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Taxonomic and functional prokaryote diversity in mildly arsenic-contaminated sediments

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Abstract

Arsenic-resistant prokaryote diversity is far from being exhaustively explored. In this study, the arsenic-adapted prokaryotic community present in a moderately arsenic-contaminated site near Sainte-Marie-aux-Mines (France) was characterized, using metaproteomic and 16S rRNA-encoding gene amplification. High prokaryotic diversity was observed, with a majority of Proteobacteria, Acidobacteria and Bacteroidetes, and a large archaeal community comprising Euryarchaeaota and Thaumarchaeota. Metaproteomic analysis revealed that Proteobacteria, Planctomycetes and Cyanobacteria are among the active bacteria in this ecosystem. Taken together, these results highlight the unsuspected high diversity of the arsenic-adapted prokaryotic community, with some phyla never having been described in highly arsenic-exposed sites.
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Keywords: Environmental genomics; Arsenic; Mining; Biodiversity; Metaproteomics

1. Introduction

Arsenic is distributed ubiquitously throughout the world: its abundance on earth is around 1.5–3 mg kg^{-1} and it is the 20th most abundant element. Arsenic levels present in soil and sediments usually range from 0.1 to 50 mg kg^{-1}, giving a mean value of 5–6 mg kg^{-1} (Mandal and Suzuki, 2002). Arsenic concentrations present in soils and sediments actually vary considerably from one geographical region to another depending on the geochemical characteristics of the soil. For example, arsenic contents have been reported to range from 10 to 196 mg kg^{-1} in West Bengali sediments (India), from 0.01

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to 626 mg kg^{-1} in soil and sediments from China and from 1 to 72 mg kg^{-1} in soil originating from the United States (Mandal and Suzuki, 2002). High levels of arsenic can be due either to natural contamination by the parent rock (India) or human activities (anthropogenic forms of contamination) (Mandal and Suzuki, 2002). In natural waters, less than 10 μg l^{-1} is usually present, but arsenic levels of 5000 μg l^{-1} have been observed in contaminated waters in the USA, South America, India and Bangladesh (Smedley and Kinniburgh, 2002). Arsenic-rich waters occur naturally in geothermal regions or because of the geochemical characteristics of the soils. Anthropogenic contaminations are observed principally in mining regions due to acid mine drainages (AMDs) efflux characterized by high sulfate, iron and other metal concentrations (Coupland et al., 2004; Hallberg and Johnson, 2005). In these aquatic environments, arsenic occurs mainly in the form of the inorganic species arsenate (As(V)) and arsenite (As(III)), which are generally more toxic than the organic forms to living organisms (Sharma and Sohn, 2009). As(III) is more soluble, and usually thought to be more toxic than As(V).

Several studies have shown that bacterial metabolic processes such as arsenic oxidation and reduction play an important role in arsenic speciation in the environment and affect its biodisponibility (Lièvremont et al., 2009). However, knowledge available about arsenic-resistant bacteria was restricted until quite recently to results of studies on bacteria grown in vitro (Lièvremont et al., 2009; Tsai et al., 2009). To explore the diversity of arsenic-metabolizing prokaryotes, several laboratories have developed environmental genomic approaches such as meta-genomic, meta-transciptomic and meta-proteomic approaches (Bertin et al., 2008; Denef et al., 2010). Functional studies have been performed on highly arsenic-contaminated AMDs (VerBerkmoes et al., 2009; Denef et al., 2010; Mueller et al., 2010; Bruneel et al., in press). However, arsenic-tolerant prokaryote diversity is far from being explored, since most of those previous studies focused on highly contaminated sites or on AMD-exposed sites which combine several abiotic stresses such as low pH and high heavy metals concentration, where a low level of bacterial diversity was observed.

The aim of this study was to further investigate arsenic-adapted prokaryote diversity. For this purpose, a study site located in the vicinity of the Gabe-Gottes mine, which was worked until 1940 in the Sainte-Marie-aux-Mines Valley (France), was chosen (Fig. 1). The material extracted initially consisted of silver ore and, more recently, of arsenic ore. Currently, waters are percolating from the mine walls and accumulate in a creek. Preliminary experiments showed that the arsenic present in the creek sediments amounts to about 500 mg kg^{-1}, which constitutes a moderate but significant level of contamination. In this study, the physical and chemical characteristics of this site, the microbial community inhabiting the creek sediments and the main proteins expressed were analyzed using environmental genomic/proteomic approaches.

2. Materials and methods

2.1. In situ sampling procedure and chemical analyses

The sampling site was at a creek formed by the waters from a former mine located 40 m from the mine entrance, at

