-1} total and 59 µg kg^{-1} water soluble). This suggests a wide distribution of arsenic oxidizing bacteria in the environment.

Most identified As(III)-oxidizing bacteria reported in the literature belonged to the genera of *Agrobacterium* (Salmassi et al. 2002), *Alcaligenes* (Osborne and Ehrlich 1976), *Ancylobactor* (Garcia-Dominguez et al. 2009), *Bosea* (Liao et al. 2011; *Herminiimonas* (Weeger et al. 1999), *Hydrogenophaga* (Garcia-Dominguez et al. 2009), *Sinorhizobium* (Kinegam et al. 2008), *Thiomonas* (Dastidar and Wang 2009) and *Thermus* (Gihring and Banfield 2001). Some strains of *Stenotrophomonas* sp. have been isolated as arsenic resistant bacteria (Drewniak et al. 2007; Cai et al. 2009) as well as hyper-resistant to As(V) with reducing ability (*S. maltophilia* SA Ant 15, Botes et al. 2007). However, to our knowledge no As(III)-oxidizing strain of this group has been identified so far. The isolated strain MM-7 is thus the first report of As(III)-oxidizing bacterium from the genus, *Stenotrophomonas*. To date, no Gram-positive strains have been reported to either oxidize As(III) or to have a gene similar to arsenite oxidase gene.

Previous studies reported that the As(III)-oxidizing bacterium *Alcaligenes* sp. strain RS-19 completely oxidized 1 mM As(III) to As(V) within 40 h with a cell density of 10^7 cells mL^{-1} (Yoon et al. 2009), *Agrobacterium albertimagni* AOL15 oxidized 585 µM within 24 h (Salmassi et al. 2002), *Herminnimonas arsenicoxidans* strain ULPAs showed total oxidation of 1.33 mM As(III) within 2.5 h of incubation with a cell density of 10^9 CFU mL^{-1} (Weeger et al. 1999). In this study, only 1.5×10^7 cells mL^{-1} of strain MM-7 completely oxidized 500 µM As(III) to As(V) within 12 h of incubation in MS medium during the exponential growth phase. These results suggest that the new As(III)-oxidizing strain

