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Diversity of arsenite transporter genes from arsenic-resistant soil bacteria

Asma Rokbani Achour [a,*], Pascale Bauda [a], Patrick Billard [a,b]

[a] *Laboratoire Ecotoxicité et Santé Environnementale (ESE), UMR7146, CNRS, Université Paul Verlaine, Campus Bridoux, rue du Général Delestraint, 57070 Metz, France*

[b] *Laboratoire des Interactions Microorganismes Minéraux Matière Organique dans les Sols (LIMOS), UMR 7137, CNRS, Université Henri-Poincaré Nancy I, Faculté des Sciences, BP 239, 54506 Vandoeuvre-lès-Nancy, France*

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Abstract

A PCR approach was developed to assess the occurrence and diversity of arsenite transporters in arsenic-resistant bacteria. For this purpose, three sets of degenerate primers were designed for the specific amplification of approximately 750 bp fragments from *arsB* and two subsets of *ACR3* (designated *ACR3*(*1*) and *ACR3*(*2*)) arsenite carrier gene families. These primers were used to screen a collection of 41 arsenic-resistant strains isolated from two soil samples with contrasting amounts of arsenic. PCR results showed that 70.7% of the isolates contained a gene related to *arsB* or *ACR3*, with three of them carrying both *arsB* and *ACR3*-like genes. Phylogenetic analysis of the protein sequences deduced from the amplicons indicated a prevalence of *arsB* in *Firmicutes* and *Gammaproteobacteria*, while *ACR3*(*1*) and *ACR3*(*2*) were mostly present in *Actinobacteria* and *Alphaproteobacteria*, respectively. In addition to validating the use of degenerate primers for the identification of arsenite transporter genes in a taxonomically wide range of bacteria, the study describes a novel collection of strains displaying interesting features of resistance to arsenate, arsenite and antimonite, and the ability to oxidize arsenite.

Keywords: Arsenic; Anion transporter; Arsenic resistance; Soil bacteria; *ACR3*; *arsB*; 16S rDNA; Diversity

1. Introduction

Arsenic is a ubiquitous toxic metalloid released in the environment either from natural weathering of rocks or through anthropogenic inputs such as mining and agricultural applications. The two most common oxidation states for soluble arsenic in nature are As(V) and As(III), present as the oxyanions arsenate (AsO_4^{3-}) and arsenite ($As(OH)_3$), respectively. Although both forms are toxic to biological systems, they induce distinct types of cellular damage. Because of its structural analogy to inorganic phosphate, arsenate can enter a cell via phosphate membrane transport systems and disrupt metabolic reactions that require phosphorylation. In contrast, arsenite is transported into the cell by aquaglyceroporins in bacteria, yeasts and mammals, and exerts its toxicity by binding thiol groups in proteins, thereby impairing their function. Arsenite is much more toxic than arsenate (for reviews, see [14,18,22]).

To counteract the deleterious effects of arsenic, microorganisms have evolved several resistance strategies, including arsenite oxidation or methylation into less toxic species, as well as active extrusion of arsenite from the cell [22]. The genes encoding the arsenite detoxification machinery (*ars* genes) are widely distributed in bacteria and archaea and can be found on plasmids or chromosomes. They most commonly consist of either three (*arsRBC*) or five (*arsRDABC*) genes arranged in a single transcriptional unit. ArsB, an integral membrane protein that pumps arsenite out of the cell, is often associated with an ATPase subunit, ArsA. ArsC is an arsenate reductase that converts arsenate to arsenite prior to efflux. ArsR is a trans-acting repressor involved in the basal regulation of the *ars* operon, while ArsD is a second repressor controlling the upper levels of *ars* genes expression [14,28].

* Corresponding author. Tel.: +33 3 87 37 85 13; fax: +33 3 87 37 85 12.
E-mail address: asma.rokbani@umail.univ-metz.fr (A.R. Achour).

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Two unrelated families of arsenite transporters have been described in bacteria [14,23]. The well-characterized ArsB family includes membrane proteins of *Escherichia coli* plasmid R773 and *Staphylococcus aureus* plasmid pI258. The ArsB permease functions as a uniporter using the membrane potential to extrude arsenite. When associated with ArsA, the permease is converted to a more efficient ATP-driven arsenite pump that provides enhanced arsenite resistance. In addition to arsenite, proteins of the ArsB family actively expel antimonite oxyanions [11].

Much less is known about the second family of arsenite carriers, Acr3p (also called Arr3p). Members of this family are found in bacteria, archaea and fungi [22,36], but have been functionally characterized in only a few species including *Bacillus subtilis*, *Synechocystis* sp., *Corynebacterium glutamicum* and *Saccharomyces cerevisiae* [9,17,27,35]. The Acr3p proteins in *B. subtilis* and *S. cerevisiae* have been reported to specifically transport arsenite, whereas the *Synechocystis* sp. Acr3p homologue appears to promote both arsenite and antimonite extrusion.