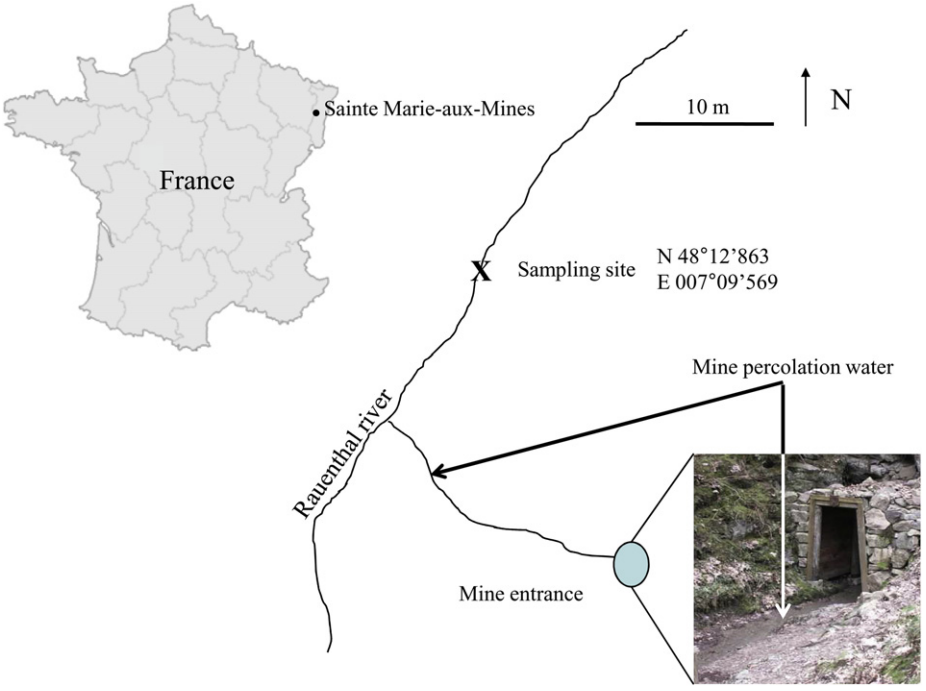


Fig. 1. Localization of the sampling site near the site of Sainte-Marie-aux-Mines (France). Samples were collected in sediment of the Rauenthal River, 40 m downstream from a mine entrance in waters percolating through the ancient mining exploitation.

Sainte-Marie-aux-Mines in France (Fig. 1). One liter of water was filtered through a 0.22 μm sterile nucleopore filter and stored at 4 °C for chemical analysis, which was performed within 48 h. The 5 cm most superficial sediments from the creek were collected in December 2007 with a plastic shovel at several points along the creek and homogenized. After being sampled, water and sediments were separated, sediments were stored at −80 °C for DNA extraction or in glycerol (25%, w/v) at −80 °C for protein extraction.

Water pH was measured using a microprocessor pH meter (pH 3000, WTW) and acid-neutralizing capacity (ANC) was determined using Gran's titration method. Conductivity was measured with a Metrohm Herisau Conductometer E518 (Herisau, Switzerland) at 25 °C. Mineralization of sediments (0.5 g dry weight) was performed in line with the French standard AFNOR NFX 31-151 prior to performing metal analysis using atomic absorption spectrophotometry methods. Organic content was determined from the loss of mass determined after treatment at 550 °C for 4 h. Metal and metalloid concentrations (after acidification with HNO_3) were determined by performing atomic absorption spectrophotometry (Analyst 100; Perkin-Elmer and Varian SpectrAA-300) and Br^-, Cl^-, SO_4^{2-}, NO_3^- and NO_2^- concentrations by performing ion chromatography (Dionex 1500i with a AS 4 A SC column; Sunnyvale, CA, USA). The suspended solids content, total phosphorus (TP), total Kjeldahl nitrogen (TKN), COD (chemical oxygen demand), BOD5 (biological oxygen demand after 5 days), and TSS (total suspended solids) were determined according to French standards relating to water analysis (AFNOR, 1994). Total phosphorus was determined in keeping with the French norm AFNOR NF T90-023. Chlorophyllic pigments were quantified spectrophotometrically (Specord 205) at 750 and 755 nm.

In order to analyze arsenic speciation in the interstitial water, part of the frozen sediment was centrifuged. The supernatant was collected, filtered (0.45 μm) and further quantified using standard additions to prevent occurrence of matrix effects with an HPLC-ICP-MS apparatus (PerkinElmer SCIEX ELAN 6100 DRC, Concord, ON, Canada) fitted with a Meinhard nebulizer and a cyclonic spray chamber. Briefly, arsenic species were separated with an anion-exchange column PRPX-100 (100 mm × 4.1 mm, Hamilton, Reno, NV, USA). Eluent was delivered online at a flow rate of 1 ml min^{-1} with a high performance liquid chromatography pump (DIONEX ICS 3000, Sunnyvale, CA, USA) coupled with the ICP-MS. Samples were injected using an autosampler (DIONEX AS50, Sunnyvale, CA, USA) through a 100 μl PEEK injection loop attached to an injection valve. The outlet of the HPLC column was directly connected to the nebulizer of the ICP-MS via 20 cm of PEEK capillary tubing (0.17 mm i.d.). Concentration values and uncertainties involved were calculated from values determined in duplicate. To measure arsenic species concentrations in sediments, some of the frozen samples were lyophilized and ground prior to the extraction process. Extractions were performed in duplicate. The extraction method used was adapted from (Thomas et al., 1997). 10 mL of H_3PO_4 1 M were added to 0.4 g of sample in a Teflon vessel. The mixture was heated with a closed microwave system (CEM model MARS, Charlotte, NC, USA) at 120 °C for 20 min. The remaining solution was diluted to 50 ml with ultrapure water and then analyzed by performing HPLC-ICP-MS.

2.2. Metagenomic DNA extraction and 16S rRNA gene analysis of the whole community

DNA was extracted directly from the sediment community using a PowerMax soil DNA isolation kit in line with the manufacturer's recommendations (MoBio Laboratories, Inc.), concentrated by precipitation in 2.5 v/v 100% ethanol and 0.1 v/v sodium acetate (3 M, pH 5.6) and stored at −20 °C until further processing.

