Water Air Soil Pollut (2009) 200:237–243
DOI 10.1007/s11270-008-9907-5

Enumeration and Characterization of Arsenic-Tolerant Diazotrophic Bacteria in a Long-Term Heavy-Metal-Contaminated Soil

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Received: 25 June 2008 / Accepted: 20 October 2008 / Published online: 9 November 2008

Abstract The abundance of arsenic-tolerant diazotrophic bacteria was compared in a long-term contaminated soil versus a non-contaminated one. In addition, the characterization of tolerant diazotrophic bacteria was carried out. Differences in the number of heterotrophic N_2 fixers were found between soils. Contaminated soil showed a decrease in the microbial population size of about 80%, confirming the great sensitivity of this group of soil bacteria to metals. However, quantitative analysis of the response to increased doses of arsenic reveals that the proportion of the culturable diazotrophic community tolerant to arsenic was identical for both soils (contaminated and non-contaminated). Twenty-two arsenic-tolerant diazotrophic isolates were obtained and further characterized. 16S ribosomal DNA sequence analysis revealed that these bacterial isolates were distributed among four taxons (*Actinobacteria*, *Firmicutes*, γ-*Proteobacteria*, and β-*Proteobacteria*). Most genera recovered from the contaminated soil were also found in the uncontaminated soil.

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Keywords Arsenic · Diazotrophic bacteria · Soil bacteria · Heavy metals

1 Introduction

The spread of metals in the terrestrial environment is largely attributable to anthropogenic activities. In industrialized areas, high concentrations of arsenic and heavy metals have been often found in soils and wastes, establishing a serious ecological risk (Singh and Steinnes 1994). The metals affect microorganisms by reducing their number, biochemical activity, diversity, and changing the community structure (Kandeler et al. 2000; Ellis et al. 2001). However, metal exposure also leads to the establishment of tolerant microbial populations (Jackson et al. 2005b). In contaminated sites, these populations may be involved in the alteration of mobility of metals through their reduction, accumulation, and in situ immobilization by extracellular precipitation (Collard et al. 1994; Roane 1999).

Arsenic is a naturally occurring toxic element that is also used in a number of industrial processes. Background soil concentrations of arsenic are typically below 15 mg/kg, but can exceed 2,000 mg/kg in some contaminated areas (Smith et al. 1998). Despite its toxicity, a number of microorganisms are capable of using either the oxidized form of inorganic arsenic (arsenate) or the reduced form (arsenite) in their

metabolism, and even more microorganisms are capable of resisting arsenic toxicity through the *ars* genetic system (Jackson et al. 2003; Oremland and Stolz 2003). In many studies, these arsenic resistant microorganisms have been obtained from arsenic-contaminated soils (Macur et al. 2004; Anderson and Cook 2004).

Microbial interactions with metals may have several implications for the environment. Microbes may play a large role in the biogeochemical cycling of toxic metals, as well as in cleaning up or remediating metal-contaminated environments. In order to increase the success of these bioremediating processes, it is important to have a better understanding of how microbial populations respond to elevated metal concentrations. The responses analyzed should be related to some important soil biological processes, for example, those involved in C and N cycling (Martensson and Torstensson 1996). Soil N cycling processes, particularly N_2 fixation, are sensitive to metal additions. For example, the N_2-fixing potential of heterotrophic biological N_2-fixing microorganisms has been found to be sensitive to small concentrations of heavy metals (Lorenz et al. 1992; Martensson 1993; Martensson and Torstensson 1996).

The long-term effects of metals or other pollutants added to soil are very difficult to assess, as there are few such experiments and, consequently, few data (McGrath et al. 1995; Speir and Ross 2002). In this study, we selected soil samples from an area with known pollution problems, namely arsenic, where pollutants have been emitted by the industry for nearly 40 years (Oliveira and Pampulha 2006). This site was chosen as an example of a post-industrial habitat presumably containing interesting populations of microorganisms which could tolerate high concentrations of metals. The objective of our investigation was to compare the arsenic sensitivity of the asymbiotic nitrogen-fixing population in a long-term contaminated soil versus a non-contaminated soil. In addition, isolation and identification of dominant isolates was done by 16S rDNA sequence analysis.

