Arsenite-induced changes in abundance and expression of arsenite transporter and arsenite oxidase genes of a soil microbial community

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Abstract

We describe a real-time PCR assay for the quantitative detection of arsB and ACR3(1) arsenite transporter gene families, two ubiquitous and key determinants of arsenic resistance in prokaryotes. The assay was applied in batch growth experiments using a wasteland soil bacterial community as an inoculum to investigate the effect of increasing arsenite [As(III)] concentrations on genes and transcript abundances. The aioA gene encoding the large subunit of arsenite oxidase was monitored in parallel. Results showed that arsB and ACR3(1) gene abundances correlated positively with the As(III) concentration. Both genes showed similar transcription patterns and strong upregulation by arsenic. Microbial As(III) oxidation occurred in As(III) spiked cultures and was associated with expression of the aioA gene in most cases. However, aioA was also expressed in several non-amended culture replicates. Analysis of cDNA clone libraries revealed that Pseudomonas was the dominant metabolically active genus whatever the As(III) concentration. Expressed arsB and ACR3(1) gene sequences were also affiliated with those from Pseudomonas, while expressed aioA sequences were more taxonomically diverse. The study suggests that arsenite transporter genes are appropriate biomarkers of arsenic stress that may be suitable for further exploring the adaptive response of bacterial communities to arsenic in contaminated environments.

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Keywords: Bacterial community; Arsenic resistance; Arsenite oxidation; Gene expression; Biomarker

1. Introduction

Arsenic is a widespread toxic metalloid present in the upper crust of the Earth but also in atmospheric dust, seawater and sometimes fresh water, which is a major issue of public health. Its presence in the environment is naturally due to the geochemical background, i.e. the weathering of parent material

and volcanic eruptions, but the main contamination sources are anthropogenic activities such as mining and the metalworking industry (Mukhopadhyay et al., 2002). Arsenic occurs in the environment primarily as inorganic forms of pentavalent arsenate As(V) and trivalent arsenite As(III), with the latter being considered the most mobile and toxic form. Due to its structural similarity to phosphate, As(V) exerts its toxicity by uncoupling oxidative phosphorylation, thereby disrupting ATP synthesis, while As(III) binds to thiol groups, which can result in enzyme inhibition (Oremland and Stolz, 2003).

Because of the environmental ubiquity of arsenic, bacteria have evolved various detoxifying mechanisms that mainly rely on redox reactions and/or efflux systems. The most common pathway for arsenic detoxification is encoded by the ars genes,
which can be either chromosomal or plasmid-borne (Rosen, 2002). This system basically involves the enzymatic reduction of As(V) by ArsC to As(III), which is pumped out of the cell by a membrane transport protein from ArsB or ACR3 unrelated families. Members of the ArsB family are prevalent among prokaryotes, but are not found in eukaryotes, and the ACR3 family is even more widespread, with members found in bacteria, archaea and fungi (Achour et al., 2007; Fu et al., 2009; Mansour et al., 2007). The expression of the ars operon is controlled by ArsR, an arsenite-responsive transcription regulator that represses gene expression under non-stress conditions and is released from the promoter in response to arsenic exposure, leading to rapid accumulation of As detoxifying enzymes in the cell (Rosen, 1999).

Bacterial oxidation of As(III) has long been regarded as a detoxification process by which As(III) is converted to the less toxic and less mobile form As(V) through an arsenite oxidase, AioAB (formerly AoxAB, AroAB or AsoAB) (Lett et al., 2012). However, As(III) also serves as an electron donor for energy generation in a few chemolithotrophic microorganisms (Silver and Phung, 2005; Stolz et al., 2006). Compared to the ars system, the expression of which is controlled only by the arsenic concentration, the regulation of aioAB genes appears to be more complex, involving multiple control determinants that may differ depending on the strain (Slyemi and Bonnefoy, 2011).