1.5 kb bacterial and 571 bp archaeal 16S rRNA-encoding genes were amplified using metagenomic DNA as a matrix and universal bacterial and archaeal primers W01/W02 (5′-AGAGTTTGATCMTGGCTC-3′ and 5′-GNTACCTTGTTACGACTT-3′) and ARC344F/ARC915R (5′-ACGGGGYGCAGCAGGCGCGA-3′ and, 5′-GTGCTCCCCCGCCAATTCCT-3′), respectively (Stahl and Amann, 1991; Raskin et al., 1994). All PCR reaction mixtures contained 50 ng DNA template, 5 μl PCR buffer (5 PRIME buffer 10×), 1 μl of deoxynucleotide mix (Mix 5 PRIME, 10 mM of each dNTP) 0.2 mM of each primer and 2.5 U Taq DNA polymerase (5 PRIME) in 50 μl volume. Bacterial 16S rRNA gene PCR amplification reactions were carried out with a Mastercycler gradient (Eppendorf) with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation (40 s at 95 °C), a 30 s annealing step at 52 °C, a 45 s elongation step at 68 °C and a final 10 min denaturation step at 68 °C. Archaeal 16S rRNA gene cycling conditions consisted of a 5 min denaturation step at 94 °C followed by 35 denaturation cycles of 1 min at 94 °C, a 1 min annealing step at 71−61 °C with a 0.5 °C decrement per cycle during the first 20 cycles and a 1 min elongation step at 72 °C, followed by a final 10 min elongation step at 72 °C.

The fragments obtained were cloned into plasmids pCR®2.1 using the TOPO® TA cloning kit in line with the manufacturer's recommendations (Invitrogen Laboratories). 240 clones containing bacterial 16S rRNA-encoding genes and 83 clones containing archaeal 16S rRNA encoding genes were sequenced using primers ARC344F and W01, respectively (Stahl and Amann, 1991; Raskin et al., 1994; Achour et al., 2007). Sequences harboring more than 600 bp in the case of bacterial 16S rRNA genes and more than 300 bp in that of achaeal 16S rRNA genes were used for phylogenetic analysis. Indeed, these sequences were compared with those in the GenBank database by performing BLAST searches (Altschul et al., 1997) and those in the RDP database by performing SEQ MATCH searches (Cole et al., 2009). Chimeras were checked using pintail online (Ashelford et al., 2005). Sequences that corresponded to chimera were removed for further analysis.

Archaeal 16S rRNA gene homologs were collected from the nr database at NCBI, using the BLAST program (Altschul et al., 1997) with default parameters. The same procedure was used on the environmental database at NCBI, using each clone as a seed in order to enrich the samples. Sequences were

aligned using the Muscle program (Edgar, 2004) with default parameters. Based on preliminary phylogenetic trees, only a few sequences representative of archaeal diversity were selected, giving a dataset of 152 sequences for final analysis. For similarity search, 445 unambiguously aligned positions were selected. A maximum likelihood tree was obtained using the PhyML program (Guindon and Gascuel, 2003) with a GTR model, 4 evolutionary rates, a calculated proportion of invariant sites and calculated nucleotide frequencies (default parameters). Statistical likelihood at nodes was calculated via a likelihood-ratio test (Anisimova and Gascuel, 2006).

Partial prokaryotic sequences were aligned using ClustalW (Thompson et al., 2002) separately on bacterial and archaea samples, and the respective distance matrices were obtained using DNADIST from the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html). The resulting matrices were fed into DOTUR (Schloss and Handelsman, 2005) in order to calculate the number of OTUs at 97% and 85% level. These percentage identities OTUs served for generating rarefaction curves (Fig. 2).

2.3. Metaproteomic analyses

In order to identify the main proteins expressed in situ by the sediment community, prokaryotic cells were recovered from sediment as follows: 8 × 12.5 g of frozen sediment were washed with 8 × 12.5 mL of salt solution (0.15 g l^{-1} Na_2SO_4, 0.45 g l^{-1} $(NH_4)_2SO_4 \cdot 10H_2O$, 0.05 g l^{-1} KCL, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.05 g l^{-1} KH_2PO_4, 0.014 g l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$) and gently shaken overnight at 4 °C. After a 30 min decantation step, the supernatant (8 × 7.5 ml) was added to Nycodenz solution without any mixing (8 × 17.5 mL, 65%, w/v) (Axis-Shield, Dundee, Scotland), and centrifuged for 3 h at 10 000 g. The cellular fraction was removed and washed by adding 3 volumes of NaCl 0.9% and centrifuged for 1 h at 10 000 g at 4 °C. Cells were resuspended in NaCl 0.9% and an aliquot was stained using the LIVE/DEAD BacLight Bacterial Viability kit (L13152, Molecular Probes, Invitrogen) as recommended by the manufacturer, in order to assess the proportion of dead cells. The preparation was examined using an epifluorescent microscope Nikon Eclipse TE2000 S. Images were taken with an integrated Hamamatsu digital camera (C4742-95; Nikon UK).

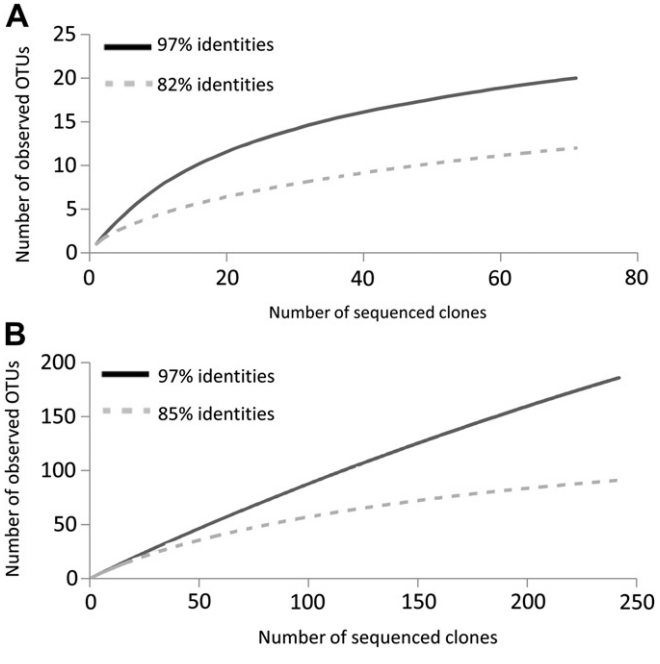


Fig. 2. Rarefaction analysis of the (A) archaeal 16S rRNA and (B) bacterial sequences from Sainte-Marie-aux-Mines sediments. The total number of sequenced clones is plotted against the number of OTUs observed in the same library. OTUs were defined at the 97% identity level (species level) for both and at 82% and 85% (class level) for Archaea and Bacteria, respectively. For archaeal analysis, the DOTUR program did not calculate the 85%, but the 82% value.