2 Materials and Methods

2.1 Soil Samples

Two composite soil samples were collected from the top layer (0–10 cm) of an arable field in the center of Portugal (Estarreja region). One sample was collected near an industrial effluent channel (not impermeable and rarely overflowing), and the other sample was collected 10 m away. Both soil samples were of identical texture (sandy loam) and had similar organic matter content (39 and 30 g/kg) and pH (5.4). Soil samples were obtained in December 2003 and 2004 and were stored in loosely fastened plastic bags at 4°C in the dark. The extractable heavy metal concentrations in both soil samples were measured by atomic absorption spectrometry after extraction with aqua regia. Hg content was determined by adapting the method described by Stewart and Bettany (1982). Arsenic content determination was based on Environmental Protection Agency method 3052, by total microwave digestion (Table 1).

2.2 Enumeration of Arsenic-Tolerant Heterotrophic Nitrogen-Fixing Microorganisms

The estimation of the numbers of nitrogen-fixing bacteria in soil was based on the most probable number technique using combined carbon medium (CC; Rennie 1981), a semi-solid nitrogen-free medium. This medium fulfilled the requirements of a wider range of nitrogen-fixing bacteria existing in our Mediterranean zone (Oliveira and Pampulha 1998). Bacterial counts were performed in five replicate tubes per dilution. Tubes exhibiting both microbial growth and acetylene reduction were scored positive for the presence of nitrogen-fixing bacteria. Acetylene reduction was detected using a gas chromatograph Varian

Table 1 Soil sample characteristics and concentration of metals (mg/kg dry soil)

Soil characteristic	Contaminated	Uncontaminated
pH	5.39	5.39
Organic matter (g/kg)	39	30
Texture	Sandy-loam	Sandy-loam
Fe	8,800	5,200
Mn	40.0	102.0
Cd	1.1	0.4
Cr	1.2	4.7
Cu	132.9	6.9
Ni	1.6	5.1
Pb	270.8	33.6
Zn	165.5	36.6
Hg	109.0	0.5
As	1558	14

3800GC (Varian Analytical Instruments, Mitchell Drive, Walnut Creek, USA) fitted with 1mx1/8″ column packed with Porapak T (80–100 mesh; Varian Analytical Instruments) using a flame ionization detector. Numbers of arsenic-tolerant bacteria were estimated using the technique described; CC medium was amended with arsenic(III)–chloride in order to obtain As concentrations of 20, 40, 80, 120, 140, 160, and 200 μg/ml.

By dividing the number of bacteria growing on the metal supplemented medium by the total population for each soil, the proportion of the culturable diazotrophic community tolerant to the noted arsenic concentrations was calculated (Fig. 1).

2.3 Isolation and Identification of Arsenic-Tolerant Diazotrophic Bacteria

Enrichment of arsenic-tolerant N_2-fixing bacteria was carried out in As-supplemented CC medium. After incubation for 4 to 5 days at 25°C, vials were used to inoculate new sterile CC medium for a second incubation. Bacteria from cultures with highest nitrogenase activity were spread on As-supplemented tryptone soya agar (Oxoid) to isolate bacterial components. The purification steps had to be repeated at least twice before the majority of the isolates could be considered pure. Isolates were picked up, purified, and stored in 25% glycerol at −80°C.

A total of 42 isolates were initially obtained (21 form uncontaminated soil and 21 from contaminated soil) isolates were picked up, purified and stored in 25% glycerol at −80°C.

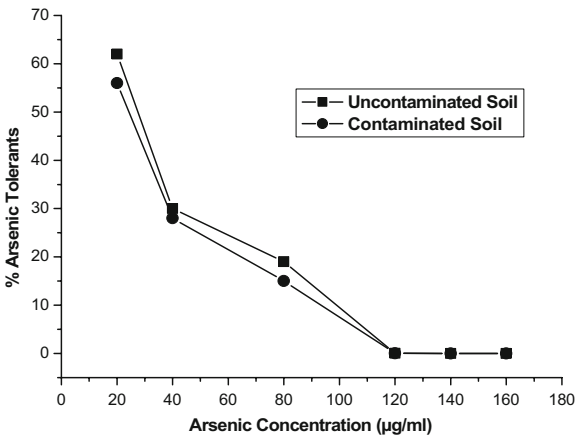

Fig. 1 Percentage of culturable diazotrophic bacteria tolerant to different arsenic concentrations in contaminated and uncontaminated soils

After the purification steps, all these isolates were tested for their ability to fix nitrogen (using the acetylene reduction assay); only seven isolates from uncontaminated soil and 15 from contaminated soil were scored as positive, and only these were considered for identification.