The soil constitutes a sensitive ecosystem whose functional stability is guaranteed by a large variety of microorganisms involved in a wide range of processes. Numerous studies have shown that metal contamination could significantly affect the structure of soil microbial communities and associated activities; however, major gaps remain in understanding how microorganisms respond to metal exposure in soil (Giller et al., 2009). In the last decade, real-time quantitative PCR (qPCR) has emerged as a sensitive and accurate method for measuring the abundance of phylogenetic or functional gene markers in complex microbial communities. Furthermore, coupling qPCR with reverse transcription (RT-qPCR) provides a powerful means of detecting and quantifying specific messenger RNA (mRNA) transcripts corresponding to the activity of interest in environmental samples (Saleh-Lakha et al., 2011; Smith and Osborn, 2009). Until now, qPCR-based approaches have been used successfully for quantitative detection of functional groups of bacteria involved in biogeochemical cycling of nitrogen, sulfur or carbon, but also of functional genes encoding key enzymes in biodegradation of organic pollutants (Saleh-Lakha et al., 2011; Smith and Osborn, 2009). However, reports on specific molecular markers of metal(loid) stress in the microbial community remain scarce (Bouskill et al., 2007; Sun et al., 2004).

In the present study, we aimed to determine whether the abundance and/or expression of the arsB, ACR3 and aioA genes could serve as indicators of arsenic stress and provide a means to assess the impact of arsenic contamination on bacterial communities. A novel qPCR method targeting arsB and ACR3(1) genes using a set of designed previously primers (Achour et al., 2007) was developed and applied, together with an aioA-targeting qPCR assay (Quemeneur et al., 2010), to investigate the dynamics of arsenite transporter and arsenite oxidase gene abundance and expression in a soil bacterial community exposed to increasing As(III) concentrations in batch growth cultures.

2. Material and methods

2.1. Soil samples

The soil used in this study is an industrial soil from a former coking plant site (Neuves-Maison, France) contaminated by a mixture of PAHs, heavy metals and arsenic. Soil characteristics have been described in previous studies (Cebron et al., 2008). Total arsenic content was 80 ppm. Soil was collected in September 2010 from four plots colonized by spontaneous vegetation (NM-SV) in a long-term field experiment conducted at Homécourt (France; Cebron et al., 2009). Soil samples were combined in a composite sample, sieved to <5 mm and stored field-moist at 4°C until use.

2.2. Preparation of inoculum and incubation conditions

Indigenous microbial cells were recovered from a total of 100 g of soil by Nycodenz density gradient centrifugation (Lindahl and Bakken, 1995). Briefly, 10 g soil samples were suspended in 30 ml of 0.85% NaCl and homogenized with 10 g glass beads for 1 h at room temperature. The obtained soil slurry (20 ml) was placed in a centrifuge tube above 10 ml of Nycodenz (Abcys SA; density = 1.3 g ml⁻¹) and the tube was centrifuged for 1 h at 10,000 g. The cell fraction was recovered and diluted in sterile water before centrifugation for 20 min at 4000 g to remove Nycodenz. The cell pellet was then suspended in 10 ml of 0.85% NaCl. This bacterial suspension was inoculated to a final concentration of 3 × 10⁵ cells ml⁻¹ into Erlenmeyers flasks (250 ml) containing 35 ml Bushnell Haas broth (Difco, pH = 7.4) supplemented with 0.4% glucose, 75 µg ml⁻¹ cycloheximide (to inhibit fungal growth) and spiked or not with 10 µM, 100 µM, 1 mM or 10 mM arsenite (NaAsO₂, Merck). These concentrations were chosen to encompass, as far as possible, As(III) concentrations previously shown to robustly induce expression of ars genes (e.g. Diorio et al., 1995; López-Maury et al., 2003; Ordoñez et al., 2005; Tauriainen et al., 1997) and the broad range of As(III) resistance levels in bacteria. Cultures of each condition were conducted in triplicate. An additional flask with non-inoculated medium was used as negative control for each arsenite test concentration. The flasks were incubated at 24°C on a rotary shaker (150 rpm) for 7 days. Samples (8 ml) were taken after 2, 3, 5 and 7 days of incubation for the determination of microbial growth, glucose consumption, pH, arsenic speciation and extraction of nucleic acids.

2.3. Analytical methods

The glucose concentration was measured using a glucose oxidase-peroxidase method (glucose GOD-PAP, BioLabo) according to the manufacturer’s instruction, with absorbance...
reading at 505 nm using an FLX Xenius spectrofluorometer (SAFAS, Monaco). Changes in culture optical density were monitored at 600 nm.

