Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Arsenic-resistant bacteria isolated from agricultural soils of Bangladesh and characterization of arsenate-reducing strains

S.P. Bachate*, L. Cavalca* and V. Andreoni

Department of Food Science and Microbiology, University of Milan, Milan, Italy

Keywords
agricultural soil of Bangladesh, arsenate reduction, arsenic resistance, *ars* genes, 16S rRNA gene.

Correspondence
Vincenza Andreoni, Department of Food Science and Microbiology, University of Milan, Via Celoria 2, I-20133 Milan, Italy.
E-mail: vincenza.andreoni@unimi.it

*Both authors contributed equally to this work.

2008/1292: received 25 July 2008, revised 28 November 2008 and accepted 30 November 2008

doi:10.1111/j.1365-2672.2009.04188.x

Abstract

Aims: To analyse the arsenic-resistant bacterial communities of two agricultural soils of Bangladesh, to isolate arsenic-resistant bacteria, to study their potential role in arsenic transformation and to investigate the genetic determinants for arsenic resistance among the isolates.

Methods and Results: Enrichment cultures were performed in a minimal medium in the presence of As(III) and As(V) to isolate resistant bacteria. Twenty-one arsenic-resistant bacteria belonging to different genera of Gram-positive and Gram-negative bacteria were isolated. The isolates, with the exception of *Oceanimonas doudoroffii* Dhal Rw, reduced 2 mmol l^{-1} As(V) completely to As(III) in aerobic conditions. Putative gene fragments for arsenite efflux pumps were amplified in isolates from Dhal soil and a putative arsenate reductase gene fragment was amplified from a *Bacillus* sp. from Rice soil.

Conclusions: Phylogenetically diverse arsenic-resistant bacteria present in agricultural soils of Bangladesh are capable of reducing arsenate to arsenite under aerobic conditions apparently for detoxification purpose.

Significance and Impact of the Study: This study provides results on identification, levels of arsenic resistance and reduction of arsenate by the bacterial isolates which could play an important role in arsenic cycling in the two arsenic-contaminated soils in Bangladesh.

Introduction

Arsenic is a toxic element released into the environment either by natural phenomena (weathering, volcanic activity) or by anthropogenic activities (Cullen and Reimer 1989). Arsenic mainly occurs in two inorganic forms, arsenate As(V) and arsenite As(III) (Neff 1997). The accumulation of arsenic in groundwater is a serious problem in many parts of the world, notably Bangladesh, where arsenic-contaminated groundwater is used for drinking by over 40 million peoples (Smedley and Kinniburgh 2002). Due to the natural abundance of arsenic in the environment, representatives from various bacterial genera have developed different resistance mechanisms for arsenic compounds (Mukhopadhyay *et al.* 2002; Rosen 2002). While arsenate enters into the microbial cells via transmembrane phosphate transport proteins (Cervantes *et al.* 1994), arsenite enters at neutral pH via aquaglyceroporins (Wysocki *et al.* 2001). The bacterial resistance with regard to reduction of arsenate or oxidation of arsenite can be divided into two basic categories consisting of either detoxification reactions that confer arsenic resistance, or redox reactions that conserve the energy gained by the reactions for cell growth (Stolz *et al.* 2002; Silver and Phung 2005). The most widespread resistance mechanisms found in bacteria are *ars* operons which are either chromosomally or plasmid encoded. The two most common types of these operons contain either five (*arsRDABC*) or three (*arsRBC*) genes. The *ars* operon in *Escherichia coli* has both plasmid and chromosomal loci (Oremland and Stolz 2003). The *arsR* gene encodes a transcriptional regulator, *arsB* encodes a membrane-bound arsenite carrier that exports arsenite and *arsC* encodes a reductase that converts arsenate to arsenite

(Mukhopadhyay and Rosen 2002). In addition, the *arsA* gene codes for a ATPase subunit (ArsA) which increases the efficiency of arsenite carrier protein, and *arsD* codes for an arsenic metalo-chaperone that delivers As(III) to ArsA (Lin *et al.* 2006). Arsenate respiration and arsenate detoxification are both microbial processes that contribute to arsenic mobilization in soil and groundwater (Oremland and Stolz 2003). Migration of As(III) through soil to groundwater is of great concern, due to its mobility and higher toxicity than As(V). Arsenite has the ability to bind to sulfhydryl groups of proteins and dithiols. Arsenate, on the other hand is a toxic analogue for inorganic phosphorylating activities (Summers and Silver 1978).

Bacterial populations associated with arsenic transformations have been characterized from diverse environments such as in oxic environments (Macur *et al.* 2004) and in anoxic sediments of lakes and rivers naturally contaminated with arsenic (Cummings *et al.* 1999; Oremland *et al.* 2005). The role of dissimilatory arsenate-reducing bacteria in arsenic release into groundwater of sedimentary aquifers of Bengal delta has been proved recently (Islam *et al.* 2004). Similarly, As(V)-reducing bacteria have been also found to mediate the reduction of As(V) under highly aerobic conditions resulting in enhanced mobilization of arsenic from limed mine tailings (Macur *et al.* 2001).

In Bangladesh, agricultural fields are frequently irrigated with arsenic-contaminated groundwater, particularly the rice paddies (*Oryza sativa*). A survey on arsenic contamination of paddy field soils in Bangladesh reports the presence of up to 1·7 μg g^{-1} of arsenic in rice grains (Mehrag and Rahman 2003). The ingestion of arsenic-contaminated rice and vegetables represents the second most common route of arsenic poisoning with ingestion of contaminated water as first (Chowdhury 2004). The arsenic transformation capabilities of bacteria of these soils can be of great concern because rice plants have been found to take up different arsenic species present in soil, in particular, As(III) at higher rate than As(V), dimethylarsinic acid (DMAA) and monomethylarsonic acid (MMAA) with uptake rates of 147, 126, 12·7 and 5·7 nmol g^{-1} fresh weight per h for As(III), As(V), MMAA and DMAA, respectively (Abedin *et al.* 2002).

Our understanding of the aerobic arsenic-resistant bacteria in agricultural soils in Bangladesh and their possible role in arsenic transformation and mobilization is scarce. The aim of present work was (i) to isolate and identify the culturable arsenic-resistant bacterial populations present in arsenic-contaminated paddy rice and lentil field soils of Bangladesh; (ii) to evaluate the arsenic transformations performed by the arsenic-resistant isolates and (iii) to check for the presence of genetic determinants for arsenic resistance among isolates to understand the resistance mechanism involved.