Proteins were extracted from these cells and separated by 1D-SDS-PAGE using Laemmli's method (Laemmli, 1970) with a 12% gradient slab gel (PROTEAN II, Bio-Rad laboratories). Electrophoresis was carried out at 200 V. Proteins were stained with Coomassie Brilliant Blue, bands were systematically cut every 1.5 mm and the 29 gel pieces were stored at −20 °C before performing mass spectrometry analysis.

In-gel digestion of gel bands was performed as previously described (Weiss et al., 2009). The resulting peptides were analyzed by nanoLC-MS/MS on nanoACQUITY Ultra-Performance-LC (UPLC, Waters, Milford, MA, USA) coupled to an SYNAPT hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Waters, Milford, MA, USA). The capillary voltage was set at 3500 V and the cone voltage at 35 V. In tandem MS experiments, the system operated with automatic switching between MS and MS/MS modes. The 3 most abundant peptides, preferably doubly and triply charged ions, were selected from each MS spectrum for further isolation and CID fragmentation with 2 energies set using collision energy profile methods. The system was entirely controlled by MassLynx 4.1 (SCN 566, Waters, Milford, MA). Raw data collected during nanoLC-MS/MS analyses were processed and converted with a ProteinLynx Browser 2.3 (23, Waters, Milford, MA) into .pkl peak list format.

MS/MS data were analyzed using the MASCOT 2.2.0. algorithm (Matrix Science, London, UK). Spectra were searched for with a mass tolerance of 30 ppm for MS and 0.1 Da for MS/MS data, allowing a maximum of one missed trypsin cleavage site and taking carbamidomethylation of cysteins and oxidation of methionines as variable modifications. Spectra were first searched for against a target-decoy version of a subset of the NCBInr database restricted to the bacteria found in 16S rRNA gene community analysis (see Results). Common contaminants (keratins and trypsin) were added to the database. Protein identification was confirmed when at least two peptides with high quality MS/MS spectra (less than 20 points below Mascot's threshold identity score at 95% confidence level) were detected. All spectra that did not satisfy these criteria were exported using Scaffold software program 2.2.0 (Proteome Software, Portland, USA) and used to search using the same database. Protein identification was confirmed when one peptide with a very high quality MS/MS spectrum (more than 5 points above Mascot's threshold identity score at 95% confidence level) was detected. After removing common contaminants,

these thresholds led to protein identification with a false discovery rate of less than 3%.

3. Results

3.1. Chemical characterization of the sampling site

The physico-chemical composition of sediment and water samples from the Gabe-Gottes mining site is shown in Table 1. The pH of the water was neutral. The low biological oxygen demand (BOD) and chemical oxygen demand (COD) values as well as the low amounts of suspended solids detected suggested that organic matter was not abundant in this water. In addition, the organic content measured in the sediments themselves was only 2%. Sulfate levels and water conductivity were also low, which suggest that ionic species were not abundant. The stream trophic state was assessed by measuring total nitrogen, phosphorus and chlorophyll content, which showed the presence of photosynthetic organisms (Dodds et al., 1998). Nitrogen and phosphorus compound contents were in the same range as levels detected in oligotrophic streams (<700 and 25 μg l^{-1}, respectively) (Dodds et al., 1998). The chlorophyll a (Chlo a) content was lower in these samples than values found in mesotrophic streams (<10 μg l^{-1} chlorophyll) (Dodds et al., 1998), which suggests that photosynthetic organisms were present but not very abundant. Based on these characteristics, this system was considered to be an oligotrophic ecosystem.

Table 1
Physical and chemical characteristics of water and sediment samples.

	Water	Sediments
General characteristics		
pH	6.9	NT
Density (kg l^{-1})	NT	2.07
Moisture 20 °C (%)	NT	18
Moisture 105 °C (%)	NT	19.3
Fraction <2 mm (%)	NT	57.8
Fraction >2 mm (%)	NT	42.2
OM on <2 mm (%)	NT	2
Conductivity (μS cm^{-1} at 25 °C)	50.4	NT
Alkalinity (meq l^{-1})	0.18	NT
TSS (mg l^{-1})	0.7	NT
VSS	100%	NT
Chemical characteristics		
Total P (mg l^{-1} in water; % in sediments)	0.03	0.08
PO_4 (mg P l^{-1})	0.02	NT
COD (mg O l^{-1})	5	NT
BOD5 (mg O l^{-1})	0.4	NT
NH_4 (mg N l^{-1})	0.02	NT
NO_2 (mg N l^{-1})	<0.01	NT
NO_3 (mg N l^{-1})	1.2	NT
TKN (mg N l^{-1})	<0.1	NT
Cl^- (mg l^{-1})	2.1	NT
SO_4 (mg l^{-1})	6.2	NT
Br (mg l^{-1})	0.004	NT
Chlo a (μg l^{-1})	0.3	NT
Pheo (μg l^{-1})	0.2	NT
Metals and metalloids (μg l^{-1} in water, μg g^{-1} in sediments)		
Al	98	15 150
As	6	320[a]
Cd	ND	0.3
Cr	ND	40
Cu	1	103
Fe	ND	23 745
Mn	ND	510
Ni	ND	23
Pb	ND	49
Zn	2	88

ND: not detected; NT: not tested; OM: organic matter; TKN: total Kjeldahl nitrogen; COD: chemical oxygen demand; BOD5: biological oxygen demand after 5 days; Chlo a: chlorophyll a; Pheo: pheophytine; TSS: total suspended solids; VSS: volatile suspended solids.