The arsenate concentration was determined by ion chromatography using an ICS-3000 HPLC system (Dionex, USA) equipped with an AS autosampler eluent generator, column oven and conductivity detector. An anion-exchange column (IonPac AS11, Dionex) equipped with a guard column (IonPac AG11, Dionex) was used to separate anions.

The total arsenic concentration was determined with ICP-OES after microwave digestion. The concentration of As(III) was determined by calculating the difference between values obtained for total As and As(V).

2.4. DNA and RNA co-extraction and cDNA synthesis

Nucleic acids were extracted from 6 ml cultures using the FastRNA Pro Soil-Direct kit (MP Biomedicals) following the manufacturer’s recommendations. After suspension in 200 μl DEPC water (kit protocol step 17), the DNA-RNA mix was divided into two fractions of 150 μl and 50 μl. The former served for RNA purification using the same kit at step 18, while DNA was purified from the 50 μl fraction with the Fast DNA spin kit (MP Biomedicals), starting at step 5. To remove residual DNA, all RNA samples were treated with RNase-free DNase I and cleaned-up with the RNeasy mini-kit (Qiagen). Purified RNA was stored at -80 °C until processing.

First-strand cDNA was synthesized from approximately 800 ng RNA using random hexamer primers and Superscript III first-strand synthesis system for RT-PCR (Invitrogen). Control reactions lacking reverse transcriptase were performed on triplicate samples. Samples were scored as positive if detected in the two runs. Samples testing positive in one replicate were scored as possible positive only if detected in the two runs. Samples testing positive

2.6. Real-time PCR assay

Standards for real-time PCR were prepared as follows: partial arsB, ACR3(1) and aioA gene fragments were amplified by PCR using the appropriate primer pair from plasmids UPM3 (Mobley et al., 1983), pFI (Achour-Rokbani et al., 2010) and Alcaligenes sp. YI013H genomic DNA (Quemeneur et al., 2008), respectively, and cloned into the pGEM-T vector. Plasmid standard for 16S rRNA genes was prepared as described by Cebron et al. (2008). All plasmids were linearized at a unique BamHI site to prevent erroneous calibration due to coiled DNA. The constructs were serially diluted to 10⁸ to 10 copies μl⁻¹ in double-distilled water and used to generate the standard curves. The DNA concentration was quantified by spectrophotometry and the gene copy number was calculated according to the pGEM-T and cloned gene sequence lengths, assuming a molecular mass of 660 Da for a base pair. qPCR of aioA and 16S rRNA genes was done as described by Quemeneur et al. (2010) and Cebron et al. (2008), respectively. For qPCR of arsB and ACR3(1), primer pairs darsB1F/darsB1R (5'-GGGTGGAA CATCCTCTGAAAGCANC-3'/5'-CAGGCGTACACCCACGARTACATNCC-3') and dacr1F/dacr1R (5'-GCCATCGCG CCTGATCGTNATGATGTAYCC-3'/5'-CGGGCGATGGGCCAC GTCYAYTTYT-3') (Achour et al., 2007) were used. The same amplification conditions were applied to both genes. Reactions were performed in a total volume of 20 μl containing 1 x iQ SYBR Green Supermix (Bio-Rad), 0.4 μM of each primer, 0.6 μl bovine serum albumin, 0.2 μl of dimethylsulphoxide, 0.08 μl of T4 bacteriophage gene 32 product (QBiogene) and 1 μl of template DNA or distilled water for the negative control. Cycling conditions consisted of 5 min of denaturation at 95 °C followed by 50 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 53 °C and 30 s of primer extension at 72 °C and a final extension step of 7 min at 72 °C. A melting curve analysis was performed at the end of each run to check amplification specificity. PCR inhibition was assessed on a random selection of DNA extracts by spiking samples with known amounts (10⁴ to 10⁷ copies) of ACR3(1) plasmid standard. None of the tested samples showed PCR inhibition. All runs were performed in an iCycler iQ (Bio-Rad) associated with iCycler Optical System Interface software (version 2.3; Bio-Rad). Amplicons obtained from real-time PCR assays were cloned and sequenced as described above for conventional PCR products.

2.7. Data analysis

The real-time PCR data presented in the following sections are the means (n = 6) from two independent runs, each performed on triplicate samples. Samples were scored as positive if targeted genes were quantified into two or three replicates. Samples testing positive in one replicate were scored as positive only if detected in the two runs. Samples testing positive
in only one of the two runs were considered ambiguous and not included in subsequent analyses.