Materials and methods

Soil characteristics and bacterial counts in soils

Soil samples (0–20 cm) were collected from a paddy rice field (Rice soil) and lentil field (Dhal soil) from the Satkhira District (Bangladesh) and the samples were chemically analysed by Martin *et al.* (2007). The various forms of arsenic content of the soils (total, water-soluble and oxalate-extractable) were determined by following the sequential extraction method of Wenzel *et al.* (2001).

For microbiological analysis, eight samples of each soil were randomly collected and then mixed together to form two composite samples. Each composite sample was put into plastic bags, transported to the laboratory where they stored moist in the dark at 4°C in polyethylene bags for 2 weeks until the analyses.

An initial soil extraction was performed by adding 45 ml of sodium pyrophosphate solution (2 g l^{-1}) to 5 g of wet soil. Serial tenfold dilutions of soil samples were prepared in 1 : 20 saline solution (Jensen 1962) for each soil sample (Pochon and Tardieux 1962). Heterotrophic bacteria were determined by plating aliquots (1 ml) of the various dilutions into 1 : 10 diluted Tryptic Soy Agar (TSA/10) medium in double sets of pour plates. Cycloheximide (0·1 g l^{-1}) was added to the media to inhibit fungal growth. To analyse the arsenic tolerance level of bacterial community in the soil (Kunito *et al.* 1999), the number of arsenic-tolerant bacteria was determined by plating 1 ml of the various dilutions of soil on TSA/10 medium supplemented with 0·77 mmol l^{-1} either of sodium arsenate, As(V), or sodium arsenite, As(III) mixed in the medium prior to pouring the plates. The arsenic tolerance of the heterotrophs was expressed as percentage of growth on TSA/10 without arsenic addition. All incubations were carried out at 30°C for 10 days. The data were subjected to analysis of variance (ANOVA), and treatment means were compared by Duncan's multiple-range test. All analyses were performed at the $P \leq 0{\cdot}05$ level.

Enrichment cultures and isolation of arsenic-resistant strains

To evaluate the arsenic resistance, soil bacteria were grown in the presence of high concentration of arsenic to determine the response to arsenic stress. Soil samples (10 g) were added to flasks containing Tris mineral medium (TMM) at low phosphate content (Mergeay *et al.* 1985), supplemented with 6 g l^{-1} of sodium gluconate (TMMG) and with 20 mmol l^{-1} of As(V) or 5 mmol l^{-1}

of As(III). Flasks were incubated under shaking (150 rev min^{-1}) at 30°C for 5 days and 10 ml of each of these enrichment cultures were transferred into fresh medium and then incubated under the same conditions. After three subsequent transfers, aliquots of cell suspensions (15 ml) were centrifuged (10°C for 20 min at 13 000 ***g***) and cell pellets were then subjected to DNA extraction as specified below. To isolate arsenic-resistant bacteria, appropriate dilutions of enrichment cultures were plated on TSA/10 containing 20 mmol l^{-1} of As(V) or 5 mmol l^{-1} of As(III) and the plates were incubated at 30°C for 5 days. After incubation, colonies with different morphologies were selected from the plates. Single colonies were streaked to purity on the same medium. Strains were maintained in glycerol stocks at -70°C. Prior to use, the strains were grown to mid-exponential phase in TMMG at 30°C with shaking.

Arsenic resistance and transformation of arsenite and arsenate by isolates

Resistance to As(V) and As(III) was determined for each isolate by growing them separately in 20 ml TMMG liquid medium amended with increasing concentrations of As(V) (from 0 to 650 mmol l^{-1}) or As(III) (from 0 to 80 mmol l^{-1}). Two vials for each concentration were inoculated with appropriate cell suspension grown in TMMG without arsenic to obtain cell density of approx. 10^6 CFU ml^{-1}. The growth was evaluated by measuring the $OD_{600\ nm}$ after 5 days incubation at 30°C. Yeast extract (0·2 g l^{-1}) was added to TMMG for isolates Dhal F, Rice D, Dhal P, Dhal Rr and Dhal G as these isolates did not grow without yeast extract after the first transfer. To test the ability of the isolates to reduce As(V) or oxidize As(III), the strains were grown to mid-exponential phase in TMMG without arsenic and then inoculated into two vials each containing 20 ml of TMMG either with 2 mmol l^{-1} As(V) or 1 mmol l^{-1} As(III) to obtain an $OD_{600\ nm}$ of 0·05. Control flasks without inoculum were incubated to check abiotic transformation of arsenic. At each sampling time, 1 ml of suspension was removed to measure cell growth by $OD_{600\ nm}$ and to determine As(V) and As(III) concentration by spectrophotometric analysis according to method described by Cummings *et al.* (1999). As(V) concentration was determined by acidifying 100 μl sample in 100 μl of HCl (24 mmol l^{-1}). A 100 μl of the acidified sample was then added to 900 μl of the reaction mixture containing the following: ammonium molybdate (6 g l^{-1}), ascorbic acid (10·8 g l^{-1}), potassium antimonyl tartrate (0·136 g l^{-1}) and concentrated H_2SO_4 (67·3 ml l^{-1}). Each component was stored as a separate solution. Samples were heated in a water bath at 78°C for 10 min and placed on ice for 5 min. The absorbance at 865 nm was compared to acidified As(V) standards. As(III) concentration was determined by oxidizing a second sample in 100 μl of KIO_3 (5 mmol l^{-1}) and HCl (48 mmol l^{-1}) for 10 min and then reading the $OD_{865\ nm}$. Blanks of milli-Q water were used to calibrate the spectrophotometer. New plastic wares were used each time to avoid contamination from excess phosphate from detergents. Standard curves were prepared for concentrations of 0–100 μmol l^{-1} for both As(V) and As(III). The difference between oxidized and unoxidized samples represented the concentration of As(III). For testing the potential of the strains to respire As(V), the isolates were aerobically grown in TMMG liquid medium without arsenic and then transferred into vials with butyl rubber stoppers containing TMM with sodium lactate (10 mmol l^{-1}) and As(V) (10 mmol l^{-1}) under an atmosphere of $N_2/CO_2/H_2$ (85 : 5 : 10). The vials were incubated in static conditions at 30°C for 7 days. After incubation, the samples were analysed for growth by $OD_{600\ nm}$ and arsenic concentration as described above.

