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Diversity of arsenite transporter genes from arsenic-resistant soil bacteria

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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].

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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].