The ratios of arsenic genes (arsB, ACR3(1) and aioA) relative to 16S rRNA genes and of transcripts relative to target genes were analyzed through two-way analysis of variance (ANOVA) with \( \alpha = 0.05 \). Correlations between gene abundance and arsenite concentration were tested using the Pearson correlation coefficient. All statistical analyses were carried out using XLSTAT 2011 software (Addinsoft).

3. Results

3.1. Efficiency of real-time PCR primers targeting arsB and ACR3(1)

Two primer pairs enabling separate amplification of arsB and ACR3(1) families of arsenite transporter genes were applied to real-time PCR. The amplification of the two genes was found to be optimal with an annealing temperature of 53 °C. We also included an extension step at 72 °C, as the two primer pairs targeted a rather large region of approximately 750 bp. When amplifying arsB from the standard plasmid, a positive log linear correlation (\( R^2 = 0.990 \) to 0.998) was observed between the copy number and PCR threshold cycle number, covering 6-log units between 10^2 and 10^8 copies/reaction (Fig. S1). Similar quantitative linearity (\( R^2 = 0.990 \) to 0.997) was reproducibly shown from 10^1 to 10^8 copies per reaction for ACR3(1) calibration curves. The mean qPCR efficiencies were 100.6% for arsB and 97.1% for ACR3(1). In both cases, melting curves showed a single peak, indicating specific amplification and no significant primer dimer formation. In subsequent analyses of microbial community DNA or cDNA, all qPCR products yielded single bands of the expected size on agarose gel, confirming the specificity of the two primer pairs.

3.2. Impact of arsenite on the growth and arsenite oxidation activity of a Nycodenz-extracted soil bacterial community

Bacterial growth in batch experiments was affected by As(III) in a dose-dependent manner. At the lowest As(III) concentration (10 μM), the growth curve did not differ from the control, while initial growth rates were reduced at 100 μM and 1 mM As(III) (Fig. 1A). The maximal inhibitory effect was observed at 10 mM As(III), where the onset of growth was delayed for 48 h. Correlatively, the rates of glucose consumption and of the resulting medium acidification were reduced as the As(III) concentration increased (Fig. 1B and C). The inoculum displayed As(III) oxidation activity, even at low As(III) concentrations (10 μM and 100 μM), where As(V) was detected in the medium after 48 h incubation. In flasks spiked with 1 mM As(III), conversion to As(V) also started after 48 h and was complete after 120 h, while it was delayed and incomplete at the end of incubation in the presence of 10 mM As(III) (Fig. 1D). It should be noted that for the latter concentration, large variations in As(III) oxidizing activity, but also pH and glucose consumption, were seen among the three replicates, suggesting selection of different bacterial populations, likely due to As(III) toxicity. The observed chemical modifications of the medium were attributable to microbial activity, as evidenced by the absence of significant variation in pH.
the initial medium characteristics in non-inoculated assays (abiotic control).

3.3. Abundance and expression of \textit{arsB}, \textit{ACR3(1)}, \textit{aioA} and 16S rRNA genes

In order to investigate whether bacterial arsenite transporter and arsenite oxidase genes were overexpressed during mid-term As(III) exposure, culture aliquots were taken for DNA and RNA co-extraction at different incubation times after inoculation, and subsequent quantification of \textit{arsB}, \textit{ACR3(1)}, \textit{aioA} and 16S rRNA gene fragments and respective transcripts was performed by real-time PCR.

Both arsenite transporters and arsenite oxidase-encoding genes were detected in the inoculum. \textit{ACR3(1)} genes were present at $8.2 \times 10^{-3} \pm 3.2 \times 10^{-4}$ copies per 16S rRNA gene copy, while \textit{arsB} and \textit{aioA} genes were significantly less abundant, with $2.4 \times 10^{-3} \pm 1.9 \times 10^{-4}$ and $1.6 \times 10^{-3} \pm 4.1 \times 10^{-4}$ copies per 16S rRNA gene copy, respectively.