DNA extraction methods

DNA from enrichment cultures was extracted by a conventional not organic solvent method (G-NOME BIO 101, QBiogene, Illkirch Cedex, France). Cell suspension solution (1·85 ml) was added to the pellet (50 mg of fresh weight) obtained from freshly grown liquid culture and 50 μl of RNase was added to the sample. Then the sample was incubated at 55°C for 2 h in the presence of 100 μl cell lysis solution and 25 μl of Proteinase mix. Samples were precipitated in the presence of 8 ml 100% ethanol and the DNA pellet was resuspended in 200 μl TE (10 mmol l^{-1} Tris HCl pH 8·0 and 1 mmol l^{-1} Na_2EDTA pH 8·0). DNA from pure strains was extracted from TMMG overnight-grown liquid cultures by Proteinase K digestion (1 mg ml^{-1}) according to Cavalca *et al.* (2004). The DNA-containing supernatant was withdrawn and put in sterile microtubes.

Identification of isolates and 16S rRNA gene analysis

The complete 16S rRNA gene was amplified from 20 ng DNA using eubacterial primers P27f and P1495r (Weisburg *et al.* 1991). PCR reactions were performed in a final volume of 50 μl containing 10 ng of DNA, 1·5 U of *Taq* polymerase, 0·2 μmol l^{-1} of each primer, 0·2 mmol l^{-1} of dNTPs, 1·75 mmol l^{-1} $MgCl_2$, and 1× PCR buffer. DNA amplification conditions were, initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 40 s, 72°C for 1 min 40 s and then a final extension step at 72°C for 10 min. Isolates were grouped on the basis of amplified ribosomal DNA restriction analysis (ARDRA)

by using restriction enzyme *Hha*I. One representative isolate of each group was selected and its 16S rRNA gene was sequenced.

Amplification of hypervariable region V3 internal to the 16S rRNA gene amplified from enrichment cultures was performed using a nested PCR approach: the 16S rDNA PCR products were 100-fold diluted and then used as template for a second amplification step of the V3 hypervariable region with primer pair P341f and P534r. PCR reactions (final volume 50 μl) contained the following: 10 ng of 16S rDNA, 1 U of *Taq* polymerase, 0·2 μmol l^{-1} of each primer, 0·2 mmol l^{-1} of dNTPs, 1·75 mmol l^{-1} $MgCl_2$ and 1× PCR buffer. The samples were first denatured for 3 min at 94°C and then subjected to 19 cycles consisting of 1 min 20 s at 94°C, 1 min 30 s at 65°C and 1 min 10 s at 72°C; the annealing temperature was decreased by 1°C every second cycle until touchdown at 55°C, at which temperature seven additional cycles were carried out. The last step included an extension of 5 min at 72°C.

Cloning

Clone libraries of hypervariable V3 16S rRNA regions of the DNA from As(V) (20 mmol l^{-1}) and As(III) (5 mmol l^{-1}) enrichment cultures of both soils were prepared in order to describe species composition. V3 fragments were amplified as described above and PCR products were purified by QIAquick PCR purification columns (Qiagen, Germany) and ligated into pCR®II-TOPO TA cloning vector (Invitrogen). After chemical transformation into library efficiency DH5α™ competent cells (Invitrogen), 50 white colonies from each experiment were screened for the presence of the correct size insert by colony PCR using vector-specific primers (M13 forward and reverse). Sequence analysis was performed on representative clones showing different *Hha*I (GE Healthcare, Sweden) restriction fragment length polymorphisms. Analogously, PCR products of eubacterial 16S rRNA and arsenic resistance gene fragments amplified from isolated strains were cloned into pCR®II-TOPO TA cloning vector. All the selected clones were directly sequenced by using M13 forward and reverse primers. Sequence reactions were performed by using the *Taq* Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, USA) and analysed by automatic DNA sequencer 310A apparatus (Applied Biosystems).

Primer design for arsenic resistance genes and PCR conditions

All the primers used for analysis of arsenic resistance genes are reported in Table 1. Four different primer sets were used to amplify genes for arsenate reductase (*arsC*) and arsenite transporter (*arsB*). A total of 50 bacterial gene sequences involved in arsenic resistance were obtained from GenBank. Preliminary phylogenetic analyses classified the gene sequences into categories corresponding to the different *arsC* genes of bacterial phylogenetic groups. Taking into account these sequence variations and the presence of different bacterial genera among Rice and Dhal soil isolates, in the present work degenerate primer pair P52f and P323r was designed on the basis of the consensus sequence of *arsC* genes of 16S rRNA-Group 1 of *Bacillus* species (i.e. *B. cereus* ATTC 10987 acc. num. AE017194, *B. thuringiensis* 97-27 acc. num. AE017355 and *B. anthracis* Ames Ancestor acc. num. AE017334). Primer sets darsB1F–darsB1R, dacr1F–dacr1R and dacr5F–dacr4R were described by Achour *et al.* (2007) were used to amplify arsenite transporter (*arsB* and *ACR 3p*) genes.

PCR reactions for *arsB* and *arsC* gene fragments selected in this work were performed in a final volume of 25 μl containing: 10 ng DNA, 0·2 mmol l^{-1} of dNTPs, 1·75 mmol l^{-1} $MgCl_2$, 0·4 μmol l^{-1} of each primer, 2 U of *Taq* polymerase and 1× PCR buffer. DNA amplification conditions for P52f-P323r were: initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 30 s and then a final extension step at 72°C for 7 min. DNA amplification conditions for primer sets darsB1F–darsB1R, dacr1F–dacr1R and dacr5F/dacr4R were as described by Achour *et al.* (2007). PCR products were cloned and sequenced as described above.

