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Abstract Acid mine drainage of the Carnoulès mine (France) is characterized by acid waters containing high concentrations of arsenic and iron. In the first 30 m along the Reigous, a small creek draining the site, more than 38% of the dissolved arsenic was removed by co-precipitation

with Fe(III), in agreement with previous studies, which suggest a role of microbial activities in the co-precipitation of As(III) and As(V) with Fe(III) and sulfate. To investigate how this particular ecosystem functions, the bacterial community was characterized in water and sediments by

16S rRNA encoding gene library analysis. Based on the results obtained using a metaproteomic approach on sediments combined with high-sensitivity HPLC-chip spectrometry, several GroEL orthologs expressed by the community were characterized, and the active members of the prokaryotic community inhabiting the creek sediments were identified. Many of these bacteria are β -proteobacteria such as *Gallionella* and *Thiomonas*, but γ -proteobacteria such as *Acidithiobacillus ferrooxidans* and α -proteobacteria such as *Acidiphilium*, *Actinobacteria*, and *Firmicutes* were also detected.

Introduction

Acid Mine Drainage (AMD) is one of the most serious forms of water pollution in industrial and post-industrial areas worldwide [38]. AMD is generated when the wastes from the mining and processing of sulfide ores (such as pyrite or arsenopyrite) come into contact with oxygenated water [5]. AMD is often characterized by pH values of 2–4. Such waters generally contain high levels of iron, toxic metals (such as aluminum, manganese, lead, cadmium, and zinc), and metalloids (arsenic) [5, 32, 48]. AMD can still occur hundreds of years after mine closure and tens of thousands of kilometers of groundwater, streams, lakes, and estuaries throughout the world have been directly impacted [40]. In several cases of AMD, natural remediation has been observed, as for example at the Carnoulès site in France and the Rio Tinto site in Spain [18, 53]. In such AMD, toxic compounds are accumulated in sediments consisting of a variety of iron (oxyhydr)oxides and hydroxysulfates such as jarosite, schwertmannite, and ferrihydrite [48]. Natural remediation of metal pollutants is generally due to the occurrence of abiotic reactions and/or microbial activities that make these toxic compounds insoluble and lead them to accumulate in sediments [32, 40]. This toxic compound precipitation processes mainly involve the oxidation and precipitation of iron, which is often the main soluble metal present in AMD, and the adsorption of other metals and metalloids by the ferric minerals formed [32, 51]. Indeed, many elements such as Sr, Cs, Pb, U, and As show a strong affinity for solid iron oxide [18, 27, 48]. Abiotic oxidation of Fe(II) proceeds very slowly in acidic (pH 3.5) waters [51]. In contrast, iron-oxidizing bacteria catalyze the reaction and thus accelerate the formation of solid iron oxide [32, 41, 51]. In addition, several bacteria contribute to the immobilization of arsenic via their ability to oxidize this metalloid [6, 14, 18, 22, 48], arsenate (As(V)) being adsorbed more strongly than arsenite (As(III)) by Fe(III) oxides and hydroxides at acidic pH levels [10]. *Thiomonas* strains show a high arsenite oxidation capacity, and these metabolic activities have been extensively analyzed under laboratory conditions [6, 14, 22].

However, to be able to develop remediation processes and/or optimize existing processes, further knowledge is required about how these bacteria function in situ. In particular, it is of crucial importance to determine which bacteria are viable and active in such ecosystems.