Recently, different studies have focused on the detection of *ars* genes in environmental samples, to correlate their presence with the arsenic resistance phenotype and/or arsenic-transforming capacities of bacterial isolates [1,7,10,25] or to use these genes as potential molecular biomarkers of arsenic contamination [5,30]. Depending on the purpose, *arsA*, *arsB* and/or *arsC* were chosen as the target genes in these studies. However, the *ACR3* gene family has never been taken into account in such analyses, even though it appears to be widespread among bacteria [36]. In this study, we chose to focus on arsenite transporter genes, since their presence is observed in most *ars* operons [23]. For this purpose we designed different sets of degenerate primers that allow for the specific amplification of the two bacterial arsenite carrier gene families. These primers were used to investigate the occurrence and diversity of *arsB*- and *ACR3*-related genes in arsenic-resistant bacteria isolated from soils.

2. Materials and methods

2.1. Isolation of arsenic-resistant strains

The sampling site was a forest in the commune of Longeville-les-St-Avold, France that exhibits variable local background levels of arsenic and lead. Using a field portable X-ray fluorescence apparatus (Niton) (kindly provided by BRGM Nancy), two soil samples that differ in their contamination levels were selected from this site. After removal of the litter, soil samples were collected in sterile polyethylene bottles and stored at 4 °C until microbiological analysis (within 16 h), or further desiccated at 45 °C overnight before soil characterization. Soil A, referred to as "uncontaminated soil", contained 12.2 mg/kg (SD = 7.4) of arsenic (As) and 253 mg/kg (SD = 8.3) of lead (Pb), whereas soil B, referred to as "contaminated soil", had higher levels of As [135 mg/kg (SD = 23.9)] and Pb [2880 mg/kg (SD = 30)].

One gram of each soil was shaken for 30 min at 150 rpm in 9.5 ml of 0.1% (wt./vol.) sodium pyrophosphate solution. The mixture was centrifuged at 500 × *g* and 0.1 ml of serial dilutions of the supernatant were plated separately onto Luria–Bertani (LB) agar plates supplemented with 20 mM arsenate As(V) or 7 mM arsenite As(III). After 96 h of incubation at 25 °C, colonies were picked and streak-purified at least twice on the same medium. Purified single colonies were inoculated in LB broth, cultured for 24–48 h and stored in 25% glycerol at −80 °C. Bacterial colonies were grouped on the basis of colony morphology and Gram stain. For each soil, one representative isolate of each group was selected to test the primer sets designed.

2.2. Bacterial strains and growth conditions

Alcaligenes eutrophus AE126 (later called *Ralstonia metallidurans* and now *Cupriavidus metallidurans* [32]), was cultivated at 30 °C in a Tris-buffered minimal medium supplemented with 0.2% sodium gluconate [12]. *Cenibacterium arsenoxidans* ULPAs1 was grown at 25 °C in CDM medium [33]. All the other reference strains were grown in Luria–Bertani (LB) medium at 30 °C (*C. glutamicum* RES167 [34], *C. glutamicum* ArsB1-B2 [17], *Shewanella* sp. ANA-3 [24] and *Shewanella oneidensis* MR-1 [16]) or 37 °C (*Mycobacterium smegmatis* mc^2155 [29], *Escherichia coli* W3110 [2], *E. coli* AW3110 [4] and *E. coli* DH10B (Gibco BRL)).

Resistance to arsenic and antimonite was determined on solid medium (LB agar). Stationary phase cultures of the isolates or reference strains were adjusted to an OD_{600} of 0.3. Five microliters of these suspensions were spotted on LB plates supplemented with increasing concentrations of sodium arsenite (1.75–112 mM), sodium arsenate (20–640 mM) or potassium antimonyl tartrate (0.1–12.8 mM). The MICs, defined as the lowest metalloid concentration that inhibited growth on plates, were determined after 72 h incubation at 25 °C for isolates and at 30 or 37 °C for reference strains. Arsenite oxidase activity was detected in CDM agar plates after 48 h of incubation at 25 °C using the $AgNO_3$ method [15]. The *C. arsenoxidans* ULPAs1 strain was used as positive control [33].

2.3. DNA preparation

DNA was extracted from the isolates by a modification of the method of Pitcher et al. [19]. Briefly, freshly isolated colonies were inoculated in 5 ml LB and incubated at 25 °C. Bacterial pellets were resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA [pH 8.0]) containing lysozyme (50 mg/ml) and RNase (50 μg/ml). After incubation at 37 °C for 1 h, the bacteria were lysed by the addition of the GES reagent (guanidine thiocyanate, EDTA, sarcosyl). Ammonium acetate was added to the solution, followed by 10 min of incubation on ice. Chloroform was added to extract the proteins, and then the DNA was precipitated with isopropanol and washed with 70% ethanol. The DNA was resuspended in Tris–HCl buffer [10 mM, pH 8.0].