Table 1 Primers used in this study for the amplification of arsenic detoxification-related genes: arsenite transporters (*arsB, ACR 3p*) and arsenate reductase (*arsC*)

Primer name	Primer sequence (5′–3′)	PCR product (bp)	Reference
darsB1F	TGTGGAACATCGTCTGGAAYGCNAC		
darsB1R	CAGGCCGTACACCACCAGRTACATNCC	*arsB* (750)	Achour *et al.* (2007)
dacr1F	GCCATCGGCCTGATCGTNATGATGTAYCC		
dacr1R	CGGCGATGGCCAGCTCYAAYTTYTT	*ACR 3*(*1*) (750)	Achour *et al.* (2007)
dacr5F	TGATCTGGGTCATGATCTTCCCVATGMTGVT	*ACR 3*(*2*) (750)	Achour *et al.* (2007)
dacr4R	CGGCCACGGCCAGYTCRAARAARTT		
P52F	AGCCAAATGGCAGAAGC		
P323R	GCTGGRTCRTCAAATCCCCA	*arsC* (275)	This study

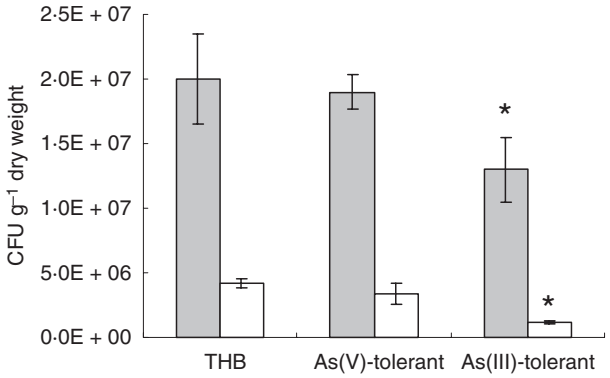


Figure 1 Counts of heterotrophs, As(V)-tolerant (0·77 mmol^{-l}) and As(III)-tolerant (0·77 mmol^{-l}) bacteria expressed as CFU g^{-1} soil dry weight in Rice and Dhal soils (gray = Rice, white = Dhal). THB, total heterotrophic bacteria grown on TSA/10. Data are expressed as mean ± standard deviation of four replicates. *, statistically significant values when compared with the THB and given in text (χ^2, $P \leq 0{\cdot}05$).

All reagents were from Invitrogen (UK), unless otherwise stated. PCR reactions were performed on T-Gradient Biometra thermocycler (Germany). The PCR products were checked on 2% agarose gel and visualized by ethidium bromide staining by using the Gel Doc image analyzer system (Bio-Rad, USA).

Sequence analysis and phylogenetic tree construction

16S rRNA (600–800 bp) and *ars* gene sequences were searched for homology by using the NCBI-BLAST2 – Nucleotide and Protein Database Query program (http://www.ebi.ac.uk/blastall/index.html). Phylogenetic analysis of ArsC, ArsB and ACR 3p deduced amino acid sequences were performed using MEGA version 3.1 software (Kumar *et al.* 2004). Phylogenetic trees were constructed using the neighbour-joining distance method based on p-distance. A total of 500 bootstrap replications were calculated. Sequences obtained in the present work were deposited in the EBI database. The accession numbers from FM163604 to FM163615 are for 16S rRNA genes and accession numbers AM983535, AM990469, AM990470 and AM990471 are for *ars* genes.

Results

Arsenic content and bacterial counts in soils

The two study soils showed similar pH values, between 8·4 (Rice soil) and 8·6 (Dhal soil). Both soils were calcareous and had low organic carbon content which was double in the Rice soil (0·97%) than in the Dhal soil (0·44%). The amount of total arsenic (10·1 ± 0·42 *vs* 7·8 ± 0·81 mg kg^{-1} dry weight) was similar in the two study soils (Martin *et al.* 2007). The soils did differ in the labile forms, in particular, Rice soil contained approximately double the amount of water-soluble arsenic and oxalate-extractable arsenic than Dhal soil (6·2 ± 0·24 μg kg^{-1} *vs* 3·5 ± 0·14 μg kg^{-1} and 2·51 ± 0·43 μg kg^{-1} *vs* 1·13 ± 0·3 μg kg^{-1}, respectively).

The abundance of heterotrophs and arsenic-tolerant bacteria in Rice and Dhal soils are shown in Fig. 1. The counts of total heterotrophs and of As(V)- and As(III)-tolerant bacteria were high in the Rice soil than in the Dhal soil, probably due to higher organic carbon content in the Rice soil (Monod 1950). No significant difference in number of heterotrophs and As(V)-tolerant bacteria was observed in the two soils at the As(V) level examined (0·77 mmol l^{-1}) probably for the low toxic effect of the As(V) amount added to TSA/10 medium and the low

Table 2 Identification of culturable isolates from Rice and Dhal soils and their As(V)/As(III) resistance levels

Isolate	Closest GenBank match (% identity)*	Enrichment culture isolated from	Arsenic resistance† (mmol l^{-1})	
			As(V)	As(III)
Dhal A	*Arthrobacter* sp. (99)	As(V)	>650	70
Dhal L	*Alcaligenes* sp. (99)	As(V)	350	70
Dhal N	*Alcaligenes* sp. (99)	As(III)	350	70
Dhal Rw	*Oceanimonas doudoroffii* (97)	As(V)	>650	1
Dhal D	*Arthrobacter* sp. (98)	As(V)	300	20
Dhal E	*Arthrobacter* sp. (98)	As(III)	300	20
Dhal G	*Arsenicicoccus bolidensis* (100)	As(III)	250	20
Dhal S	*Arsenicicoccus bolidensis* (100)	As(V)	250	20
Dhal B	*Microbacterium hydrocarbonoxydans* (100)	As(III)	150	30
Dhal C	*Microbacterium hydrocarbonoxydans* (100)	As(III)	150	30
Dhal P	*Kocuria erythromyxa* (100)	As(III)	100	5
Dhal Q	*Staphylococcus succinus* (100)	As(III)	100	5
Dhal F	*Variovorax paradoxus* (99)	As(III)	30	15
Dhal Rr	*Arthrobacter agilis* (100)	As(V)	100	5
Rice A	*Bacillus megaterium* (100)	As(III)	200	30
Rice B	*Bacillus megaterium* (100)	As(III)	200	30
Rice H	*Bacillus megaterium* (100)	As(V)	200	30
Rice C	*Bacillus* sp. DT10 (99)	As(V)	200	20
Rice D	*Microbacterium hydrocarbonoxydans* (100)	As(III)	150	30
Rice E	*Microbacterium hydrocarbonoxydans* (100)	As(III)	150	30
Rice F	*Microbacterium hydrocarbonoxydans* (100)	As(V)	150	30

*The 16S rRNA identity of the isolates was determined by analysing 600–800 bp.