The AMD of Carnoulès mine in Southern France is a highly suitable site for analyzing how microorganisms contribute to the transformation of metals and metalloids in situ, since efficient natural remediation processes are known to occur at this site [12, 18]. This former mine generated around 1.2 Mt of tailings containing 0.7% Pb, 10% FeS₂, and 0.2% As. Water percolating through the tailings forms the head of the Reigous creek. This creek is acidic (pH around 3) and highly contaminated with As (100 to 350 mg L⁻¹). The behavior of As and Fe in the Reigous creek has been intensively studied [18, 23, 48]. In the creek spring, As(III) is the main As species present and Fe occurs in the form of Fe(II) [18]. Along the first 30 m of the creek (about 1 h residence time), the oxidation of Fe(II) leads to the co-precipitation of more than 38% of the dissolved As [18, 23]. Arsenic accounts for up to 22% of the total dry weight of the sediments formed along the first 10 m along the creek. In the wet season, approximately 30 m downstream of the spring, these sediments are mainly composed of As(III)–Fe(III) oxyhydroxysulfates, whereas As(V)–Fe(III) oxyhydroxysulfates compounds predominate during the dry season [48]. Because of the very high molar As/Fe ratio (up to 0.3) existing in the dissolved phase of the Carnoulès creek, the mineralogical content of the sediments differs significantly from that classically observed at most AMD [47], especially along the first 50 m of the creek. Several strains of *Thiomonas* and *Acidithiobacillus ferrooxidans* have been isolated from Reigous creek waters, and based on the results of laboratory experiments, it has been suggested that these bacteria may contribute to the oxidation of Fe(II) and the co-precipitation of As [14, 18, 21–23, 48]. Preliminary analyses have shown that the DNAs of both bacteria are present in the Reigous creek, as well as that of *Gallionella* sp., *Thiobacillus* sp., and some sulfate-reducing bacteria [12]. Archaea have also been found to occur in the Reigous creek (*Ferroplasma acidiphilum* and sequences affiliated to uncultured *Thermoplasmatales* archaeon) as well as a eukaryotic microorganism, *Euglena mutabilis* [12, 13, 15]. However, the bacterial population inhabiting the As-rich Reigous sediments has never been characterized so far. It, therefore, seemed to be necessary not only to identify the bacteria present in this creek but also to determine which members of this community are viable and, therefore, perform metabolic activities in situ.

The aim of this study was to describe the bacterial populations occurring in both the sediments and waters at the disused Carnoulès site and to identify the bacteria at work. For this purpose, three complementary approaches

were used. First, chemical and mineralogical studies were performed in order to determine the arsenic species present. A 16S rRNA encoding gene library was then analyzed in order to identify the bacterial population present in the creek sediments and waters. Lastly, based on the findings obtained using a metaproteomic approach combined with high-sensitivity mass spectrometry methods, the active species inhabiting the sediments were identified.

Methods

Sampling and Analysis

Samples were collected from Reigous creek in April 2006 at COWG station located 30 m downstream from the spring. This sampling was part of a long-term monitoring of the physicochemistry of the Reigous Creek water [23]. The main physicochemical parameters (pH, temperature, and dissolved oxygen concentrations) were measured in situ at this sampling point. The 5-cm deep sediments on the bottom of the creek and a thin column (less than 10 cm) of running water covering the sediments were sampled. Solid samples were removed with a sterile spatula from the surface of the sediments. Water samples (300 ml) were immediately filtered through 0.22 μm Millipore membranes fitted on Sartorius polycarbonate filter holders (for water chemical analysis) or through sterile 0.22- μm Nucleopore filters that were then transferred to a collection tube (Nunc), frozen in liquid nitrogen, and stored at -80°C until DNA extraction (for 16S rRNA encoding gene analysis). Sampling was repeated three times. For total Fe and As determination, filtered water was acidified to pH=1 with HNO_3 (14.5 M) and stored at 4°C in polyethylene bottles until analysis. For As and Fe speciation, a 10 μl aliquot of filtered sample water was added to either 0.5 ml of 5% (v/v) 0.25 M EDTA solution for As speciation [7] or a mixture of 0.5 ml acetate buffer (pH 4.5) and 1 ml of 1,10-phenanthroline chloride solution for Fe speciation [50]. The vials were completed to 10 ml with deionized water. The samples used for arsenic speciation and Fe(II) and sulfate determination were stored in the dark and analyzed within 24 h.

Chemical Analysis

The determination of total dissolved As was performed by ICP-MS using Thermo X7 series with a conventional external calibration procedure. Indium was used as internal standard to correct for instrumental drift and possible matrix effects. It was not necessary to correct interference with chloride because of the extremely high As levels present. Certified reference material SLRS-4 (freshwater

samples) was used to check analytical accuracy and precision. The results showed that the recovery rate obtained was within $\pm 5\%$.