2.4. Degenerate primer design

Three sets of primers were designed to probe arsenite carrier-related genes in bacterial isolates. A total of 75 bacterial, archaeal and fungal protein sequences known to be responsible for arsenite efflux and putative homologues obtained from genome sequencing projects were acquired from GenBank and aligned using Clustal W. A preliminary phylogenetic analysis (not shown) confirmed previous observations by Wysocki et al. [36], i.e. arsenite transporters could be classified into two broad categories corresponding to the ArsB and Acr3p families, and the Acr3p-like proteins could be further divided into two branches which we designated as the Acr3(1)p and Acr3(2)p subgroups. The Acr3(1)p branch contains a fungal subgroup which was excluded from the analysis. Conserved regions in each of the three subgroups (ArsB, Acr3(1)p and Acr3(2)p) were identified using Block Maker and used to design COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) [21]. The three degenerate primer pairs, darsB1F/darsB1R (5′-GGTGTGGAACATCGTCTGGAAYGCNAC/5′-CAGGCCGTACACCACCAGRTACATNCC), dacr1F/dacr1R (5′-GCCATCGGCCTGATCGTNATGATGTAYCC/5′-CGGCGATGGCCAGCTCYAAYTTY TT) and dacr5F/dacr4R (5′-TGATCTGGGTCATGATCTTCCCVATGMTGVT/5′-CGGCCACGGCCAGYTCRAARAARTT), were selected for the detection of *arsB*, *ACR3*(*1*) and *ACR3*(*2*) genes, respectively. Degenerate nucleotide sites are indicated by standard ambiguity codes as follows: N = A, C, G, or T; R = A or G; V = A, C, or G and Y = C or T.

2.5. PCR amplification and sequencing

Purified plasmid pUM3 [13] and genomic DNA from reference strains listed in Table 1 were used as templates to optimize amplification conditions and to test the specificity of the degenerate primers. For PCR amplification of nearly full-length 16S rDNA gene, we used universal primers w01 (5′-AGAGTTTGATCMTGGCTC) and w02 (5′-GNTACCTTGTTACGACTT). All PCR reaction mixtures contained approximately 50 ng DNA template, 1 × PCR buffer, 0.2 mM of each deoxyribonucleoside triphosphates, 0.2 μM of each primer and 1 U Taq DNA polymerase (Eppendorf) in 25 μl volume. Amplifications were performed in a 2700 thermal cycler (Applied Biosystems). Cycling conditions for all PCRs consisted of 5 min of denaturation at 94 °C followed by 35 cycles of 45 s of denaturation at 94 °C, 45 s of annealing at 57–52 °C with a 0.5 °C decrement per cycle during the first 10 cycles and 30 s of primer extension at 72 °C. This was followed by a final extension reaction at 72 °C for 7 min. PCR products were purified with a Nucleospin gel extraction kit (Macherey-Nagel). Purified *arsB*/*ACR3* amplicons and 16S rDNA genes were ligated into a TA cloning vector (pCR2.1, Invitrogen). The products were used to transform *E. coli* DH10B competent cells. White colonies were screened for the presence of the correct size insert by colony PCR using vector specific primers and selected recombinant plasmids were purified (Perfectprep Plasmid Mini kit, Eppendorf) for sequencing analysis. DNA sequencing was performed by MWG-Biotech (Ebersberg, Germany), *arsB*/*ACR3* nucleotide sequences were determined on both strands.

2.6. Sequence analysis and phylogenetic tree

16S rDNA and *arsB*/*ACR3* gene sequences were compared to the entire GenBank nucleotide and amino acid databases using BlastN and BlastX programs, respectively (http://www.ncbi.nlm.nih.gov/Blast/). Phylogenetic analysis of 16S rDNA sequences and ArsB/Acr3p deduced amino acid sequences were performed using MEGA version 3.0 software [8]. The neighbor-joining distance method based on *p*-distance was used to construct phylogenetic trees and the validity of the branches was ascertained with 250 bootstrap replicates.

2.7. Nucleotide sequence accession numbers

The nucleotide sequences determined in this study are available in EMBL database under accession no. AM283001–AM283032 (partial *arsB*/*ACR3* genes) and AM284983–AM285023 (partial 16S rDNA genes).

3. Results

3.1. Identification and characterization of arsenic-resistant isolates

The abundance and diversity of arsenic-resistant bacteria was investigated in two soil samples with different levels of arsenic contamination. Resistance to arsenic was defined as the ability to grow on LB plates containing either 7 mM As(III) or 20 mM As(V) at 25 °C. Both culturable bacterial counts and percentage of arsenate-resistant bacteria were comparable between the uncontaminated (9.5×10^4 CFU/g soil and 10.5%, respectively) and contaminated (6.2×10^4/g soil and 8.3%, respectively) soil samples. The percentage of arsenite-resistant bacteria in the uncontaminated soil was 1.1%, while no arsenite-resistant colonies could be recovered from the contaminated soil. One representative of each phenotypic group was selected for further characterization, yielding a total of 41 arsenic-resistant strains (33 from soil A and 8 from soil B), of which 22 were Gram-positive and 19 were Gram-negative (Table 1).

These isolates were identified by partially sequencing the 16S rDNA gene and classified into 22 genera distributed between five major bacterial lineages (Fig. 1.): *Actinobacteria* (14 isolates), γ-*Proteobacteria* (12 isolates), *Firmicutes* (8 isolates), α-*Proteobacteria* (5 isolates) and β-*Proteobacteria* (2 isolates). Even though the low number of isolates in soil B does not allow for accurate statistical analysis, the relative abundance of each clade in the two soils appeared to be similar. Most genera recovered from the arsenic-contaminated soil B (i.e. *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Arthrobacter* and *Micrococcus*) were also found in the uncontaminated soil A. The latter sample, however, displayed higher bacterial