†The resistance to As(V) and As(III) was evaluated by a tenfold increase in $OD_{600\ nm}$ of the inoculum.

ratio of arsenic to phosphate, i.e. 0·77–1·6 mmol l^{-1} (Cervantes and Chavez 1992). Both soils contained high percentage of As(III)-tolerant bacteria and their percentage was higher in Rice soil (65%) than in Dhal soil (29%).

Identification of isolates and their arsenic resistance pattern

A total of 21 arsenic-resistant bacteria were isolated from the enrichment cultures, 14 isolates were from Dhal soil and 7 from Rice soil. The isolates were identified by partially (600–800 bp) sequencing the 16S rRNA gene. The isolates from Rice soil belonged to the genera *Bacillus* and *Microbacterium*; in contrast, those from the less polluted Dhal soil belonged to eight different genera as *Arthrobacter*, *Bacillus*, *Alcaligenes*, *Kocuria*, *Staphylococcus*, *Microbacterium, Variovorax* and *Oceanimonas* (Table 2).

Resistance to As(V) and As(III) of the isolates was determined by measuring the $OD_{600\ nm}$ after 5 days growth in TMMG broth amended with various concentrations of As(V) and As(III). The isolates varied in their arsenic resistance pattern showing from moderate to high resistance to As(V) and As(III) (Table 2). Isolates *Arthrobacter* sp. Dhal A, *Alcaligenes* sp. Dhal L and Dhal N exhibited the highest resistance to arsenic resisting up to 70 mmol l^{-1} of As(III) and up to 350 mmol l^{-1} of As(V). Other isolates of *Arthrobacter* genus (Dhal Rr, Dhal D and Dhal E) exhibited a different and lower resistance pattern when compared with isolate Dhal A. An isolate, *Oceanimonas doudoroffii* Dhal Rw, was highly resistant to As(V) (>650 mmol l^{-1}) but scarcely grew in the presence of 1 mmol l^{-1} As(III). The isolates (Dhal B, Dhal C, Rice D, Rice E, Rice F and Rice G), all represented by *Microbacterium* genus, although derived from the two soils, had the same arsenic resistance pattern and all resisted up to 150 mmol l^{-1} As(V) and up to 30 mmol l^{-1} As(III). A group of Gram-positive bacteria which included Dhal G, Dhal S, Dhal P, Dhal Q, Rice A, Rice B, Rice H and Rice C resisted from 5 to 30 mmol l^{-1} As(III) and from 100 to 250 mmol l^{-1} As(V). Finally, *Variovorax paradoxus* Dhal F, although showing high resistance to As(III), was the bacterium with the lowest resistance to As(V).

Clone libraries of enrichment cultures

The V3 regions amplified from the cultures enriched in presence of 20 mmol l^{-1} As(V) and 5 mmol l^{-1} As(III) of both soils were cloned and sequenced in order to describe the species highly resistant to stress imposed. The comparison of the cloned sequences with GenBank database revealed the presence of different populations (Table 3). The majority of cloned sequences obtained from the Dhal soil culture enriched on 20 mmol l^{-1} As(V) belonged to *Diaphorobacter* sp. (58% of total clone library) and *Arthrobacter* sp. (39% of total clone library), while *Methylobacterium* sp. was present in lower percentage, which was 3% of total clone library. Cloned sequences obtained from 5 mmol l^{-1} As(III) enrichment culture of Dhal soil belonged to *Bacillus litoralis* (88·8% of total clone library), *Paenibacillus thiaminolyticus*, *Strenotrophomonas*

Table 3 Identification of cloned 16S rRNA sequences obtained from Rice and Dhal soil enrichment cultures

OUT*	Closest GenBank match	Accession No.	Identity (%)	Abundance (% of total clone library)
Dhal soil enrichment culture in 5 mmol l^{-1} As(III)				
Clone D1As(III)	*Stenotrophomonas maltophilia*	DQ466574	100	2·22
Clone D2As(III)	*Paenibacillus thiaminolyticus*	AJ320490	100	4·44
Clone D3As(III)	*Arthrobacter oxydans*	X83408	100	4·44
Clone D4As(III)	*Bacillus litoralis*	AY608605	100	88·8
Dhal soil enrichment culture in 20 mmol l^{-1} As(V)				
Clone D1As(V)	*Diaphorobacter* sp.	DQ987613	97	58
Clone D2As(V)	*Arthrobacter eutrophus*	M32021	100	39
Clone D3As(V)	*Methylobacterium* sp. *AC72a*	AY776209	100	3
Rice soil enrichment culture in 5 mmol l^{-1} As(III)				
Clone R1As(III)	*Stenotrophomonas maltophilia*	X 95924	100	3·77
Clone R2As(III)	*Paenibacillus thiaminolyticus*	Y16129	99	67·9
Clone R3As(III)	*Ancylobacter* sp. *OL1*	DQ986318	98–100	28·3
Rice soil enrichment culture in 20 mmol l^{-1} As(V)				
Clone R1As(V)	*Bacillus benzovorans*	Y14693	99–100	96
Clone R2As(V)	*Pseudoxanthomonas mexicana*	AY124375	100	2
Clone R3As(V)	*Bacillus* sp. *MDS01*	EU236670	100	2

*OUT, operational taxonomic unit.

