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Short Communication

Diversity and community structure of culturable arsenic-resistant bacteria across a soil arsenic gradient at an abandoned tungsten–tin mining area

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ABSTRACT

We studied the bacterial diversity at a single location (the Terrubias mine; Salamanca province, Spain) with a gradient of soil As contamination to test if increasing levels of As would (1) change the preponderant groups of arsenic-resistant bacteria and (2) increase the tolerance thresholds to arsenite [As(III)] and arsenate [As(V)] of such bacteria. We studied the genetic and taxonomic diversity of culturable arsenic-resistant bacteria by PCR fingerprinting techniques and 16S rRNA gene sequencing. Then, the tolerance thresholds to As(III) and As(V) were determined for representative strains and mathematically analyzed to determine relationships between tolerances to As(III) and As(V), as well as these tolerances with the soil contamination level. The diversity of the bacterial community was, as expected, inversely related to the soil As content. The overall preponderant arsenic-resistant bacteria were *Firmicutes* (mainly *Bacillus* spp.) followed by γ-*Proteobacteria* (mainly *Pseudomonas* spp.), with increasing relative frequencies of the former as the soil arsenic concentration increased. Moreover, a strain of the species *Rahnella aquatilis* (γ-*Proteobacteria* class) exhibited strong endurance to arsenic, being described for the first time in literature such a phenotype within this bacterial species. Tolerances of the isolates to As(III) and As(V) were correlated but not with their origin (soil contamination level). Most of the strains (64%) showed relatively low tolerances to As(III) and As(V), but the second most numerous group of isolates (19%) showed increased tolerance to As(III) rather than to As(V), even though the As(V) anion is the prevalent arsenic species in soil solution at this location. To our knowledge, this is the first study to report a shift towards preponderance of Gram-positive bacteria (*Firmicutes*) related to high concentrations of soil arsenic. It was also shown that, under aerobic conditions, strains with relatively enhanced tolerance to As(III) predominated over the most As(V)-tolerant ones.

1. Introduction

Arsenic is a toxic trace element that occurs in the soil solution mainly as arsenate [As(V)] and arsenite [As(III)] anions. As(V) is the predominant arsenic form in well-oxidized soils, while As(III) occurs predominantly in reduced environments; but both forms can coexist because of the relatively slow speed of redox transformations (Chang et al., 2008). Arsenic geochemistry is complex, and many variables play a role in determining speciation, solubility and bioavailability of arsenic in soils, such as pH, CEC, texture (clay mineralogy), amorphous Fe–Al oxides, organic matter, sulfur content, phosphorus concentration, and soil redox conditions (Voigt et al., 1996; Fitz and Wenzel, 2002; Anawar et al., 2006; Anawar et al., 2008; Khan et al., 2009; Moreno-Jiménez et al., 2010). Most risk from arsenic to living organisms is associated with the relative distribution of its different chemical forms that are bioavailable.

Bacteria are considered as efficient bioindicators of soil quality because they respond faster and are more sensitive to subtle environmental changes than higher organisms. Either functional or structural bacterial diversity can be used as bioindicators of soil pollution (Nielsen et al., 2002). Many bacteria have evolved mechanisms enabling them to cope with high arsenic concentrations, and even the possibility that particular bacteria may substitute arsenic for phosphorus to sustain its growth has been raised recently (Wolfe-Simon et al., 2010). Some bacteria are able to use arsenic as either an electron donor or an electron acceptor, altering the redox state of arsenic. Due to such activities, arsenic-resistant bacteria play an important role in controlling the speciation and

Abbreviations: RAPD, Random amplified polymorphic DNA; TP-RAPD, Two primers random amplified polymorphic DNA.

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Table 1
Physicochemical characteristics of As-contaminated soil samples from the mining site of Terrubias (Salamanca province, Spain).[a]

Soil sample	Total As ($mg\ kg^{-1}$)	Water-soluble As(III) ($\mu g\ kg^{-1}$)	Water-soluble As(V) ($\mu g\ kg^{-1}$)	pH	OM[b] (%)	CEC[c] ($meq\ 100\ g^{-1}$)	Sand (%)	Silt (%)	Clay (%)
AS1	943	54	154	5.89	1.12	8.37	24.0	25.5	49.0
AS2	170	27	55	6.26	1.74	7.46	23.8	27.5	46.5
AS3	102	17	42	6.55	1.61	7.61	19.5	31.4	47.6

[a] Water extraction of soil arsenic was done according to García-Sánchez et al. (2002). Concentration of As(III) and As(V) in solution were determined by HG-AAS following the method of Glaubig and Goldberg (1988). The remaining physicochemical parameters were analyzed as described in Anawar et al. (2008).
[b] Organic matter (OM).
[c] Cation exchange capacity (CEC).

bioavailability of arsenic, participating actively in its environmental cycling (Oremland and Stolz, 2003; Páez-Espino et al., 2009). Moreover, arsenic-resistant bacteria can have important applications in bioremediation strategies (Mateos et al., 2006; Feo et al., 2007; Yan et al., 2010). Because of these reasons, the relationships between soil arsenic and bacterial function and composition are an active field of research in soil microbiology.