Overall, the abundance of \textit{arsB} gene did not change over time, but was affected by the As(III) concentration, with higher values observed at 1 mM and 10 mM As(III) (Fig. 2A). The \textit{ACR3(1)} gene copy number showed a similar trend with respect to arsenic concentration, i.e. it was at a maximal level at 1 mM As(III), but was also altered over time (Fig. 2B). In contrast to arsenite transporter genes, the abundance of \textit{aioA} was significantly lower at high arsenite concentrations (1 mM and 10 mM As(III)). However, the \textit{aioA} copy number also changed over time, as observed for \textit{ACR3(1)} (Fig. 2C). There was no significant interaction between arsenite concentration and time for the latter genes. Interestingly, Pearson’s correlation analysis revealed that both \textit{arsB} and \textit{ACR3(1)} gene abundances were correlated with the As(III) concentration, while no such relationship was noted for \textit{aioA} (Table 1).

The transcriptional activity of \textit{arsB}, as measured by gene-copy-normalized transcript abundance (\textit{arsB} mRNA/DNA ratio), was undetectable in the As(III)-free control as well as at a low As(III) concentration (10 μM throughout the experiment (Fig. 3A)). Incubation with higher As(III) concentrations led to dramatic increases in \textit{arsB} transcriptional activity after 72 h. In cultures spiked with 100 μM As(III), maximal gene expression occurred after 72 h and was followed by a progressive decrease to an undetectable level after 168 h. In contrast, at 1 mM and 10 mM As(III), expression rose to maximal levels later, at 120 h, and subsequently decreased. The transcription dynamics of the second arsenite transporter-encoding gene \textit{ACR3(1)} showed striking similarities with that of \textit{arsB} (Fig. 3B). The only noticeable differences were in higher levels of \textit{ACR3(1)} expression at 10 mM As(III) and a more rapid drop in expression at 100 μM As(III). As observed for arsenite transporter genes, transcripts of \textit{aioA} were first detected after 72 h of incubation in As(III) spiked cultures, but also in one of the three As(III)-free replicate controls (Fig. 3C). This transcription was detected in a second As(III)-free replicate after 120 h and remained at a level similar to that of As(III) spiked cultures. \textit{aioA} transcripts were never detected at 10 mM As(III).

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Fig. 2. Time evolution of the relative abundance (gene copy number/16S rRNA gene copy number ratios) of (A) \textit{arsB}, (B) \textit{ACR3(1)} and (C) \textit{aioA} genes in arsenite spiked cultures and arsenite-free controls, determined by real-time PCR. Data are mean values ± SEM (n = 3). The tables below each chart show results of two-way ANOVA, with different letters indicating significant differences ($p < 0.05$). Arrows (>) indicate relative abundance of each target gene in the inoculum. Values below the limit of detection are indicated by an asterisk (*).
We also calculated the 16S rRNA to rDNA ratios to estimate the growth state of bacteria during batch experiments. These ratios sharply increased between 48 h and 72 h of incubation during the exponential growth phase and remained at a maximal level or slightly decreased when entering into stationary phase (Fig. 3D). Comparison of the dynamics of gene expression showed that high transcript levels of \( {\text{arsB}}, {\text{ACR3(1)}}, {\text{aioA}} \) occurred mostly at high 16S rRNA to rDNA ratios, i.e. during the period of active cell growth or shortly thereafter.

3.4. Diversity of expressed \( {\text{arsB}}, {\text{ACR3(1)}}, {\text{aioA}} \) and 16S rRNA genes

16S rRNA clone libraries were prepared from 120-h old cultures exposed to 1 mM and 10 mM As(III), as well as from unspiked control cultures, to investigate the diversity of metabolically active populations. The phylogenetic analysis of 46 clones revealed that they were all affiliated with the genus \( {\text{Pseudomonas}} \) except for one clone related to the genus \( {\text{Enterobacter}} \) (Table 2). The two dominant \( {\text{Pseudomonas}} \)-related sequences were recovered from both the control and arsenic-spiked cultures, while the Enterobacter-related sequence was detected only in As(III)-free cultures. Consistently, cDNA clone library analyses of arsenite transporter genes also indicated sequences most similar to that of \( {\text{Pseudomonas}} \) species. In the case of \( {\text{aioA}} \), a single sequence affiliated with \( {\text{Pseudomonas}} \) was expressed in the absence of arsenic. This sequence turned out to be present at 1 mM As(III), but was also accompanied by four other \( {\text{aioA}} \)-like sequences showing diverse taxonomic affiliations.