Table 1
Bacterial isolate characteristics

Strains and isolates[a]	MIC (mM)			*arsB/ACR3*[b]		
	As(V)	As(III)	Sb(III)	*arsB*	*ACR3(1)*	*ACR3*(2)
Gram-positive bacteria						
Low G + C (*Firmicutes*)						
A12 *Bacillus* sp. (99%)	40	7	>12.8	+		
A14 *Bacillus* sp. (99%)	320	7	>12.8	+		
A22 *Bacillus* sp. (99%)	80	7	1.6			
A17 *Bacillus* sp. (99%)	40	7	0.4		+	
A13 *Bacillus massiliensis* (97%)	320	1.75	0.8			
A20 *Sporosarcina* sp. (99%)	160	14	1.6	+		
A08 *Planococcus* sp. (98%)	>640	14	1.6			
B05 *Bacillus* sp. (99%)	80	14	>12.8	+		
High G + C (*Actinobacteria*)						
A15 *Tsukamurella strandjordae* (99%)	160	1.75	0.1		+	
A11 *Zimmermannella faecalis* (99%)	640	14	0.4		+	
A06 *Pseudoclavibacter helvolus* (99%)	640	3.5	0.1		+	
A19 *Plantibacter agrosticola* (99%)	80	1.75	0.1			
A33 *Microbacterium* sp. (99%)	640	56	3.2		+	
A01 *Arthrobacter* sp. (99%)	160	1.75	0.8	+		
A03 *Arthrobacter* sp. (98%)	320	14	0.1		+	
A05 *Kocuria* sp. (99%)	640	7	0.1		+	
A24 *Micrococcus luteus* (99%)	640	3.5	0.1		+	
A02 *Micrococcus luteus* (99%)	640	7	0.1		+	
A21 *Streptomyces* sp. (99%)	40	1.75	0.1		+	
B03 *Rhodococcus* sp. (99%)	320	14	0.8		+	
B02 *Arthrobacter* sp. (98%)	160	3.5	0.1		+	
B01 *Micrococcus* sp. (99%)	640	7	0.1		+	
Gram-negative bacteria						
Alphaproteobacteria						
A16 *Sinorhizobium* sp. (99%)	160	1.75	0.4			+
A26 *Phyllobacterium myrsinacearum* (99%)	320	3.5	0.4			+
A27 *Aminobacter aminovorans* (99%)	160	3.5	1.6			+
A10 *Paracoccus* sp. (99%)	160	3.5	0.8		+	
B04 *Ensifer adhaerens* (100%)	160	1.75	0.4			+
Betaproteobacteria						
A25 *Variovorax paradoxus* (99%)	160	7	0.8			
A31 *Variovorax* sp. (99%)	320	3.5	0.4			
Gammaproteobacteria						
A23 *Pseudomonas* sp. (99%)	640	14	0.4	+		
A30 *Pseudomonas putida* (100%)	320	3.5	0.8	+		
A29 *Pseudomonas fluorescens* (100%)	160	7	1.6	+	+	
A07 *Pseudomonas* sp. (99%)	320	7	1.6	+		+
B06 *Pseudomonas* sp. (99%)	320	1.75	0.8	+	+	
B08 *Pseudomonas* sp. (99%)	320	14	0.4	+		
A04 *Acinetobacter lwoffii* (99%)	320	14	1.6			
A32 *Acinetobacter* sp. (99%)	320	14	3.2			
B07 *Acinetobacter lwoffii* (99%)	40	3.5	1.6			
A28 *Stenotrophomonas* sp. (99%)	320	3.5	1.6			
A18 *Stenotrophomonas* sp. (99%)	320	1.75	1.6			
A09 *Stenotrophomonas* sp. (100%)	320	14	0.8			
Strains used as reference						
E. coli W3110	160	7	>12.8	+		
E. coli AW3110	20	1.75	0.4			
E. coli DH10B/pUM3	320	28	>12.8	+		
C. glutamicum RES167	>640	28	0.2		+	
C. glutamicum ArsB1-B2	20	3.5	0.2			
Shewanella sp. ANA-3	640	14	0.8	+		+
S. oneidensis MR-1	80	1.75	0.8			+
M. smegmatis mc^2155	40	1.75	0.2		+	

[a] Isolates were identified based on 16S rDNA sequence analysis. For each isolate, we indicated: (i) the bacterial strain having the closest 16S rDNA gene sequences, as determined by the Blast program, and (ii) the corresponding percentage of identities (in brackets). The size of 16S rDNA sequences used in the analysis is variable (564 -912 bp). A01-A33 isolates are from soil A and B01-B08 are from soil B.

[b] Arsenite transporter sequences were detected by PCR using the relevant group-specific primer pair.