Analyses of inorganic arsenic species (As(III), As(V)) were carried out using anion-exchange chromatography (25 cm $\times$ 4.1 mm i.d. Hamilton PRP-X100 column with Varian ProStar gradient solvent delivery system) coupled to a hydride generation (VGS 200, FISON, France) with an atomic fluorescence spectrometry detector (Excalibur, PSA analytical, GB) [17]. The detection limit obtained was 172 ng L^{-1} for As(III) and 458 ng L^{-1} for As(V), with a precision better than 5%. Total dissolved Fe was determined by flame atomic absorption spectrometry. Fe(II) concentration was determined using colorimetry at 510 nm after complexation with 1,10-phenanthroline chloride solution in buffered samples (pH 4.5) [50] (detection limit: 11 $\mu\text{g L}^{-1}$; precision better than 5%). Sulfate concentration was determined after precipitation of BaSO_4 with BaCl_2 and spectrophotometric measurement at 650 nm [50].

Solid Sample Characterization

XAFS data were gathered on the laboratory samples and the sample taken at COWG on April 2006, at 10 K in transmission mode on a bending magnet D44 at the LURE synchrotron (Orsay, France), and in fluorescence mode on the 11-2 wiggler beamline at SSRL (Stanford, CA), respectively. Experiments and data reduction were previously reported [48, 49].

DNA Isolation, 16S rRNA Encoding Gene Cloning, Restriction Analysis, and Sequencing

Genomic DNA was extracted in triplicate from filtered water and sediments using the UltraClean Soil DNA Isolation Kit according to the manufacturer's recommendations (MoBio Laboratories Inc., USA). These triplicates were pooled before PCR amplification. All the genomic DNA samples extracted were stored at -20°C until further processing. Bacterial diversity was analyzed by cloning PCR amplified 16S rRNA encoding genes. Bacterial 16S rRNA encoding genes were amplified with 8F (5'-AGAGTTTGA TCCTGGCTCAG-3') and 1489R primers (5'-TACCTTGT TACGACTTCA-3') [43, 57], as previously described [12]. These PCR products were cloned into *Escherichia coli* TOP 10 strain using the pCR2.1 Topo TA cloning kit (Invitrogen, Inc.). Cloned 16S rRNA encoding gene fragments were re-amplified using the primers TOP1 (5'-GTGTGCT GGAATTCGCCCTT-3') and TOP2 (5'-TATCTGCAGA ATTCGCCCTT-3') that anneal to the vector and surround the inserted PCR fragment and then digested with *Hae*III or *Hinf*I enzymes. Restriction profiles were analyzed using 2.5% agarose gel electrophoresis (small fragment resolution

agarose; QA agarose, QBiogène, Inc.). Around 200 clones from each library were analyzed and grouped according to the RFLP patterns obtained. PAST (Paleontological STatistics v 1.19) software from the website <http://folk.uio.no/ohammer/past/> was used to calculate different diversity indices (rarefaction analysis, taxa, total clones, singletons, dominance, coverage, shannon, equitability, and simpson, Table 1) for each clone library. The total number of clones obtained compared with the number of clones representing each unique phylotype was used to produce the rarefaction curves. Coverage values were calculated to determine how efficiently the libraries described the complexity of a theoretical community such as an original bacterial community. The coverage [29] value is given as $C = 1 - (n_1/N)$ where n_1 is the number of clones that occurred only once in the library. Rarefaction analysis showed that the curves generated were near saturation (data not shown) and consistent with the high coverage values of the two clone libraries (97.8 for the sediment and 98.6 for the water). This indicated that the clone libraries were sufficiently sampled.

Partial sequences of the clones from dominant groups were determined by the dideoxy nucleotide chain-termination method using the BigDye 3.1 kit (Applied Biosystems) on an ABI PRISM 3730XL Genetic analyzer (Applied Biosystems). The PINTAIL program [4] was used to check the presence of chimerae. Sequences were also examined manually for chimerae, which were excluded from further analyses. These sequences were compared with known sequences (NCBI database) using the BLAST, ALIGN, and CLUSTALW programs [1, 26, 55]. All sequence data obtained were submitted to the EMBL databases under accession numbers (FR676963-FR677013; AM988784-AM988794; AM988796; AM988798; AM988801-AM988805; AM988807-AM988809).