[a] As(III) and As(V) concentrations measured in interstitial water were 14.6 and 135 μg l^{-1}, respectively.

Concentrations of Pb, Al and Fe in these sediment samples were 5, 190 and 500 times higher, respectively, than the average metal concentrations in the surrounding soils (http://www.stats.environnement.developpement-durable.gouv.fr/acces-thematique/sol/le-sol/la-contamination-des-sols-par-les-elements-traces.html). Total arsenic contamination measured in the water column was lower (6 μg l^{-1}) than the 10 μg l^{-1} limit recommended for drinking water. On the other hand, in sedimentary interstitial water, significantly higher As(III) and As(V) concentrations were measured (14.6 (±0.3) μg l^{-1} and 135 (±5) μg l^{-1}, respectively), whereas none of the methylated forms searched for, i.e. DMA and MMA, were detected. The arsenic concentration observed in the sediment samples (320 mg kg^{-1} dry weight) was six times higher than the average geochemical content in this region (48 mg kg^{-1} in the Vosges, http://infoterre.brgm.fr) and the As(V) species was 10 times more concentrated than the As(III) species. All in all, the physical and chemical characteristics determined here showed that this site contained neutral oligotrophic water with a moderately high level of heavy metal contamination.

3.2. Structure of the prokaryote community in sediments based on 16S rRNA genes

An inventory of the whole bacterial and archaeal community was drawn up by performing 16S rRNA-based community analysis. Total DNA extracted from the sediment was used as template for 16S rRNA gene amplification. Partial sequencing of 240 bacterial and 83 archaeal 16S rRNA genes and RPD analysis (see Materials and Methods) led to the identification of 193 and 20 OTUs (calculated with 97% identity) belonging to 14 bacterial and 2 archaeal phyla, respectively. Concerning the bacterial library, rarefaction curves calculated at the class level (85% identities) reached a plateau (Fig. 2). As the class level is the rank usually used for representing the bacterial community with less ambiguity, we limited our analysis to these clones, even though the rarefaction curves calculated at the species level (97% identity) did not reach a plateau. The rarefaction curves produced for archaeal sequence libraries reached a plateau which confirmed that the archaeal clone library was sufficiently sampled.

The bacterial 16S rRNA gene sequences were found to be affiliated at the order, family or genus level, and considerable diversity was observed (Fig. 3). The bacterial community was composed mainly of Bacteroidetes (13%), Betaproteobacteria (15%), Acidobacteria (17%) and Deltaproteobacteria (12%). Several phyla, such as Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, Nitrospira and Planctomycetes, accounted for 5–10% of the bacteria. Lastly, Firmicutes, Verrucomicrobia, Cyanobacteria, Gemmatimonadetes and Deinococcus/Thermus accounted for only a small proportion of the bacteria (less than 5%) (Fig. 3). At the order level, Sphingobacteriales, Rhizobiales, Burkolderiales, Myxococcales and Planctomycetaceae were present. Interestingly, 12% of the clones were related to Acidobacteriaceae, the most abundant family. 14 clones amounting to 6% of the clones were related to the *Nitrospira* genus. This genus was therefore the most abundant one identified in this ecosystem. Lastly, 4 clones could not be definitively attributed to any known phylum, which suggests that unknown bacteria account for at least 1% of this community. Archaeal phylogenetic analysis (Fig. 4) showed that this population included Euryarchaeaota (29 clones, corresponding to 38% of the sample) and Thaumarchaeota (also known as group I Archaea) (DeLong, 1992; Fuhrman et al., 1992; Brochier-Armanet et al., 2008) (48 clones, corresponding to 62% of the sample). The Euryarchaeaota detected in this study site fell into four clusters (Fig. 4, I-IV), three of which (I–III) were found to be affiliated with methanogenic Archaea and were possibly from the anaerobic part of the sediment. One cluster (Fig. 4, IV) was more divergent and not closely related to any known species, but was affiliated with several 16S rDNA clones previously reported (Rudolph et al., 2004; Galand et al., 2006). A large number of clones from Thaumarchaeota were also identified (Fig. 4). Three of them (clones 64 HQ664164, 19 HQ664132, 20 HQ664133) were affiliated with the species "*Candidatus Nitrososphaera gargensis*", a moderately thermophilic Thaumarchaeon recently isolated from a terrestrial hot spring (Hatzenpichler et al., 2008). The others were affiliated with environmental sequences originating from the soil which likely represent uncultivated lineages of Thaumarchaeota (Sliwinski and Goodman, 2004; Nicol et al., 2006; Valenzuela-Encinas et al., 2008).

3.3. Metaproteomic analysis

Complementary to 16S RNA analysis, a metaproteomic approach was used to identify the main functional traits of the bacterial community. Microorganisms were separated from sediments and interstitial water by performing Nycodenz density gradient. An aliquot of these cells was stained with a live/dead BacLight kit (Invitrogen) and results showed that most cells extracted from the sediments were viable (data not shown). This finding showed that the gradient density-based bacterial recovery step did not affect cell membrane integrity. After SDS-PAGE electrophoresis, proteins were identified using an NCBInr database restricted to bacteria obtained with 16S rRNA-based community analysis (Table 2). Only 23 proteins were identified, half of them are involved in biological processes conserved within the prokaryotic kingdom, such as replication and transcription (elongation factors, transcriptional regulator), translation (ribosomal proteins), energy metabolism (ATP synthases), regulation (histone family, transcriptional regulator) and stress responses (Hsp90, GroEL). As these proteins are involved in widespread and highly conserved biological processes, they could be assigned to specific taxons. These proteins originated from Betaproteobacteria and Deltaproteobacteria, which were abundant in this community, as well as from Alphaproteobacteria, and Planctomycetes, each of which accounted for less than 10% of the community. Indeed, several of these proteins were