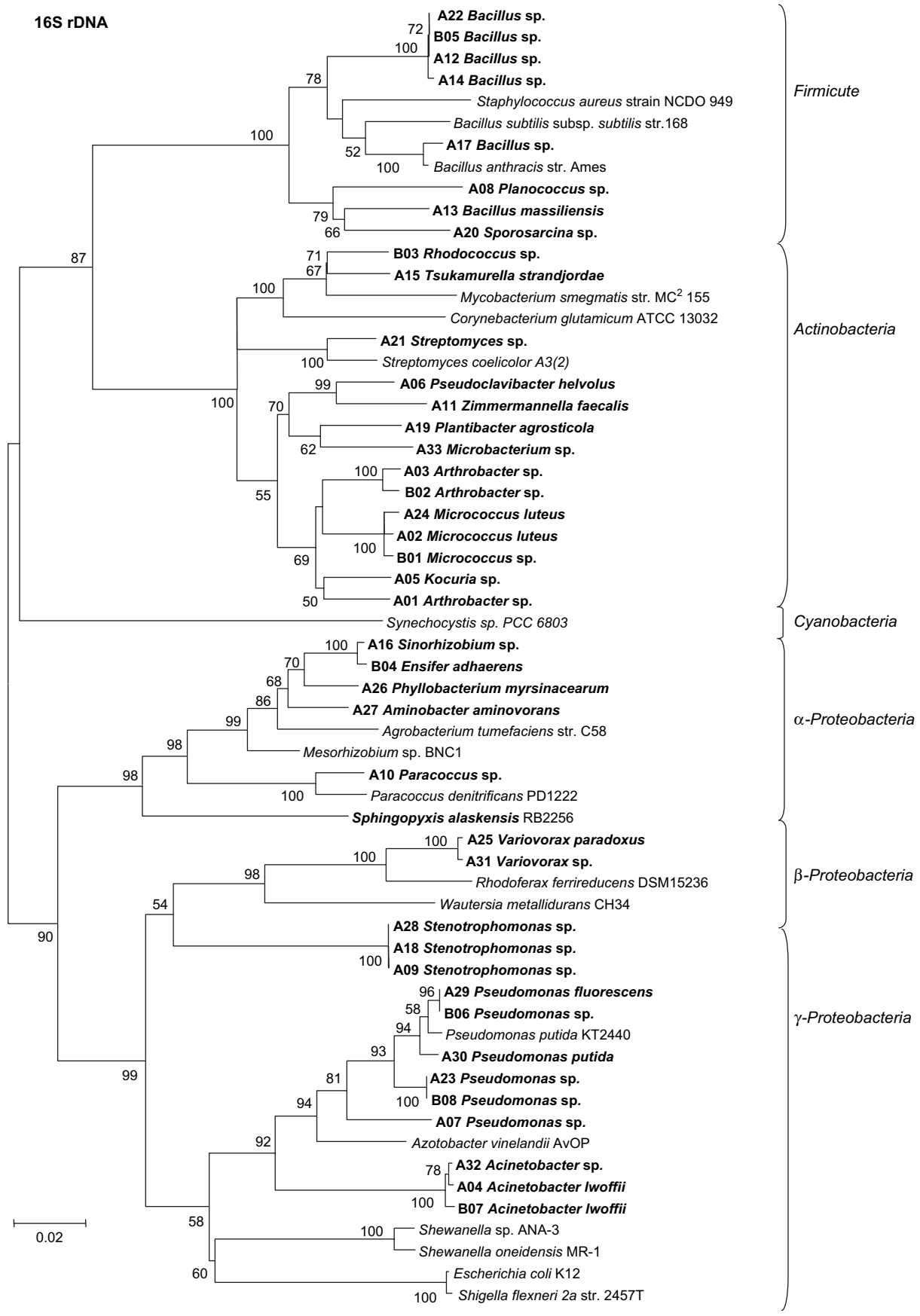

Fig. 1. Phylogeny of partial 16S rDNA genes (433 bp). Sequences from this study are in bold type. Bootstrap values over 50% are shown. The scale bars indicate substitutions per site.

diversity, with 20 different genera compared to the seven genera isolated from soil B.

The isolates were examined for their sensitivity/resistance to arsenate, arsenite and antimonite using a growth inhibition plate assay. For each metalloid, the range of minimum inhibitory concentrations (MIC) was rather large among the 41 isolates (Table 1), with relative toxicity ranking in the following order: arsenate < arsenite < antimonite.

The highest overall MICs for the three metalloid species were observed for Gram-positive isolates: A08, phylogenetically identified as *Planococcus* sp., displayed growth up to 640 mM arsenate; isolate A33, a close relative of *Microbacterium* sp., was still able to grow in the presence of 28 mM arsenite; and three isolates (A12, A14 and B05) identified as *Bacillus* sp. showed a MIC for antimonite higher than 12.8 mM. Interestingly, all the antimonite-sensitive isolates (MIC $\leq$ 0.1 mM) were found to belong to the *Actinobacteria* group. Conversely, this group contained the majority of arsenate-resistant isolates (7 out of 14 isolates with MIC up to 640 mM). Except for the latter findings, there was no apparent correlation between the metalloid resistance level of the isolates and either their phylogenetic affiliation or sample origin.

The isolates were also screened for arsenite oxidase activity. A reference arsenite-oxidizing bacterial strain (*C. arsenoxidans* ULPAs1) was included in the experiment for comparison purpose. Only three isolates responded positively to the assay, including highly arsenite-resistant *Microbacterium* isolate A33 and isolates A04 and B07 identified as *Acinetobacter*. Relative arsenite oxidase activity in these bacteria ranked in the following order: A33 < B07 = ULPAs1 < A04 (not shown).