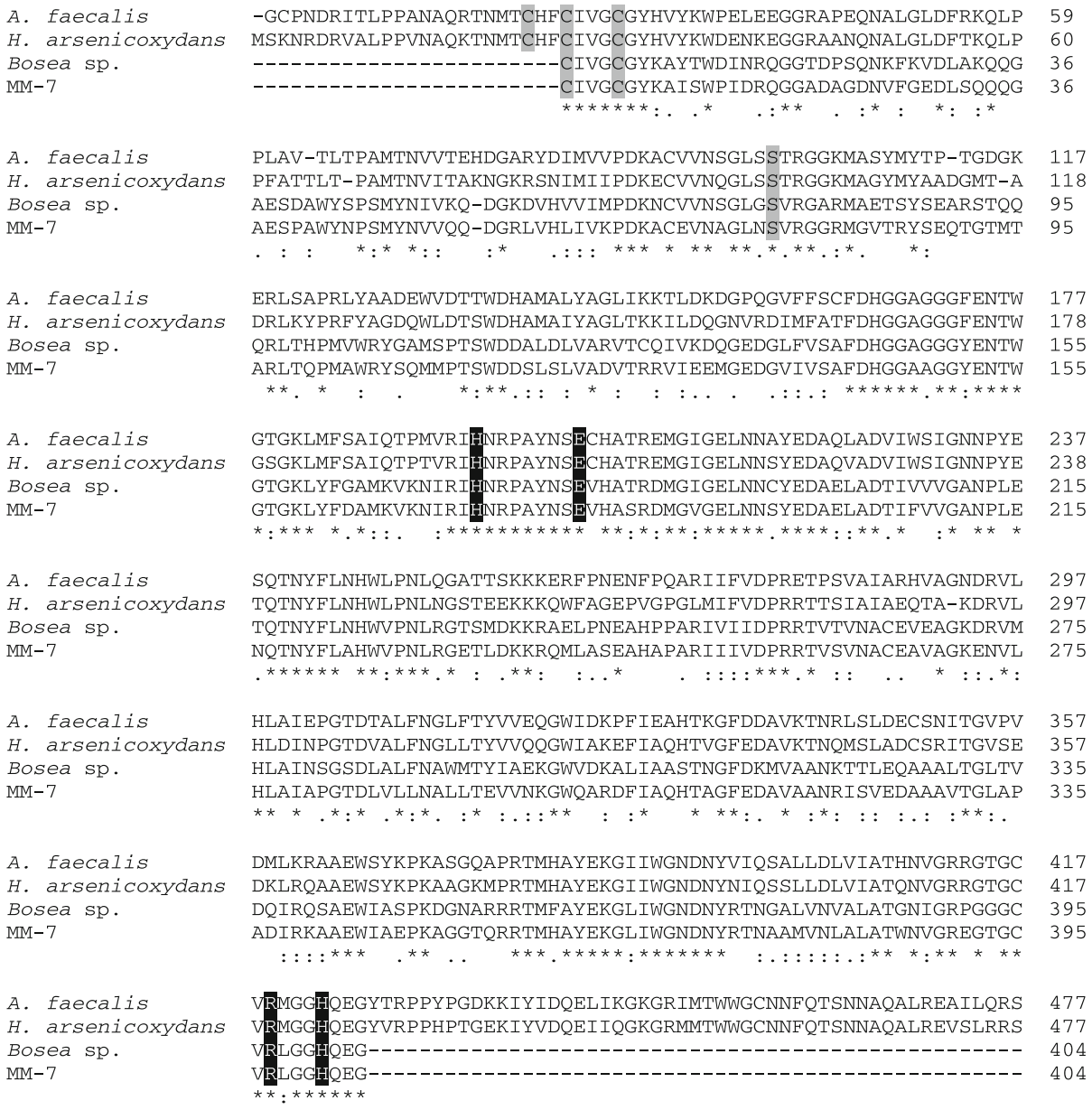

Fig. 5 Sequence alignment of the large subunit of the arsenite oxidases of *Alcaligenes faecalis*, *Herminiimonas arsenicoxydans*, *Bosea* sp. and the putative protein deduced from the DNA sequence of *Stenotrophomonas* sp. MM-7 *aoxB* gene. Residues involved in binding the Fe−S clusters are *grey shaded* and the amino acids interacting with the molybdenum centre (arsenite binding sites) are indicated by *white letters* on a *solid background*. *Asterisk* indicates identical amino acids, *colon* indicates conserved amino acids and *dot* indicates similar amino acids

Stenotrotrophomonas sp. MM-7 isolated in this study has a superior capacity of As(III) oxidation over other bacterial strains. The strain MM-7 could grow and oxidize As(III) in the MS medium containing dextrose, lactose, glucose, maltose, mannitol and sucrose as sole carbon source at pH 7 and at 25 °C. No autotrophic growth was observed in the absence of organic carbon source, even in the presence of As(III). Thus, the oxidation of As(III) does not suggest involvement in energy metabolism in this strain.

Microbial oxidation of As(III) occurs over a wide range of pH depending on the type of species. Some species are able to oxidize As(III) at low pH ($\leq$4), i. e. *Sulfolobus acidocaldarius* (Sehlin and Lindström 1992) and *Thiomonas arsenivorans* (Dastidar and Wang 2009). However, most of the known As(III)-oxidizing species demonstrated the optimum oxidation at the near neutral range of pH (Osborne and Ehrlich 1976, Salmassi et al. 2002; Suttigarn and Wang 2005). The optimum pH range for the oxidation of As(III) by *Stenotrophomonas* sp. MM-7 was found to be at 5–7. The longer time required for the complete oxidation at a higher pH range 8–10 could be due to the decrease in pH with the progress of As(III) oxidation and when it reached a near neutral range the oxidation was completed. Suttigarn and Wang (2005) also reported the optimum pH 7 for As(III) oxidation by *Alcaligenes faecalis* strain O1201, but Dastidar and Wang (2009) found the optimum pH to be 6 in the case of *Thiomonas arsenivorans* strain b6. Battaglia-Brunet et al. (2002) isolated an autotrophic As(III)-oxidizing population (CASO1) which exhibited significant As(III) oxidation activity between pH 3–8. Therefore, these differences in optimum pH value suggests that pH modification is important for different cultures in order to achieve the maximum As(III) oxidation in the bioremediation of As(III).