4. Discussion

The adaptive response of microbial communities to arsenic in contaminated environments is still poorly understood. In this study, we describe SYBR-Green-based real-time PCR assays to explore this response through measurement of the abundance and expression of arsenite transporter and arsenite oxidase genes, two key components of bacterial tolerance to this metalloid.

### Table 1

Pearson’s correlation analyses of bacterial gene abundance with total arsenic concentration at sampling times.

<table>
<thead>
<tr>
<th>Gene abundance</th>
<th>Pearson’s correlation coefficient (( p )-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As-48 h</td>
</tr>
<tr>
<td>16S rDNA/( m )g DNA</td>
<td>0.041 (0.899)</td>
</tr>
<tr>
<td>( {\text{arsB}} )/( m )g DNA</td>
<td>0.712 (0.009)</td>
</tr>
<tr>
<td>( {\text{arsB}}/16S ) rRNA</td>
<td>0.499 (0.099)</td>
</tr>
<tr>
<td>ACR3(1)/( m )g DNA</td>
<td>0.727 (0.007)</td>
</tr>
<tr>
<td>ACR3/16S rRNA</td>
<td>0.676 (0.016)</td>
</tr>
<tr>
<td>( {\text{aioA}}/m )g DNA</td>
<td>–0.457 (0.135)</td>
</tr>
<tr>
<td>( {\text{aioA}}/16S ) rRNA</td>
<td>–0.722 (0.008)</td>
</tr>
</tbody>
</table>

* Significant (<0.05) \( p \)-values for the correlations are indicated in bold. Note that abundances of the the \( {\text{ACR3(1)}} \) gene at 10 mM As(III) were not included in the analysis.

Fig. 3. Time evolution of the expression (mRNA copy number/gene copy number ratios) of (A) \( {\text{arsB}} \), (B) \( {\text{ACR3(1)}} \), (C) \( {\text{aioA}} \) and (D) 16S rRNA genes in arsenite-spiked cultures and arsenite-free controls, determined by real-time PCR. Data are mean values ± SEM (\( n = 3 \)). Relative expression values determined on one or two of three replicates are marked by one (○) or two (○○) circles, respectively. Values below the limit of detection are indicated by an asterisk (*).

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Table 2
Origin and nearest GenBank neighbors of partial 16S, ACR3(1), aioA and 16S rRNA gene sequences expressed in batch growth cultures.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Clone ID (accession no.)</th>
<th>Number of clones recovered*</th>
<th>Nearest BLAST match (accession no.)</th>
<th>Nucleotide identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>As(III) 1 mM</td>
<td>As(III) 10 mM</td>
<td>Pseudomonas brassicacearum NFM321 (DQ178233)</td>
</tr>
<tr>
<td>16S</td>
<td>NM16S_1 (JX63389) 8</td>
<td>8 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NM16S_2 (JX63390) 10</td>
<td>10 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NM16S_3 (JX63391) 1</td>
<td>1 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NM16S_4 (JX63392) 1</td>
<td>1 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>arsB</td>
<td>NMarsB_1 (JX63398) 3</td>
<td>3 4</td>
<td>Uncultured bacterium clone ArsBCarn_43 (FR847155)</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>NMarsB_2 (JX63399) 1</td>
<td>1 1</td>
<td>Pseudomonas putida HS–N24 (EF100617)</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>NMarsB_3 (JX63400) 1</td>
<td>1 1</td>
<td>Enterobacter aerogenes PSB20 (HQ242733)</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>NMarsB_4 (JX63401) 2</td>
<td>2 2</td>
<td>Uncultured bacterium clone AioA_1 (EU304312)</td>
<td>90</td>
</tr>
<tr>
<td>aioA</td>
<td>NMAioA_1 (JX63393) 10</td>
<td>10 3</td>
<td>Uncultured bacterium clone P. putida (JX63400)</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>NMAioA_2 (JX63394) 3</td>
<td>3 3</td>
<td>Uncultured bacterium clone AioA_3 (EU304310)</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>NMAioA_3 (JX63395) 1</td>
<td>1 1</td>
<td>Uncultured bacterium clone AioA_4 (EU304275)</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>NMAioA_5 (JX63397) 1</td>
<td>1 1</td>
<td>Uncultured bacterium clone AioA_5 (EU304276)</td>
<td>89</td>
</tr>
<tr>
<td>ACR3(1)</td>
<td>NMACR3_1 (JX63402) 2</td>
<td>2 9</td>
<td>P. putida N6 (CP003588)</td>
<td>90</td>
</tr>
</tbody>
</table>

* Sequences sharing more than 97% identity were considered to be in the same relatedness group.