3.2. Arsenite transporter gene sequences from arsenic-resistant isolates

Three sets of degenerate primers targeting the *arsB* and *ACR3* gene families of arsenite transporters were designed with the CODEHOP program. The specificity of the primers for *arsB* sequences was tested with purified pUM3 plasmid and genomic DNA from *Shewanella* sp. ANA-3. The ability of the primers to target *ACR3(1)* genes was tested by amplification from an *M. smegmatis* DNA extract. Genomic DNA from *C. metallidurans* AE126 as well as from *S. oneidensis* MR-1 were used as templates to check primer specificity for *ACR3(2)* genes. Genomic DNA from *E. coli* AW3110, a mutant strain lacking the *ars* genes [4], was used as a negative control. A single amplicon of the expected size (approximately 750 bp) was obtained with positive controls (data not shown). Cross-amplification of *Shewanella* sp. ANA-3 genomic DNA with the dacr5F/dacr4R primer pair generated a single PCR product, indicating that this strain contains an *ACR3(2)*-like gene in addition to the previously characterized *arsB* gene [24].

To assess the efficiency of our primers, the arsenic-resistant isolates characterized above were screened for the presence of *arsB*/*ACR3*-like genes. Of the 41 isolates, 29 showed successful amplifications with either one or two of the three specific primer sets (Table 1), with the following distribution: 11 *arsB*+ isolates (*Firmicutes* (4), γ-*Proteobacteria* (6) and *Actinobacteria* (1)); 16 *ACR3(1)*+ isolates (*Actinobacteria* (12), *Firmicute* (1), γ-*Proteobacteria* (2) and α-*Proteobacteria* (1)); five *ACR3(2)*+ isolates (α-*Proteobacteria* (4) and γ-*Proteobacteria* (1)). Both *arsB* and *ACR3* sequences were amplified from three isolates, A29 and B06 (*arsB*+, *ACR3(1)*+) and A07 (*arsB*+, *ACR3(2)*+), that are closely related to *Pseudomonas* sp. All PCR-positive isolates produced a single amplicon with an approximate length of 750 bp, except in the case of the A01 isolate from which a single PCR product of about 850 bp was reproducibly obtained. Negative PCR responses were obtained for the 12 remaining isolates which do not represent a specific taxonomic group and displayed variable levels of resistance to As(V), As(III) or Sb(III) (Table 1).

3.3. Phylogenetic analysis

Identity of the *arsB*/*ACR3* PCR products was confirmed by cloning and sequencing. The phylogenetic analysis (Fig. 2) placed the deduced ArsB amino acid sequences into two distinct clusters containing the well-characterized ArsB proteins of γ-*Proteobacteria* and *Firmicutes*, respectively. The only exception was the ArsB sequence of *Arthrobacter* isolate A01 that appeared unrelated to previously known ArsB sequences. The most distant *arsB* sequences (A01 and A14/A12) displayed 46.9% nucleotide sequence identity.

ACR3(1) sequences were detected in *Actinobacteria*, *Firmicutes* and γ-*Proteobacteria* (Table 1). However, a large majority (75%) were *Actinobacteria* representatives. The Acr3(1)p sequences of isolates A29 and B06 identified as *Pseudomonas*, and the *Paracoccus* related isolate A10, formed a distinct cluster which also contained Acr3(1)p sequences of α-*Proteobacteria* strains. It is noteworthy that the two latter *Pseudomonas* isolates also possessed an *arsB* gene. The Acr3(1)p sequence of *Bacillus* A17 was clustered with those of other *Bacillus* strains. The third cluster was formed by 11 Acr3p sequences of *Actinobacteria* isolates and contained other Acr3(1)p of *Actinobacteria* strains such as *Streptomyces coelicolor* and *M. smegmatis*. Only the Acr3(1)p sequence of A06 (*Pseudoclavibacter*) branched outside of the *Actinobacteria* cluster. There was 51.3% nucleotide sequence identity between the most distant *ACR3(1)* sequences A06 and A17.

Five *ACR3(2)* sequences were detected in Gram-negative isolates (Table 1). Four closely related sequences were from α-*Proteobacteria*, and a more distant sequence was present in the *Pseudomonas* isolate A07, which also contained an *arsB* sequence. The nucleotide sequence identity between A07 and A26 *ACR3(2)* sequences was 66.4%.

4. Discussion

The diversity of arsenic-resistant bacteria obtained from the two screened soil samples was high. To our knowledge, about one half of the 22 genera identified based on 16S rDNA sequence data were not previously reported in the literature as either arsenic-resistant or arsenic-transforming genera. Most of them were recovered from the less contaminated sample. The lower diversity of arsenic-resistant bacteria in the