Preliminary characterization of strains was done by colony morphology and motility and *Gram* staining using standard methods.

Extraction of genomic DNA from pure cultures was performed by resuspension of a freshly isolated colony on 50 μl Milli-Q water and incubation at 95°C for 5 min. The mixture was centrifuged at 5,000×*g* for 5 min at 4°C and the supernatants used for PCR. For PCR amplification of the 16S rDNA, we used the universal primers 7f and 1510r (Lane 1991). The sequences of the primers are following: 7f, 5′-AGAGTTTGAT(CA)CTGGCTCAG-3′, 1510r, 5′-ACGG(CT)TACCTTGTTACGAC TT-3′. The reactions were conducted using 50 μl (final volume) mixtures containing 10× *Taq* buffer; each deoxyribonucleotide triphosphate was at a concentration of 0.2 mM, and each primer was at a concentration consisting of 0.5 μM and 1.25 U of *Taq* DNA polymerase. The conditions for the PCR procedure were as follows: an initial denaturation step of 94°C for 2 min followed by 35 amplification cycles of denaturation (45 s at 94°C), annealing (60 s at 50°C) and elongation (1 min at 72°C), and then a final extension step of 5 min at 72°C using thermocycler Mastercycler personal (Eppendorf, Hamburg, Germany). Amplified DNA was examined by electrophoresis in 1% agarose gel with 5 μl aliquots of PCR product. The amplified 1,500-bp product was purified by GFX PCR and gel band purification kit (GE Healthcare, Buckinghamshire, UK) and then sequenced in both directions. DNA sequencing was performed by STABVIDA (Oeiras, Portugal). Sequences of the 16S rDNA were edited and then subjected to a Genbank BLASTIN search to retrieve sequences of closely related taxa (http://www.ncbi.nlm.nih.gov/Blast/).

3 Results

The chemical characteristics of the two soil samples under study are shown in Table 1. There were no

differences in the pH, texture, and organic matter content between the two soil samples under study. However, differences were observed for heavy metal content, namely, for Pb, Zn, and Hg. The metalloid As also showed a high content in the contaminated soil samples (1,558 mg/kg dry soil). The total concentration of Hg exceeded the permissible limits established by the CEC Directive in 1986 in agricultural soil (1–1.5 mg/kg dry soil) and approached these limits in the cases of Pb (50–300 mg/kg dry soil) and Zn (150–300 mg/kg dry soil; Commission of the European Communities 1986).

Heterotrophic non-symbiotic nitrogen-fixing bacteria seem to be sensitive to heavy metal contamination undergoing a decrease in population size. The initial soil samples presented a most probable number of 1.5×10^5 and 2.5×10^4, respectively, for uncontaminated and contaminated soils, representing a decrease of about 80% in the population of N_2 fixers for the contaminated soil (Table 2).

Diazotrophic bacteria decreased, in both soils, in response to the presence of arsenic in the medium at the doses tested.

Arsenic additions affects diazotrophic bacteria in both soils. The addition of 20 μg/ml resulted in a decrease of about 43% and 38% in the population size in the contaminated and uncontaminated soil, respectively. Thus, a substantial proportion of the culturable diazotrophic community showed tolerance to this As concentration. Further increments in concentrations led to a continuous decrease in the number of diazotrophic bacteria (Table 2). The limits of As tolerance of diazotrophic bacteria seemed to be identical for both soils (120 μg/ml; Table 2). Also, when comparing the bacterial tolerance (in percent) to increased arsenic concentration, we stated an identical behavior for both soils, since the percentage of arsenic tolerants in relation to unamended soil was similar (Fig. 1).