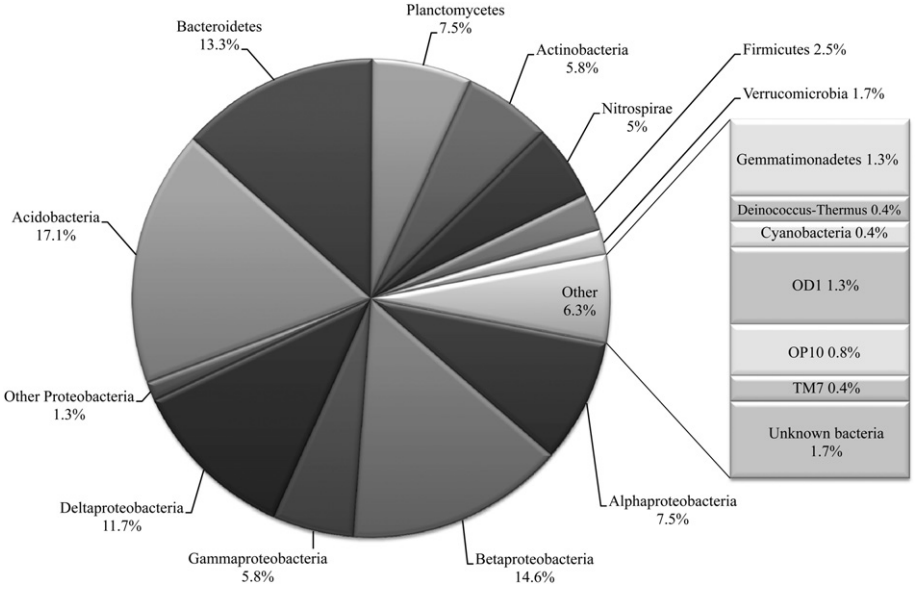


Fig. 3. Phylogenetic RDP affiliation of bacterial 16S rRNA genes (Class rank for Proteobacteria, phyla rank for the others). The most abundant phyla are shown in the circular diagram and the less abundant ones on the right.

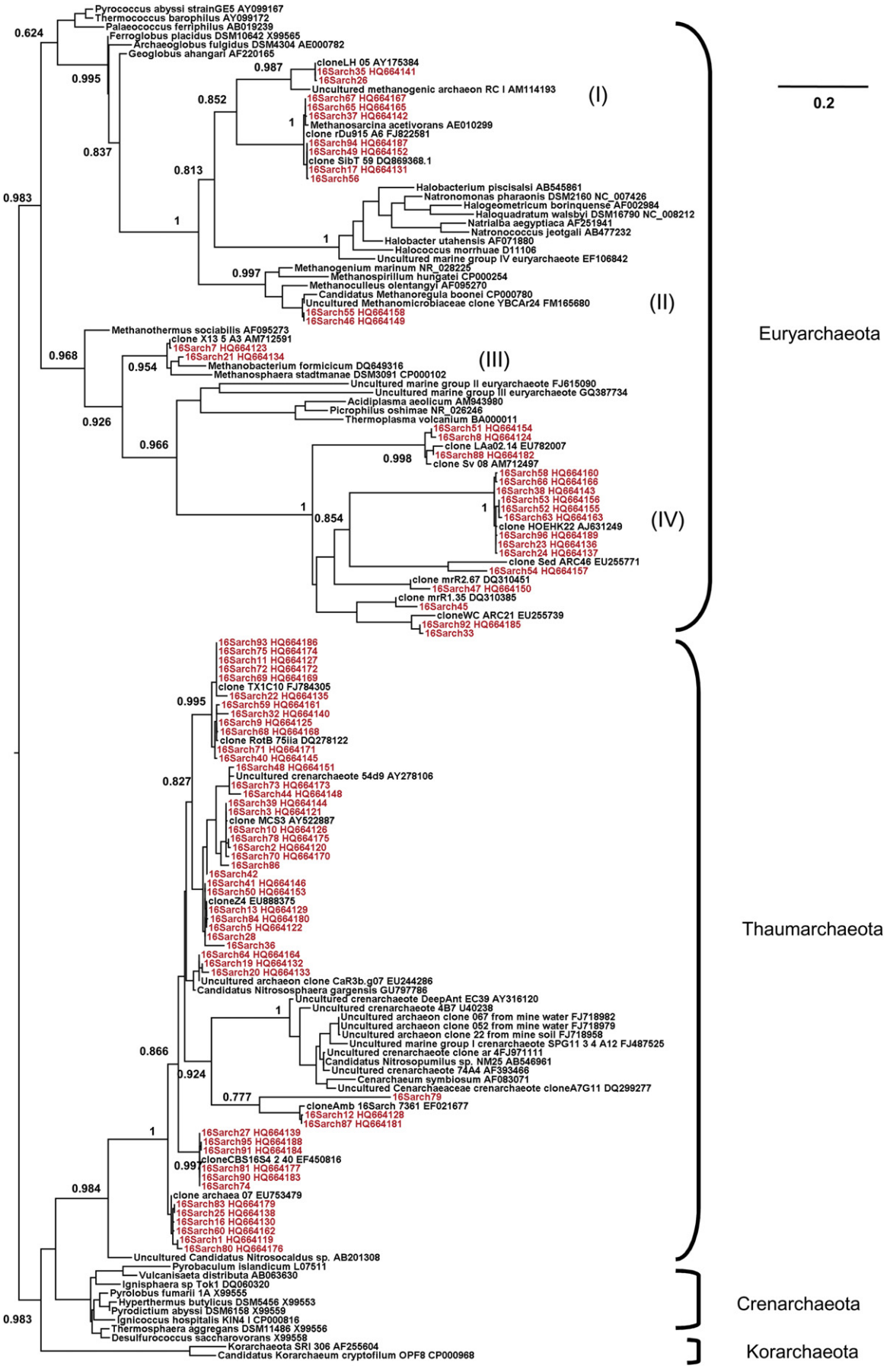


Fig. 4. Maximum likelihood tree of 16S rRNA gene homologs from the archaeal clones (in red font) along with a selection of representatives of archaeal diversity. The four main archaeal phyla are indicated. Numbers at nodes indicate aLTR (approximate likelihood-ratio test) branch support as computed by PhyML. The scale bar gives the average number of substitutions per site.