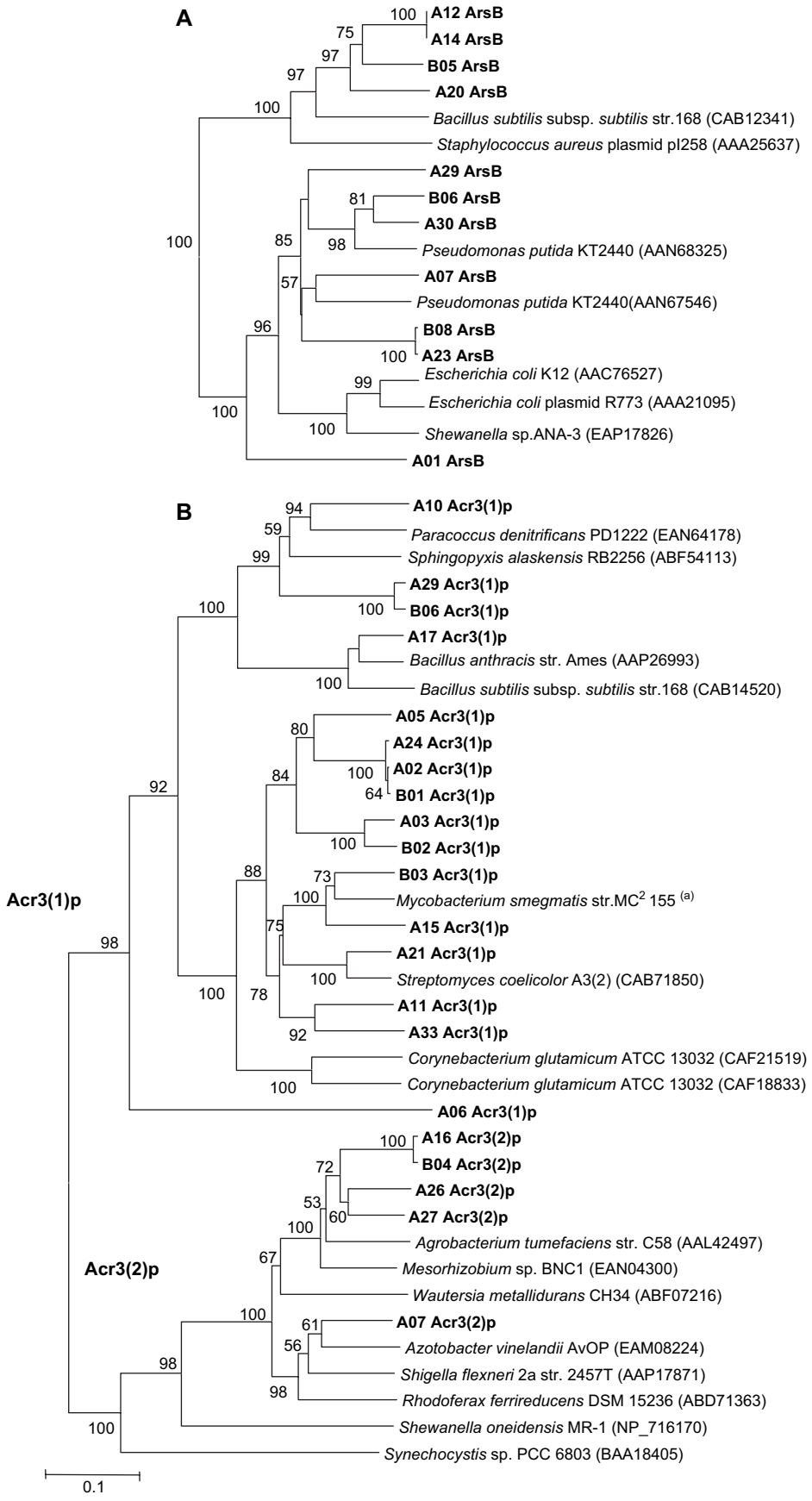

Fig. 2. Phylogeny of deduced amino acid sequences (212 amino acids) from *arsB* (A), and *ACR3(1)*/*ACR3(2)* (B) sequences. Sequences from this study are in bold type and the GenBank accession numbers for sequences obtained from the NCBI database are shown in parentheses. Bootstrap values over 50% are shown. The scale bars indicate substitutions per site. (a) *M. smegmatis* Acr3(1)p sequence was obtained from the Institute for Genomic Research (TIGR) (gnl|TIGR_246196 contig:3563: m_smegmatis).

contaminated sample was not unexpected, since high levels of metallic pollutants are likely to exert a strong selective pressure and thus reduce overall microbial diversity. In a recent study conducted on an estuary with an insignificant level of arsenic, Jackson et al. [7] isolated 37 different arsenate-resistant bacteria belonging to six major bacterial clades. These results, together with those obtained in the present study, indicated that arsenic-resistant bacteria appear to be phylogeneticaly diverse and widely distributed in the natural environment, regardless of the level of arsenic contamination. In addition to arsenate and arsenite, MICs were determined for antimonite, since tolerance to this metalloid is mediated by the *ars* operon in a variety of bacteria. A direct comparison of MIC values to those from other studies was not attempted, as differences in the method of resistance determination (growth inhibition on solid vs. liquid medium) and in the medium composition (e.g. organic constituents and phosphate concentration) may affect metalloid bioavailability and toxicity, resulting in discrepancies in the MIC values. Nevertheless, *E. coli* and other strains whose tolerance to arsenic has been previously characterized (e.g. *C. glutamicum* [17]) were included in the analysis for comparison purpose.