Metal tolerance to bacteria can be higher in complex organic growth media due to the reduced availability of metal ions which are chelated by organic ligands, when compared to the values found in minimal medium (Weeger et al. 1999). In the case of MM-7, the As(III) toxicity tests were conducted in MS medium with low metal binding capacity, thus the measured levels of metal tolerance can be considered to be realistic. The tolerance level of As(III) observed here with the *Stenotrophomonas* sp. MM-7 strain is close to the highest levels of tolerance in As(III)-oxidizing bacteria reported by Green (100 mM) and Turner (60 mM) (Green 1918; Turner 1949). The As(III) tolerance level of MM-7 is significantly higher than the previously reported arsenic-tolerant *Stenotrophomonas* strain (10 mM) (Botes et al. 2007). Therefore, the hyper-tolerant behaviour shown by the strain in this study without prior exposure to such high levels of As(III) suggests that this trait is genetically intrinsic.

Arsenite oxidase enzyme is composed of two subunits: firstly, the small subunit is a Rieske-type [2Fe−2S] cluster and secondly, the large subunit incorporates the molybdenum center and a [3Fe−4S] cluster. The large subunit of arsenite oxidase was similar to other members of the dimethylsulfoxide (DMSO) reductase family of molybdenum enzymes. It was, however, unique in having no covalent bond between the polypeptide and the Mo atom (Ellis et al. 2001). Consequently it was reported as being a new subgroup of the DMSO reductase family. The large subunit of arsenite oxidase gene of strain MM-7 was identified by PCR amplification using degenerate primers which had been later cloned and sequenced. As no autotrophic growth was observed with MM-1, this isolate oxidizes As(III) as part of a detoxification mechanism.

This paper describes the first evidence of As(III) oxidation and the presence of arsenite oxidase gene from the genus *Stenotrophomonas*. The strain was able to oxidize As(III) at an exceptionally high rate in MS medium. The best approach to remove As(III) is to oxidize it to As(V) first, which is less soluble and much easier to remove. Thus, this new strain could be an excellent candidate for application in the arsenic remediation processes.

Acknowledgments This study was conducted while the first author was receiving the International Postgraduate Research Scholarship (IPRS) supported by the Australian Government in collaboration with the Centre for Environmental Risk Assessment and Remediation, University of South Australia. The authors would like to thank Dr. Mohammad Rahman for technical assistance with the ICP analysis and Dr. Kannan Krishnan for his assistance with the molecular study.

References

Battaglia-Brunet F, Dictor M-C, Garrido F, Crouzet C, Morin D, Dekeyser K, Clarens M, Baranger P (2002) An arsenic(III)-oxidizing bacterial population: selection, characterization, and performance in reactors. J Appl Microbiol 93:656–667

Bhumbla DK, Keefer RF (1994) Arsenic mobilization and bioavailability in soils. In: Nriagu JO (ed) Arsenic in the environment. Part 1 Cycling and characterization. Wiley, New York, pp 51–82

Botes E, Heerden EV, Litthauer D (2007) Hyper-resistance to arsenic in bacteria isolated from an antimony mine in South Africa. S Afr J Sci 103:279–282

Cai L, Liu G, Rensing C, Wang G (2009) Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils. BMC Microbiol 9:4. doi:10.1186/1471-2180-9-4

Clifford D (1990) Ion exchange and inorganic adsorption. In: Pontius F (ed) Water quality and treatment, AWWA handbook. McGraw-Hill, New York, p 9

Dastidar A, Wang Y-T (2009) Arsenite oxidation by batch cultures of *Thiomonas arsenivorans* strainb6. J Environ Eng 135:708–715

Drewniak L, Styczek A, Sklodowska A (2007) Arsenic hypertolerant bacteria isolated from gold mine rocks biofilms. Adv Mater Res 20–21:576

Ellis PJ, Conrads T, Hille R, Kuhn P (2001) Crystal structure of the 100 kDa arsenite oxidase from *Alcaligenes faecalis* in two crystal forms at 1.64 and 2.03 Å. Structure 9:125–132

Garcia-Dominguez E, Mumford A, Rhine ED, Paschal A, Young LY (2009) Novel autotrophic arsenite-oxidizing bacteria isolated from soil and sediments. FEMS Microbiol Ecol 66:401–410