Table 2 Most probable number (MPN) of arsenic-tolerant diazotrophic bacteria in contaminated and uncontaminated soil samples (MPN, 10^3/g dry soil)

Medium	Contaminated soil	Uncontaminated soil
CC	25±3.2	149±15
CC + 20 μg/ml As	14.1±1.8	91.8±10
CC + 40 μg/ml As	7.1±1.0	40.2±5.0
CC + 80 μg/ml As	3.8±0.6	28.7±1.9
CC + 120 μg/ml As	0.015±0.004	0.08±0.01
CC + 140 μg/ml As	0	0
CC + 160 μg/ml As	0	0
CC + 200 μg/ml As	0	0

We attempted to isolate diazotrophic bacteria that were able to tolerate high concentrations of As. In total, 22 arsenic resistant isolates showed ability to fix nitrogen (15 from contaminated soil and seven from uncontaminated soil) All the isolates were resistant to an arsenic concentration of 120 μg/ml. Analysis of 16S rDNA revealed the presence of representatives from four major bacterial taxons (the *Actinobacteria*, *Firmicutes*, γ-*Proteobacteria*, and β-*Proteobacteria*; Table 3). However, members of β-*Proteobacteria* (*Achromobacter*) were only found in contaminated soil (four isolates).

4 Discussion

Analysis of extractable heavy metals and arsenic concentrations revealed a high level of Pb, Zn, Hg, and As. Soil arsenic concentration in the contaminated soil (1,558 mg/kg) is higher than the worldwide average (6.0 mg/kg; Tamaki and Frankenberger 1992). Some of the detected metals are considered to be toxic to biological systems.

The microflora of contaminated soil had been exposed to greater amounts of metals as a result of contamination from a nearby industrial complex (Castro et al. 2003). This contamination had occurred slowly over a long period, and it was considered that the soil population would represent one which had gradually changed in its metal tolerance characteristics in response to rising soil metal concentrations. Whatever the mechanism of population development had been operating, either selection of phenotypic groups or of individuals carrying plasmids or chromosomal mutations, those should be well represented in a more or less stable condition (Piotrowska-Seget et al. 2005). By analyzing such a population and comparing it to one which had not experienced changes in soil metal content, some insight may be obtained as to the ways in which natural soil bacterial populations respond to environmental changes.

Toxicity of metals on soil microorganism activity is well known, but at the same time, less is known about the effects on different organism groups. Species of microorganisms (Berdicevsky et al.

Table 3 Assignment of taxonomic groups of selected As-tolerant isolates based on 16S rDNA partial sequencing and closest sequence match in GenBank

Soil sample	Isolate	Taxon	Closest cultured relative in GenBank (% similarity)
Uncontaminated soil (control soil)	As1	*Actinobacteria*	*Brevibacterium* (99%)
	As2	*Firmicutes*	*Bacillus megaterium* (97%)
	As4	*Actinobacteria*	*Rhodococcus* (99%)
	As5	*γ-Proteobacteria*	*Pseudomonas* (99%)
	As6	*γ-Proteobacteria*	*Pseudomonas* (97%)
	As7	*Firmicutes*	*Bacillus sporothermodurans* (93%)
	As28	*γ-Proteobacteria*	*Pseudomonas* (97%)
Contaminated soil	As3	*Firmicutes*	*Bacillus thuringiensis* (98%)
	As8	*γ-Proteobacteria*	*Pseudomonas* (99%)
	As9	*Firmicutes*	*Staphylococcus* (99%)
	As12	*Firmicutes*	*Staphylococcus* (99%)
	As13	*Actinobacteria*	*Arthrobacter* (99%)
	As14	*Actinobacteria*	*Arthrobacter keyseri* (94%)
	As15	*Actinobacteria*	*Arthrobacter* (93%)
	As17	*Firmicutes*	*Staphylococcus* (99%)
	As18	*β-Proteobacteria*	*Achromobacter xylosoxidans* (97%)
	As19	*β-Proteobacteria*	*Achromobacter* (96%)
	As21	*Firmicutes*	*Bacillus sporothermodurans* (92%)
	As23	*β-Proteobacteria*	*Achromobacter* (97%)
	As24	*β-Proteobacteria*	*Achromobacter* (98%)
	As31	*Firmicutes*	*Bacillus* (85%)
	As32	*Firmicutes*	*Bacillus circulans* (99%)

1993), strains of the same species (Romandini et al. 1992), and also activities of the same microbial species (Giller et al. 1998) can all show considerable differences in their sensitivity to metal toxicity.