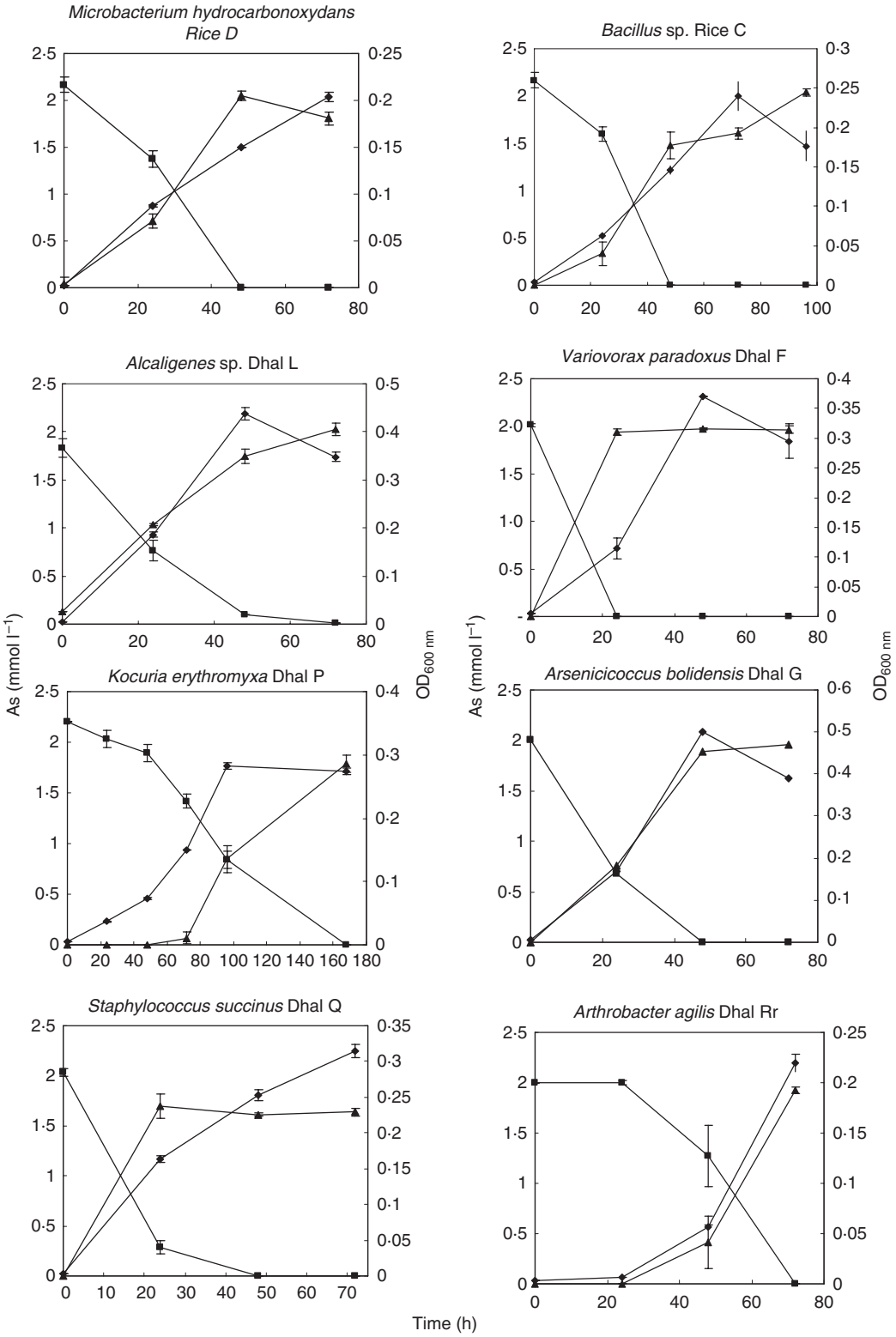
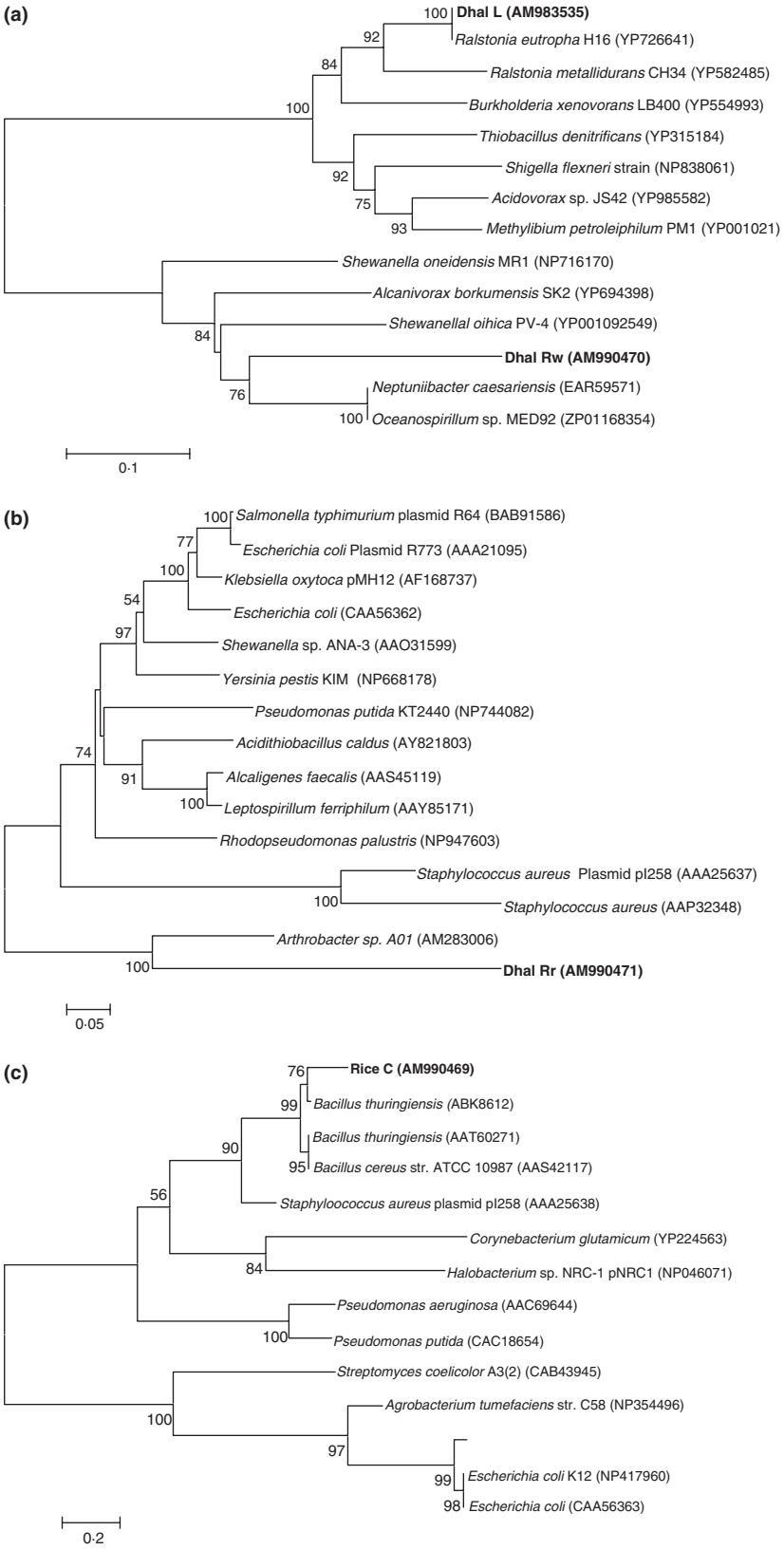