Primers targeting arsB and ACR3(1) were previously shown to be highly specific and enabled amplification of arsenite transporter gene sequences from a taxonomically wide range of bacteria (Achour et al., 2007). Further to this, the specificity of these primers was confirmed in a number of environmental studies dealing with the impact of arsenic on the structure and diversity of microbial communities (e.g. Bachet et al., 2009; Cai et al., 2009a,b; Cavalca et al., 2010; Corsini et al., 2010; Hoeft et al., 2010; Sheik et al., 2012). However, the PCR method was not quantitatively applied to environmental samples. While short amplicon lengths are generally recommended for maximizing PCR efficiency in real-time PCR, we chose to optimize cycling conditions with the initial sets of primers rather than designing new primers to produce shorter amplicons. This choice proved fruitful, as the optimized assays enabled amplification of the two 0.75 kb templates, which are larger than typically used for real-time PCR, with efficiencies close to 100% and large dynamic ranges of detection.

The response of the soil microbial community to arsenic stress was studied on Nycodenz-extracted cells instead of soil microcosms. In this way, we overcame difficulties associated with isolating RNA from soil and subsequent RT-qPCR measurements (Saleh-Lakha et al., 2011). This also avoided adsorption on soil mineral phases of As(III) and As(V) resulting from microbial oxidation, and ensured that most arsenic present in the growth medium was available to the bacteria. While the bacterial inoculum investigated here proved to be highly diverse (Cebron et al., 2009), phylogenetic analysis revealed a sharp reduction in diversity after 5 day incubation, with Pseudomonas being the dominant metabolically active genus in all tested conditions. The shift in diversity appeared to be independent of the arsenic level, but might rather be attributed to use of glucose as a sole carbon source in the culture medium. Indeed, many of the Pseudomonas species are known to be typical r-strategists (van Overbeek and van Elsas, 1997) which tend to be strongly favored under nutrient-rich conditions, e.g. as reported in soils amended with glucose (Jenkins et al., 2010). It is, however, noteworthy that the dominant aioA genes expressed shifted from single Pseudomonas-related sequences in arsenic-free cultures to more taxonomically diverse sequences at 1 mM As(III). This indicates the presence of weakly abundant non-Pseudomonad active populations that were not detected from 16S cDNA library analysis, but likely played an important role in As(III) oxidation. We also noted that Pseudomonas-related arsB sequences were more diverse than ACR3(1) sequences (4 arsB vs. 1 ACR3(1)). This might appear unexpected given the wide occurrence of both transporter families in bacteria. It should be kept in mind that gene primers targeting ACR3 in this study only amplify the ACR3(1) group of arsenite transporter genes and could not capture the whole diversity of the ACR3 gene family. Nevertheless, because 16S rRNA and functional gene sequences recovered were widely diverse, correlations between gene diversity and the level of arsenic concentration, as assessed by others (Sheik et al., 2012), were not investigated.

One purpose of this work was to examine the links between the abundance of arsenic efflux pumps and arsenite oxidase genes, their expression level and the level of arsenic stress. Such investigations on arsenic and other metal resistance genes could be conducted only in rare cases where suitable quantitative PCR methods were available (Bouskill et al., 2007; Sun et al., 2004). However, application of high-throughput molecular tools provides a growing body of evidence that metalloid efflux pumps play an important role in the adaptation of bacterial communities to metalloid stress in contaminated environments. For instance, strong linkages were established between the level of arsenic contamination, the rhizosphere of the arsenic hyperaccumulator Pteris vitata and arsenic resistance gene distribution using functional gene (Geochip) microarrays (Xiong et al., 2010). Recent analyses of microbial communities using similar Geochip arrays or metagenomics also indicated an overabundance of metal resistance genes in metal-rich environments (Hemme et al., 2010;
Using newly developed real-time PCR assays, we found strong positive correlations between changes in *arsB* and *ACR3(1)* gene abundance and arsenic levels during the entire incubation time. The fact that the two genes showed similar patterns of abundance while differing from that of the *aioA* gene underscores the importance of As(III) efflux mechanisms in short-term adaptation of bacteria to arsenic stress. Whether they contribute to the adaptive response of microbial communities to long-term arsenic exposure remains to be determined and the real-time PCR assays developed here should help in addressing this issue.