Free-living heterotrophic N_2-fixing bacteria are ubiquitous and include species which can fix nitrogen under aerobic, microaerophilic, and anaerobic conditions. In the present experiment, population of heterotrophic N_2 fixers was significantly affected in contaminated soil undergoing a decrease of about 80% (Table 1). This result is in agreement with previous studies. McGrath et al. (1995) reported that N_2 fixation by free-living heterotrophic bacteria was found to be inhibited at soil metal concentrations lower than that of our contaminated site. Significant decreases in acetylene reduction activity by aerobic and microaerophilic N_2 fixers were also reported by Brookes and McGrath (1984) in metal-contaminated soils. Märtensson and Witter (1990) found heterotrophic N_2 fixation to be severely reduced in metal-contaminated soil in Sweden, and Fließach and Reber (1991) confirmed the great sensitivity of N_2 fixation by free-living heterotrophic bacteria to metals in the old arable soil at Braunscheweig in Germany.

One method purposed to assess environmental effects of metals is to determine the number of metal-tolerant bacteria from an environment affected by heavy metals, as bacteria are capable of rapidly responding to changes in their environment (Angle et al. 1993). Theoretically, if a significant proportion of the bacterial population is tolerant to a high concentration of metal contaminant, then the judgment is made that the soil is negatively affected by the presence of the metal (Olson and Thornton 1981). Quantitative analysis of the response of diazotrophic bacteria to increased doses of arsenic (Table 1) failed to detect any significant change in arsenic tolerance of bacterial population, since both soils (contaminated and uncontaminated) present an identical behavior (level of As tolerance of 120 μg/ml).

We concluded that the percentage of arsenic tolerants was identical for both soils. There are a number of reports of an increased abundance of metal tolerance with severe soil pollution. For example, Olson and Thornton (1982) found a correlation between the proportion of metal-tolerant bacteria and the total Cd and Zn concentration of some extremely polluted soils from mining areas, and Campbell et al.

(1995) observed a higher level of metal tolerance in *Pseudomonas* isolated from soil around industrial sites compared with isolates from uncontaminated agricultural soils. However, El-Aziz et al. (1991) found no correlation between metal tolerance of isolates of *S. meliloti* and the degree of metal contamination in soils. Our results show a similar behavior in relation to arsenic tolerance. Results from Jackson et al. (2005a) also support the suggestion that arsenate-resistant bacteria are common in contaminated and uncontaminated soils. It should be noted that most media, including CC medium, support only a fraction of the total diazotrophic population. It is difficult, however, to examine the metal tolerance of bacteria that could not be cultured. Therefore, our results and conclusions apply only to the culturable diazotrophic population of soil bacteria.

The diazotrophic isolates identified in this study were 14 Gram-positives and eight Gram-negatives (Table 3). The arsenic-tolerant Gram-negative bacteria reported in this study belonged to *Pseudomonas* and *Achromobacter*. Arsenic-tolerant Gram-positives, such as *Brevibacterium*, *Bacillus*, *Rhodococcus*, *Staphylococcus*, and *Arthrobacter* were also found. Table 3 shows that Gram-positives (64%) were dominant. Also, in the study of Achour et al. (2007), Gram-positive arsenic-resistant isolates were predominant.

In a general way, the tolerant arsenic bacteria that we identified are also distributed among the same major bacterial lineages [*Actinobacteria* (five), *Firmicutes* (nine), γ-*Proteobacteria* (four), and β-*Proteobacteria* (four)]. These results appeared to differ from other studies where Gram-negative bacteria usually dominate metal-contaminated soils (Kelly et al. 2003; Piotrowska-Seget et al. 2005). Reports indicating an increase of Gram-positive bacteria in metal polluted soils are rare (Pennanen 2001; Garau et al. 2007).

With the exception of genus *Achromobacter*, only detected in the contaminated soil, most genera recovered from the contaminated soil were also found in the uncontaminated soil. This occurrence suggests that genes for arsenic resistance are widespread, and so, it is not surprising that arsenic-resistant bacteria might well be found in many environments regardless of current or past history of arsenic exposure (Jackson et al. 2005b).

This study corroborates the idea of sensitiveness of soil diazotrophic bacteria to metal contamination. However, diazotrophic arsenic-tolerant bacteria were common in both soils, contaminated and uncontaminated, showing that most diazotrophic bacteria are intrinsically tolerant to high concentrations of arsenic.

Acknowledgments This research was supported by Fundação para a Ciência e Tecnologia (FCT), Portugal under POCTI Program, contract POCTI/AGG/46707/2002.

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