Figure 2 Bacterial growth ($OD_{600\ nm}$) (♦) and corresponding reduction of As[V] (■) to As[III] (▲) by eight isolates from Dhal and Rice soil. The OD values reported are 1 : 10 dilution of original sample. Data are represented as means ± standard error, $n = 4$.



maltophilia and *Arthrobacter oxydans*. The 98% of cloned sequences obtained from 20 mmol l^{-1} As(V) Rice soil enrichment culture belonged to genus *Bacillus* and only 2% belonged to *Pseudoxanthomonas mexicana*. The 5 mmol l^{-1} As(III) enrichment culture of Rice soil showed presence of cloned sequences corresponding to *P. thiaminolyticus* (67·9% of total clone library), to *Ancylobacter* sp. (28·3% of total clone library) and to *S. maltophilia* (3·77% of total clone library).

Arsenic transformation by isolates

To verify the presence of detoxification mechanisms in arsenic-resistant bacteria, As(V) reduction and As(III) oxidation profiles were studied for 21 isolates. With the exception of *O. doudoroffii* Dhal Rw that did not reduce As(V), 20 isolates tested reduced 2 mmol l^{-1} As(V) completely to As(III) under aerobic conditions. As an example of the reducing capability of the isolates, we reported the growth and As(V) reduction profiles of one isolate representing each genus (Fig. 2). The time required for the complete reduction varied in the isolates. Isolates Rice C, Dhal L and Dhal F reduced it completely in 24–48 h, while Dhal P and Dhal Rr required longer time for complete reduction of As(V). Complete reduction of As(V) occurred when strains attained maximum cell density. As(V) reduction did not occur in controls without cells indicating that As(V) reduction was a microbial process.

None of these strains reduced As(V) in the absence of oxygen and none isolates oxidized As(III) under aerobic conditions.

Analysis of *ars* genes in isolates

Arsenic resistance genes were searched in order to investigate the presence of arsenic resistance determinants among the isolates. A 275 bp fragment of putative *arsC* gene from *Bacillus* sp. Rice C (primer set P52F/P323R), a 850 bp fragment of putative *arsB* gene from *Arthrobacter agilis* Dhal Rr (primer set darsB1F/darsB1R) and a 750 bp putative *ACR3(2)* gene fragment (primer set dacr5F/dacr4R) from *O. doudoroffii* Dhal Rw and *Alcaligenes* sp. Dhal L were successfully amplified. Phylogenetic trees were constructed to study the phylogenetic relationship of the translated amino acid sequences of *ACR3*(2), *arsC* and *arsB* genes with previously known sequences (Fig. 3). Phylogenetic analysis of inferred amino acid sequence of Acr3(2) protein of isolates *Alcaligenes* sp. Dhal L and *O. doudoroffii* Dhal Rw placed them in two distinct clusters formed by putative Acr3(2) proteins of β-proteobacteria and γ-proteobacteria respectively, except for *Shigella flexneri* which was placed together with β-proteobacteria. ArsC amino acid sequence of *Bacillus* sp. Rice C was placed in cluster formed by *Staphylococcus* Trx ArsC family together with putative arsC proteins of other *Bacillus* species. The length of *arsB* gene fragment obtained from *Arthrobacter* sp. Dhal Rr was 850 bp although expected size was 750 bp and its inferred amino acid sequence was related only with ArsB sequence of *Arthrobacter* sp. A01 characterized by Achour *et al.* (2007) but not with other known ArsB sequences.

Discussion

The two agricultural soils investigated in this study showed a moderate arsenic concentration (Huq *et al.* 2003) and the total arsenic content of the Rice soil was close to the levels usually reported in paddy rice fields in Bangladesh (Alam and Sattar 2000). The higher level of labile arsenic forms in the Rice soil than in the Dhal soil can be a consequence of the higher water requirement of rice crop. The heterotrophs were inhibited by 0·77 mmol l^{-1} of As(III) but not by As(V). The higher number of As(III)-tolerant bacteria in Rice than in Dhal soil can be ascribed to the higher content of water-soluble and oxalate-extractable arsenic in the former. Moreover, under reducing conditions that can occur in flooded soils, like the rice fields, As(III) can be the predominant arsenic species and generally less retained by soil colloids (Sadiq 1997; Bissen and Frimmel 2003). In lentil field soil, oxidizing conditions are maintained for many months in a monsoon climate and, with As(V) being the dominant species, a stronger retention of arsenic by soil colloids could be expected. These results can suggest that labile arsenic forms rather than the total arsenic content can be considered in evaluating the arsenic tolerance of bacterial communities.

Diversity in the composition of enrichment cultures was observed by comparing the arsenic-resistant isolates and clone libraries. The isolates from Rice soil belonged to two genera, while the cloned sequences corresponded to five genera. *Bacillus* species were the dominant clones in the As(V)-enriched culture and were effectively isolated. In As(III)-enriched culture from Rice soil *Paenibacillus* and *Ancylobacter* were the dominant species but these species were not isolated. The isolated strains from

Figure 3 Phylogenetic tree of inferred amino acid sequences of arsenic resistance genes (a) *ACR3(2)* genes of isolates Dhal L and Dhal Rw, (b) *arsB* gene of Dhal Rr and (c) *arsC* gene of Rice C. Sequences obtained in this study are in bold type. Numbers above nodes represent bootstrap confidence values obtained with 500 resamplings; values below 50 are not shown. The GenBank accession numbers for the corresponding arsenic resistance gene sequences are given in parentheses. Scale bar indicates substitutions per site.