Gihring TM, Banfield JF (2001) Arsenite oxidation and arsenate respiration by a new *Thermus* isolate. FEMS Microbiol Lett 204:335–340

Green HH (1918) Description of a bacterium that oxidizes arsenite and one which reduces arsenate to arsenite from a cattle-dipping tank. S Afr J Sci 14:465–467

Ilyaletdinov AN, Abdrashitova SA (1981) Autotrophic oxidation of arsenic by a culture of *Pseudomonas* arsenitoxidans. Mikrobiologiya 50:197–204

Kinegam S, Yingprasertchai T, Tanasupawat S et al (2008) Isolation and characterization of arsenite-oxidizing bacteria from arsenic-contaminated soils in Thailand. World J Microbiol Biotechnol 24:3091–3096

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 115–175

Liao VH-C, Chu Y-J, Su Y-C et al (2011) Arsenite-oxidizing and arsenate-reducing bacteria associated with arsenic-rich groundwater in Taiwan. J Contam Hydrol 123:20–29

Mandal BK, Suzuki KT (2002) Arsenic round the world: a review. Talanta 58:201–235

Muller D, Lievremont D, Simeonova DD, Hubert J-C, Lett M-C (2003) Arsenite oxidase (aox) genes from a metal-resistant β-proteobacterium. J Bacteriol 185:135–141

Ng JC, Moore MR (2005) Arsenic in drinking water: a natural killer in Bangladesh and beyond. Med J Aust 183:562–563

Nriagu JO (2002) Arsenic poisoning through the ages. In: Frankenberger WT (ed) Environmental chemistry of arsenic. Marcel Dekker, New York, pp 1–26

Osborne FH, Ehrlich HL (1976) Oxidation of arsenite by a soil isolate of *Alcaligenes*. J Appl Bacteriol 41:295–305

Rhine ED, Phelps CD, Young LY (2006) Anaerobic arsenic oxidation by novel denitrifying isolates. Environ Microbiol 8:899–908

Rhine ED, Ní Chadhain SM, Zylstra GJ, Young LY (2007) The arsenite oxidase genes (aroAB) in novel chemoautotrophic arsenite oxidizers. Biochem Biophys Res Commun 354: 662–667

Salmassi TM, Venkateswaren K, Satomi M, Newman DK, Hering JG (2002) Oxidation of arsenite by *Agrobacterium albertimagni* AOL15, sp. nov., isolated from hot creek, California. Geomicrobiol J 19:53–66

Santini JM, Sly LI, Schnagl RD, Macy JM (2000) A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: phylogenetic, physiological, and preliminary biochemical studies. Appl Environ Microbiol 66:92–97

Sehlin HM, Lindström EB (1992) Oxidation and reduction of arsenic by *Sulfolobus acidocaldarius* strain BC. FEMS Microbiol Lett 93:87–92

Simeonova DD, Micheva K, Muller D et al (2005) Arsenite oxidation in batch reactors with alginate-immobilized ULPAs1 strain. Biotechnol Bioeng 91:441–446

Smedley PL, Kinniburgh DG (2002) A review of the source, behaviour and distribution of arsenic in natural waters. Appl Geochem 17:517–568

Suttigarn A, Wang Y-T (2005) Arsenite oxidation by *Alcaligenes faecalis* strain O1201. J Environ Eng 131:1293–1301

Turner AW (1949) Bacterial oxidation of arsenite. Nature (London) 164:76–77

US EPA (2000) Technologies and costs for removal of arsenic from drinking water, EPA report 815-R-00-028. US EPA, Washington DC

Weeger W, Lievremont D, Perret M et al (1999) Oxidation of arsenite to arsenate by a bacterium isolated from an aquatic environment. Biometals 12:141–149

WHO (1993) Guidelines for drinking water quality, vol 1: recommendations, 2nd edn. WHO, Geneva. http://www.who.int/water_sanitation_health/dwq/gdwq2v1/en/. Accessed 6 June 2012

Yoon I-H, Chang J-S, Lee J-H, Kim K-W (2009) Arsenite oxidation by *Alcaligenes* sp. strain RS-19 isolated from arsenic-contaminated mines in the Republic of Korea. Environ Geochem Health 31:109–117