expressed by bacteria affiliated with *Hyphomicrobium*, *Parvimicrobium*, *Bradyrhizobium*, *Sphyngopyxis*, *Sideroxydans*, *Desulfovibrio* and *Planctomyces*. The metaproteomic approach also revealed the presence of 6 proteins involved in the photosynthetic process and expressed by Cyanobacteria, although these photosynthetic bacteria accounted for less than 1% of the bacterial community.

4. Discussion

The aim of this study was to characterize, both chemically and microbiologically, a moderately arsenic-contaminated environment and to identify the arsenic-tolerant prokaryotic bacterial species. The bacterial and archaeal diversity observed at our study site is greater than that observed at other more severely contaminated sites (Baker and Banfield, 2003; Johnson and Hallberg, 2003), at the Carnoulès AMD, for example, where less than 10 phyla were identified (Bruneel et al., 2005, 2006, 2008) and in meta-genomic studies on AMD biofilm (4–6 bacterial species) (Tyson et al., 2004). Nor was such diversity observed in geothermal spring waters (6 phyla (Hamamura et al., 2009)).

This high level of diversity makes the meta-proteomic approach rather difficult to apply, which explains why only 23 proteins were identified despite efficient bacteria and protein extraction procedures. A low number of identified proteins was also observed previously in soil in which a high level of diversity was observed (Benndorf et al., 2007; Williams et al., 2010). As pointed out by the authors of recent reviews (VerBerkmoes et al., 2009; Denef et al., 2010), meta-proteomic studies are successful when applied to communities with low levels of diversity. When a high level of diversity is observed, each protein is diluted in a complex preparation and only the most abundant proteins are therefore likely to be identified. Moreover, a large proportion of the bacteria forming this community have never been studied thus far in vitro, and their genome sequences and hence their protein sequences, which are required for MS identification purposes, are not available in public databases. For example, contrary to Proteobacteria, only a few Acidobacteria protein sequences are available in the existing protein databases. Likewise, no archaeal proteins were identified, probably because only a few protein sequences of Archaea are available. On the other hand, the meta-proteomic approach has some advantages compared to other approaches. Indeed, as proteins are more stable than RNA (especially those originating from prokaryotes), the metaproteome content was presumed to be less affected by the extraction procedure than the transcriptome and probably gives better insight into the biological functions expressed in situ. Moreover, the meta-proteomic approach gives taxonomic information that was not observed with the 16S rRNA gene-based approach. First, the diversity estimated with the 16S rRNA gene-based approach might be underestimated because of DNA extraction and PCR biases. Second, the meta-proteomic approach highlights expression of proteins involved in conserved biological processes that could be assigned to a specific taxon at the genus or species level, whereas RDP analysis allowed us to affiliate only 28% at the genus level despite the use of almost all 16S rRNA gene sequence lengths. Such a limit was in agreement with previous observations establishing that it is not always possible to affiliate certain bacteria using only 16S rRNA gene sequences (Schleifer, 2009). Indeed, some microorganisms showing very similar 16S rRNA gene sequences turned out to belong to different taxons when other phylogenetic markers were used. For these reasons, meta-proteomic data provide a valid complementary tool for use with 16S rRNA gene-based taxonomy.

Several Thermoplasmatales related to our clones (Fig. 4, cluster IV) were previously identified in arsenic-rich environments such as that present in Carnoulès (Bruneel et al., 2008) as, for example, several *Ferroplasma* (Baker and Banfield, 2003; Denef et al., 2010). On the other hand, various Thaumarchaeota present here are affiliated with environmental sequences originating from soil (Sliwinski and Goodman, 2004; Nicol et al., 2006; Valenzuela-Encinas et al., 2008). To our knowledge, none of them have been previously observed in arsenic-contaminated environments. Concerning the bacterial phyla, Bacteroidetes have rarely been observed in arsenic-rich environments (except for a few *Flavobacteria*) (Honschopp et al., 1996), whereas other phyla detected here were previously described in arsenic-contaminated samples, as for example *Sideroxydans*, *Rhodoferax*, *Variovorax*, *Flavobacterium* and *Microbacterium* (Baker and Banfield, 2003; Macur et al., 2004; Bruneel et al., 2006, 2011; Casiot et al., 2006; Achour et al., 2007; Achour-Rokbani et al., 2010). Such bacteria could impact arsenic speciation in this studied site. Bacteria related to *Sideroxydans lithotrophicus* were active in this ecosystem, as revealed by the metaproteomic approach. Such microorganisms are known to be preferentially present in a low oxygen concentration environment where they efficiently oxidize iron and may consequently have an indirect impact on the soluble arsenic concentration. Indeed, Fe(III) produced by these bacteria may co-precipitate with arsenate, leading to a decrease in soluble arsenite. Similarly, other species belonging to *Rhodoferax* are known to reduce iron under anaerobic conditions; their metabolism may therefore lead to an increased concentration of soluble arsenic compounds. Finally, some Betaproteobacteria identified using our proteogenomic approach may directly change arsenic speciation. Indeed, *Variovorax* sp. are arsenic-resistant bacteria known to reduce arsenate (Rathinasabapathi et al., 2006) and bacteria related to *Flavobacterium* were shown to methylate arsenic (Honschopp et al., 1996). Since arsenic was mainly found in an oxidized form (AsV) in this environment, it is tempting to speculate that both arsenic reduction and methylation activities were not important, in contrast to oxidation activity. Although we cannot exclude that abiotic processes also influence speciation of arsenic in this environment, arsenite oxidation may be catalyzed by bacteria affiliated with *Microbacterium*, as previously shown (Mokashi and Paknikar, 2002). Finally, other bacteria may also play a significant role in the functioning of this ecosystem. Identification of proteins originating from Plantomycetes, Cyanobacteria and Proteobacteria such as Rhizobiales revealed that these bacteria were not only present, but also viable, and physiologically active despite their low abundance, as suggested by the 16S rDNA metagenomic approach (less than 5% of the bacterial