We further established that upregulation of both *arsB* and *ACR3(1)* genes occurred after three-day exposure to As(III) concentrations above 100 μM. The upregulation in itself was expected, since the expression of most known *ars* operon is regulated by arsenic. However, transcription analyses of *ars* genes in different bacterial species indicated that derepression generally occurs at a lower As(III) concentration (in the range of 1–10 μM As; Diorio et al., 1995; López-Maury et al., 2003; Ordóñez et al., 2005; Tauriainen et al., 1997) than those observed here and within hours of exposure. A possible explanation for these discrepancies may stem from suboptimal efficiency of the reverse transcription protocol that could affect cDNA yield or length and thereby decrease the sensitivity of the totality of RT-qPCR assays. Nevertheless, our results are noteworthy since they provide first evidence of As(III)-induced changes in the transcription of arsenic resistance genes at the community level.

The abundance and expression of *aioA* were successfully quantified in batch growth cultures using a previously developed real-time PCR assay (Quemeneur et al., 2010). As might be expected for a bacterial community inhabiting long-term arsenic-contaminated soil, the *aioA* gene was abundantly present in the inoculum, suggesting a high potential for As(III) oxidation. Microbial As(III) oxidation indeed occurred in As(III)-spiked cultures and was consistently associated with expression of the *aioA* gene in most cases. The fact that four of five *aioA*-like sequences expressed at 1 mM As(III) were not detected in control cultures implies that these genes are likely regulated by arsenic, which is a characteristic feature of most As(III)-oxidizing bacteria (Slyemi and Bonnefoy, 2011). However, the *aioA* gene was also expressed in several non-amended culture replicates. Arsenite oxidase activity in the absence of As(III) has been reported in a few strains including *Thiobomona* sp. 3As (Duquesne et al., 2008), *Ralstonia* sp. 22 (Leteaut et al., 2010), the psychrotolerant arsenite oxidizer GM1 (Osborne et al., 2010) and *Agrobacterium tumefaciens* 5A (Kashyap et al., 2006); however in that latter case As(III) oxidation appeared to be regulated by quorum sensing and occurred only in the late growth phase. Our results suggest that basal expression of arsenite oxidase may also occur in some *Pseudomonas* strains, which contrasts with a previous report on *Pseudomonas* sp. TS44 that exhibits no obvious *aioA* expression in the absence of arsenic (Cai et al., 2009a,b). Also intriguing were the continuous decrease in *aioA* gene abundance over time and undetectable *aioA* transcripts at 10 mM As(III) despite strong arsenite oxidase activities. It is unclear whether this results from low sensitivity of the RT-qPCR assay or from failure to amplify an unusual arsenite oxidase gene with divergent target sequences. Together with the lack of correlation between *aioA* abundance and As(III) concentration, these results suggest that arsenite oxidase expression may not be a suitable marker for arsenic stress.

In summary, we have developed a real-time PCR assay that can be used to accurately quantify the two major families of arsenite transporter genes in natural bacterial communities. Despite biases resulting from cultivation of the community, we showed that the abundance of arsenite efflux pump genes correlated with the As(III) concentration, whereas that of *aioA* gene did not. Furthermore, only *arsB* and *ACR3(1)* displayed clear arsenic-dependent upregulation. Considering their inducibility and widespread distribution among prokaryotes, both genes appear to be promising functional biomarkers for environmental arsenic stress and bioavailability. Further studies on the abundance and expression of *arsB* and *ACR3(1)* in indigenous soil bacterial communities exposed to different levels of arsenic over various periods of time should help to gain a better understanding of how these genes contribute to the adaptation of communities to arsenic stress and their role in shaping community structure and diversity.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2013.01.012.

**References**