In addition to the influence of the soil physicochemical properties on arsenic ecotoxicity, it is well known that the diversity and species richness of native soil bacterial communities differ according to local edaphic and environmental conditions (Neufeld and Mohn, 2005; Fierer and Jackson, 2006; Roesch et al., 2007; Fulthorpe et al., 2008; Angel et al., 2010). However, most published works on arsenic's effects on soil microbiology are short-term contamination experiments under controlled conditions, studies restricted to evenly contaminated locations or comparisons between edaphically and/or environmentally dissimilar areas (i.e. Maliszewska et al., 1985; Turpeinen et al., 2004; Oliveira et al., 2009). Thus, factors other than soil arsenic content would have likely influenced the outcomes of many of these studies.

In order to avoid any biased results due to factors other than increasing soil arsenic concentration, we selected a small area in which a gradient of arsenic contamination has arisen after a pollution event that occurred about 25 years ago to test if increasing levels of arsenic would (1) change the preponderant groups of arsenic-resistant bacteria and (2) increase the tolerance thresholds to arsenite and/or arsenate of the indigenous bacteria.

2. Materials and methods

2.1. Site description, soil sample collection and isolation of arsenic-tolerant bacteria

Soil samples were collected using sterile techniques from an abandoned tungsten–tin (containing arsenopyrite) mine located in Terrubias, Salamanca province, Spain (40°49′N, 5°46′W). The mineral paragenesis mainly consists of arsenopyrite, pyrite, scheelite, wolframite and minor chalcopyrite; and the mining wastes consists of fine-grained ore minerals including arsenopyrite, and ore weathering products (Fe-oxyhydroxides and scorodite) with high arsenic contents that polluted the soils of the surrounding environment (Anawar et al., 2008). Mine tailings were deposited around 25 years ago. Since then, a gradient of arsenic concentration in the soil has been formed by rainwater going down a smooth slope that connects a heavily contaminated patch of soil with the mining pond. Three sampling sites (AS1, AS2 and AS3) were established at approximately 10 m intervals along the slope starting at the tailing area (site AS1). For each sampling site, four soil samples (0–10 cm in depth) were randomly taken inside 1 m^2 area, pooled and sealed in sterile air-tight polyethylene containers. Upon arrival at laboratory, an aliquot of each pooled sample was dried at 50 °C and sieved through a 2 mm screen, which was used to determine the main soil properties and total soil arsenic content (Table 1). Another soil aliquot was used for the extraction of water-soluble arsenic, which was carried out under anaerobic conditions in sealed centrifuge tubes flushed with N_2 to displace air. Water -soluble As(III) and As(V) determinations were made within 4 h by HG-AAS following the method of Glaubig and Goldberg (1988) (Table 1).

For each pooled soil sample, 1 g of fresh soil was shaken in 100 mL of sterile saline buffer (0.85% NaCl), serially diluted and plated on Nutrient Agar (NA, Difco) plus cycloheximide ($0.15\ mg\ mL^{-1}$) amended either with arsenate or arsenite. Arsenate ($Na_2HAsO_4{\cdot}7H_2O$) and arsenite ($NaAsO_2$) water solutions were sterilized by filtration (0.22 μm Millipore filter) and added to autoclaved NA media cooled to about 50 °C, each to obtain concentrations of 100 mg As L^{-1}. Plates were incubated for 7 d at 25 °C. An enrichment culture was carried out from the soil collected at the tailing area (sampling site AS1) because no colonies appeared after direct plating. For this, an aliquot of 1 g of soil was added to 500-mL flask containing 100 mL of nutrient broth (NB) and incubated under agitation for 7 d at 25 °C. Aliquots of this culture were then plated and incubated on the arsenic-containing media as described before. Colonies different in shape, color and margins appearing on arsenic-containing NA plates were streak purified at least three times and stored at −80 °C in 25% sterile glycerol for further analyses.

2.2. Total DNA fingerprinting and 16S rRNA gene partial sequence analyses

Essential information on the PCR-based DNA fingerprinting techniques used in this study is given here, while a more detailed description of the methodology is available as Supplementary material.

Two primers random amplified polymorphic DNA (TP-RAPD) fingerprinting was used as shortcut technique for grouping the arsenic-resistant isolates in a taxonomically meaningful way. TP-RAPD fingerprints have an advantage for grouping purposes because strain-dependent variations are minimal. Thus, strains belonging to the same bacterial taxon (species/genus) share a unique band pattern, which, in turn, is different from that of other bacterial taxa (Rivas et al., 2001). Therefore, for identification purposes, this technique is a good tool for grouping of bacteria in order to select representative strains for 16S rRNA gene sequencing, as demonstrated with a broad range of soil eubacteria (Rivas et al., 2002; Valverde et al., 2006; Velázquez et al., 2008).

The diversity of strains within each TP-RAPD group was then assessed by random amplified polymorphic DNA (RAPD) fingerprinting using a primer derived from the M13 bacteriophage (M13-RAPD). Like other PCR fingerprinting techniques (i.e. ERIC-PCR, Box-PCR, Rep-PCR), M13-RAPD fingerprinting is an effective technique to distinguish closely related bacterial strains (Huey and Hall, 1989; Valverde et al., 2006; Velázquez et al., 2010).