Table 2
Main proteins expressed by the community.

Phylum	Class	Order; family; genus; species	Protein name	Protein molecular weight (Da)	Number of unique peptides	Percentage sequence coverage (%)	Peptide sequence
Proteobacteria	Unknown	Unknown	Thioredoxin	11 540.5	1	8.33	GIPTLILFK
	Alphaproteobacteria	Rhodobacterales or Rhizobiales	F_0F_1-ATP synthase subunit beta	51 052.6	3	7.97	FTQAGSEV SALLGR IGLFGG AGVGK TVLIMELI NNVAK
			Pyrrolo-quinoline quinone	65 645.4	1	1.82	VAWTF STGVLR
		Rhizobiales; Hyphomicrobiaceae; *Hyphomicrobium*; *Hyphomicrobium denitrificans*	Conserved hypothetical protein	21 210.5	2	4.35	GPVSLPR RGPVSLPR
		Rhizobiales; Phyllobacteriaceae; *Parvibaculum*; *Parvibaculum*	Histone family protein DNA-binding protein	12 014.1	2	16.80	LTGLGILQVR NPATGEAIK
			30S Ribosomal protein S4	23 808.7	1	4.37	LSDYGVQLR
		Rhizobiales; Bradyrhizobiaceae; *Bradyrhizobium*	Heat shock protein 90	68 916.8	1	2.24	GVIDSEDL PLNISR
		Sphingomonadales; Sphingomonadaceae; *Sphingopyxis*	30S Ribosomal protein S3	27 155.2	1	3.32	LGGAEIAR
			30S Ribosomal protein S12	13 973.1	1	6.50	PTINQLVR
	Betaproteobacteria	Gallionellales; Gallionellaceae; *Sideroxydans*; *Sideroxydans lithotrophicus* ES-1	Chaperonin GroEL	57 287.8	2	7.85	AAVEEGIVPG GGVALLR AGRPLLIIAE DVDGEALA TLVVNNIR
		Burkholderiales; Comamonadaceae	Elongation Factor Tu	43 021.5	1	3.28	QVGVPYII VFLNK
			Enolase	45 992.7	2	5.61	AAAEES GLPLYR AAVPSG ASTGSR
			5-Methyl-tetrahydro-pteroyl-triglutamate/homocysteine S-methyltransferase	86 464.0	1	1.28	VLSIGIVDGR
			50S ribosomal protein L2	30 162.6	1	4.01	SAGTSAT LLAR
	Deltaproteobacteria	Desulfovibrionales; Desulfovibrionaceae; *Desulfovibrio*; *Desulfovibrio salexigens*	Periplasmic binding protein/ LacI transcriptional regulator	30 060.5	1	4.81	VIQLEGLA GTSAAR
Cyanobacteria or Firmicutes	Unknown	Unknown	Elongation Factor Tu	43 262.6	1	3.28	TTLTAAITT VLAK

(*continued on next page*)

Table 2 (*continued*)

Phylum	Class	Order; family; genus; species	Protein name	Protein molecular weight (Da)	Number of unique peptides	Percentage sequence coverage (%)	Peptide sequence
Cyanobacteria	Unknown	Unknown	Photosystem II reaction centre protein PsbD/D2	39 443.4	2	5.40	AYDFVSQELR
			Photosystem Q(B) protein 1	29 295.2	2	8.68	NILLNEGIR LIFQYAS FNNSR VINTWADIINR
			Allophycocyanin alpha chain	17 413.2	2	11.80	SIVNADAEAR YLSPGELDR
	Chroococcales	*Synechococcus*	Hypothetical protein S7335_4928	7758.3	1	10.30	LATVVTPR
	Nostocales	Nostocaceae	Photosystem Q(B) protein 1 (32 KDa thylakoid membrane protein 1); Photosystem II protein D1	39 531.8	1	6.39	TIDTWAD LLNR
Planctomycetes	Planctomycetacia	Planctomycetales; Planctomycetaceae; *Planctomyces*; *Planctomyces limnophilus*	ATP synthase F1 subcomplex beta subunit	51 974.8	2	4.99	FSQAGSEV SALLGR TVILQELIAR
		Planctomycetales; Planctomycetaceae; *Planctomyces*; *Planctomyces maris*	F_0F_1-ATP synthase subunit beta	52 729.3	1 1	4.97	TVILTELIAR FSQAGSEVS ALLGR

clones). As Cyanobacteria and Rhizobiales are known to be involved in carbon and nitrogen fixation, these organisms could play an important role in such oligotrophic ecosystems.

In conclusion, by combining proteomic and genomic approaches in a mildly arsenic-contaminated environment, the diversity of arsenic-adapted prokaryotic communities was enlarged. Such approaches can thus serve to explore the diversity and the main functional traits of a bacterial community, including those having a high level of diversity.

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