Dhal soil belonged to nine genera, while the cloned sequences corresponded to six genera. Among these, *Arthrobacter* was isolated but not the *Strenotrophomonas*, *Paenibacillus*, *Diaphorobacter* and *Methylobacter.* This discrepancy could be due to the exposure of enrichment cultures to toxic levels of arsenic which could have hampered the growth of more sensitive populations. The enrichment conditions, however, do not reproduce real conditions of an environmental sample and select the micro-organisms growing faster and adapting better to the growth conditions imposed. However, all of cloned sequences belonged to species reported to have arsenic resistance (Jackson *et al.* 2005a,b; Achour *et al.* 2007).

Twenty-one heterotrophic aerobic arsenic-resistant bacteria representing different genera in the Gram-positive and Proteobacteria groups were isolated and identified. Most of isolates belonged to Actinobacteria and Firmicutes division and only a small number of isolates belonged to Proteobacteria. On the contrary, in arsenic-free soils and in an estuary with negligible level of arsenic, Jackson *et al.* (2005a,b) found that the majority of isolates were Proteobacteria, while only few isolates belonged to Actinobacteria and Firmicutes. The less contaminated Dhal soil showed more diverse bacterial population which belonged to at least nine genera, in accordance with the results of Jackson *et al.* (2005a). Our results together with those of Jackson *et al.* (2005a,b) and Achour *et al.* (2007) confirm the wide distribution of phylogenetically diverse arsenic-resistant bacteria in the environment. The isolate Dhal Rw showed 97% 16S rDNA sequence homology with *O. doudoroffii* (Brown *et al.* 2001). None of three *Oceanimonas* species recognized up to now have been characterized in terms of arsenic resistance. All other isolates belonged to species reported to have arsenic resistance or to be involved in arsenic cycling in soils and waters (Anderson and Cook 2004; Macur *et al.* 2004; Achour *et al.* 2007). Isolates Dhal G and Dhal S showed high 16S rDNA sequence homology with *Arsenicicoccus bolidensis*, a new genus recently proposed (Collins *et al.* 2004). The type strain *A. bolidensis* has been isolated from sediments containing mine waste in Sweden and has been reported to posses both As(V) reduction mechanisms that impart resistance to As(V) toxicity or coupled to respiration of As(V) (Routh *et al.* 2007). Differently from the *A. bolidensis* type strain, Dhal G and Dhal S did not respire As(V) under anaerobic conditions.

Although arsenic content of the two soils was moderate, many isolates were highly resistant to both forms of arsenic indicating that the level of bacterial arsenic resistance can be many folds higher than the arsenic content of the soil, in accordance with other similar studies (Jackson *et al.* 2005a; Achour *et al.* 2007). Three *Arthrobacter* spp. from Dhal soil, although showing identical 16S rRNA gene sequences based on ARDRA profile, differed in their arsenic resistance (Table 2) thus confirming that variation in the resistance can occur within same species. High arsenic resistance in some isolates may be due to presence of multiple sets of *ars* operon present on chromosome (Ordóñez *et al.* 2005) or transposon (Tuffin *et al.* 2006).

Although molecular determinant sequences for arsenic resistance are known for many bacteria, we found such sequences in three isolates from Dhal soil and in one *Bacillus* sp. from Rice soil (Fig. 3). The absence of arsenic resistance genes in most of the isolates may be inferred to sequences sufficiently different from the primers used in this study and to the diversity of arsenic resistance genes (Jackson and Dugas 2003; Achour *et al.* 2007). Relatedness of amino acid sequence of the ArsB protein of *A. agilis* Dhal Rr with *Arthrobacter* sp. A01 and not with other known ArsB proteins was in agreement with the results of Achour *et al.* (2007). Moreover, a larger *arsB* gene fragment (850 bp) than the expected one of 750 bp was amplified from both, Dhal Rr and A01 strains, thus indicating the probable existence of a new subgroup of ArsB proteins among bacteria. A gene fragment of putative arsenite efflux pump (*ACR 3(2)*) was amplified from isolate Dhal Rw which was unable to reduce arsenate, suggesting that the mechanism of arsenate resistance in this bacterium relies on prevention of As(V) uptake, rather than an intracellular arsenate reduction to arsenite followed by rapid export. An alternative mechanism than reduction to As(III) and efflux can not however be excluded for this strain. Such an alternative mechanism has been proposed by Baker-Austin *et al.* (2007) for the archaeon *Ferroplasma acidarmanus* F1 that, although highly resistant to both As(V) and As(III), is unable to reduce As(V).

Twenty bacteria isolated from the arsenic-enrichment cultures were capable of reducing As(V) but not of oxidizing As(III), probably due to the absence of genetic determinants coding for the arsenite oxidase genes (Mukhopadhyay *et al.* 2002). The mechanism of As(V) reduction by isolates can be related to a detoxification purpose as in aerobic conditions the arsenate reductase, which is encoded by *arsC* gene, is involved in As(V) reduction. Further, the amplification of *arsB*, *ACR3p* and *arsC* genes in some isolates confirmed the presence of detoxification mechanism for the reduction of As(V). None of the As(V)-reducing isolates could grow under the conditions tested here for dissimilatory As(V) reduction, where lactate served as the primary carbon and energy source and As(V) as terminal electron acceptor. This behaviour is not surprising considering that the isolates were enriched under aerobic conditions. However, the detoxification pathway can occur either aerobically or

anaerobically and is not necessarily governed by prevailing redox conditions (Jackson *et al.* 2001).

The results obtained in this study give evidence of the presence of phylogenetically diverse arsenic-resistant bacteria in agricultural soils and that the resistance levels of these bacteria are regardless of the arsenic contamination. Although direct role of these bacteria in arsenic mobilization was not studied, the ability of the isolates to reduce As(V) suggests that these bacteria can potentially mediate arsenic transformations in these soils and their role in the arsenic cycling in these soils may become relevant with changing environmental conditions.

Acknowledgements

The research was supported by Programma di Interesse Nazionale (PRIN) 2004 project of Italian Ministry for University and Research. S.P.B. is the recipient of a 'Research Doctorate in Technological Innovations for Food and Environmental Sciences' research fellowship of Milan University. The authors wish to thank Dr M. Martin, Department of Valorisation and Protection of Agro Forest Resources (University of Torino, Italy), for providing soils and their chemical characterization.

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