We found that 29 out of the 41 (70.7%) arsenic-resistant isolates contained a gene or genes related to *arsB*/*ACR3*. The sequencing of all amplicons confirmed the high selectivity of the PCR approach, with each primer pair allowing specific amplification of the targeted arsenite carrier group. Several reasons can explain the PCR failure for the 12 remaining isolates. First, if we hypothesize that the *arsB*/*ACR3* genes are the primary determinants in arsenite resistance, their absence in arsenite-sensitive (MIC $\leq$ 1.75 mM) isolates such as *Bacillus* A13, *Plantibacter* A19 and *Stenotrophomonas* A18 is not surprising. A similar situation has recently been reported for *Sinorhizobium meliloti* 1021, whose *ars* operon includes an aquaglyceroporin gene (*aqpS*) in place of *arsB* and confers resistance to environmental arsenate only [37]. In the case of isolates that exhibited an arsenite resistance phenotype, unsuccessful amplification may be due to the presence of an arsenite transporter gene homologue with a highly divergent DNA sequences. Finally, alternative mechanisms could be used by these isolates to cope with arsenite toxicity. Such mechanisms could include arsenite methylation that results in volatile products that escape from the cells [20] or arsenite oxidation to produce the less toxic arsenate [28]. The latter explanation may be valid for two *Acinetobacter* isolates (A04 and B07) which apparently lacked the *arsB*/*ACR3* gene while exhibiting arsenite oxidase activity. It is noteworthy that a Blast search in the genome of *Acinetobacter baylyi* ADP1 (http://www.genoscope.cns.fr/externe/English/Projets/Projet_DY/organisme_DY.html) did not reveal the presence of any arsenite carrier gene homologue, while the *Acinetobacter baumanii* strain AYE genome was recently shown to possess an atypical *ars* operon (*arsCRCBH*) located within a genomic resistance island presumably acquired by lateral gene transfer [6]. An arsenite-resistant bacteria identified as *Acinetobacter* has also been isolated from an arsenic-contaminated site in New Zealand. However, its ability to oxidize arsenite was not tested [1].

Phylogenetic analysis showed that *arsB*/*ACR3* genes were broadly distributed among our arsenic-resistant isolates. Because early studies on *ars* operons revealed the presence of the *arsB* gene, it was previously assumed that the largest fraction of bacteria employed transporters of the ArsB family to expel arsenite from the cells. In this study, however, we found that the *ACR3* genotype was predominant over *arsB* (21 *ACR3*(+) *versus* 11 *arsB*(+)). This result is consistent with the growing number of *ACR3*-like sequences retrieved from microbial genome data and suggests that this gene family of arsenite transporters might be much more widespread than initially thought.

One limitation in the PCR approach we used lies in its inability to directly show the presence of multiple alleles of an arsenite transporter gene in single strain, as is the case in several bacteria such as *C. glutamicum* [17] or *Pseudomonas putida* KT2440 [3]. Such information can be easily obtained for individual strains via sequencing a significant number of cloned PCR products or by DNA hybridization. These experiments were not undertaken here because of the large number of isolates to be analyzed. Nevertheless, the use of group-specific primer pairs revealed the presence of both *arsB* and *ACR3* genes in three *Pseudomonas* isolates (A07, A29 and B06), a case that has been reported only in *B. subtilis* up to now [23]. The presence in *Shewanella* sp. ANA-3 of an *ACR3*(*2*)-like gene in addition to the previously characterized *arsB* gene [24] is now confirmed by analysis of the draft genome sequence data (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

Another observation that merits further investigations is the positive correlation between the antimonite sensitivity phenotype and the presence of the *ACR3*(*1*) genes. We found that 75% of *ACR3*(*1*)+ isolates belonged to the *Actinobacteria* lineage. Of these, a large majority (9 out of 12) were sensitive to the lowest tested antimonite concentration (MIC $\leq$ 0.1 mM). One possible explanation is that the Acr3(1)p proteins in these isolates are functionally similar to those of *B. subtilis* and *S. cerevisiae*, which are unable to expel antimonite out of the cell and thus provide sensitivity to this metalloid [27,35]. Consistently, disruption of the two arsenite transporter genes in *C. glutamicum*, which belong to the *ACR3*(*1*) group according to our phylogenetic analysis and that of Wysocki et al. [36], resulted in hypersensitivity to arsenite [17] but not antimonite (Table 1). Higher antimonite resistance levels observed in the other isolates may result either from the ability of the arsenite transporter to carry out antimonite export, as recognized early for the *E. coli* ArsB protein [31] and more recently for the *Synechocystis* sp. Acr3(2)p-related protein [9], from reduced antimonite uptake [26], or from a still unknown resistance mechanism.

Except for the above observation, there was no clear relationship between the different analyzed parameters, i.e. phylogenetic affiliation, metalloid resistance levels and *arsB*/*ACR3* genotype. This observation is not unprecedented and has been reported in various studies dealing with bacterial arsenic resistance in the environment. For example, Ford et al. [5] failed to correlate the level of arsenate resistance with the prevalence of *ars* genes in a set of aerobic and anaerobic

bacteria. Similarly, several authors found that closely related isolates based on their 16S rDNA sequences exhibited variable tolerance to arsenicals [7,10]. Whatever the possible reasons for such variations, they preclude the use of either phylogenetic identity or the presence of *arsB*/*ACR3* genes alone as reliable criteria to predict the behavior of bacterial communities towards arsenicals.

The primers we developed in this work proved to be highly specific, yet enabled the isolation of diverse sequences in each of the targeted arsenite transporter gene group, indicating that they could be useful to further explore the diversity of key arsenic resistance genes in a variety of bacteria. Furthermore, given that expression of most *ars* operons described to date is inducible by arsenicals, the quantification of the *ars*B/*ACR3* mRNA level would serve as a sensitive biomarker of arsenic stress and provide a means to assess the impact of arsenic contamination on bacterial communities in natural environments.

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