Preparation of Protein Extracts and Gel Electrophoresis Analysis

Prokaryotes were separated from sediments and eukaryotes using a Nycodenz density gradient. It should be noted that the main population determined from the DNA directly extracted from sediments was similar to that identified after Nycodenz treatment (data not shown), which suggests that this treatment did not result in enrichment of the sample by any particular microorganisms. Ten grams of sediments

were washed in 10 mL of saline buffer and agitated overnight at 4°C. After 10 min of decantation, 7.5 mL of supernatant were added without mixing to 17.5 mL of Nycodenz solution (Axis-Shield, Dundee, Scotland), and then centrifuged for 30 min at 10,000×g. The cellular fraction (nycodenz/sample interface) was removed and washed by adding two volumes of NaCl 0.9% and centrifuged for 15 min at 10,000×g at 4°C. Proteins were extracted from this cellular fraction as previously described [58], further purified using the 2-D Clean-up kit (GE Healthcare), and resuspended in rehydration buffer (364 g L⁻¹ thiourea, 1,000 g L⁻¹ urea; 25 g L⁻¹ CHAPS, 0.6% v/v IPG buffer Pharmalyte, 10 g L⁻¹ of DTT, and 0.01% bromophenol blue). Protein concentrations were quantified using the 2-D Quant kit (GE Healthcare). These proteins were separated by 2-D gel electrophoresis as previously described [58] and finally stained with silver nitrate. Gels were analyzed using an Image Scanner, LabScan v 3.0 (GE Healthcare), and the ImageMaster 2D platinum software program (v. 6.01, GE Healthcare). The spots selected were cut out of the 2-D gels and stored at -20°C. Eighty-one spots were analyzed by performing nanoLC-Chip-MS/MS.

In-Gel Digestion, Mass Spectrometry Analysis, and Protein Identification

Unless otherwise specified, all chemicals were obtained from Sigma (St. Louis, MO, USA). In-gel digestion of gel spots was performed as previously described [58]. The resulting peptides were analyzed by performing nanoLC-MS/MS on an Agilent 1100 Series HPLC-Chip/MS system (Agilent Technologies, Palo Alto, USA) coupled to an HCT Ultra ion trap (Bruker Daltonics, Bremen, Germany). The MS/MS data were analyzed using the MASCOT 2.2.0 algorithm giving a maximum of one missed cleavage, with a mass tolerance of 0.5 Da for MS and MS/MS data and carbamidomethylation of cysteines and oxidation of methionines were specified as the variable modifications. MS/MS data searches were performed against two in-house generated databases. The first database was composed of the protein sequences of all the organisms related to the groups identified by performing 16S rRNA encoding gene analysis on the Reigous creek sediments and water (α -, β -, δ -, and γ -*proteobacteria*, *Bacilli*, *Clostridia*, *Actinobacteria*,

Table 1 Diversity indices calculated from the two clone libraries in sediment and water at the Carnoulès mine drainage creek

Clone library	Taxa	Total no. of clones	Singletons	Dominance (D)	Coverage (C)	Shannon (H)	Equitability	Simpson (1-D)
Sediments	15	229	5	0.5503	97.8	1.146	0.4231	0.4497
Water	18	221	3	0.2551	98.6	1.949	0.6744	0.7449

Nitrospira), as well as unclassified bacteria from <http://beta.uniprot.org/>, *Thiomonas* sp. from <http://www.genoscope.cns.fr/> (FP475956–FP475957), *Euglenozoa*, and *Viridiplantae*. The second database included all bacterial and archaeal GroEL chaperonins (12501 and 291 sequences, respectively) extracted from the Uniprot database (<http://www.uniprot.org/uniprot>).

To assess the false positive rate in the protein identification, a target-decoy database search was performed [25]. With this approach, peptides are matched against a database consisting of the native protein sequences detected in the database (target) and the sequence-reversed entries (decoy). Protein identification was confirmed when at least two peptides with a minimum Mascot ion score of 30 were detected. In the case of one-peptide hits, the score of the unique peptide had to be greater than the 95% significance Mascot threshold level. All the proteins identified were added to the “InPact” proteomic database developed at our laboratory (<http://inpact.u-strasbg.fr/~db/>) [8].

Phylogenetic Analyses

A search for GroEL homologs and 16S rRNA encoding sequences was carried out in the Uniprot and NCBI databases, respectively. A total number of 530 reviewed GroEL bacterial sequences were retrieved from the RefSeq database. These sequences were 500–550 amino acids in length. Only one sequence representative of each genus (259 sequences in all) was kept. GroEL and 16S rRNA encoding sequences were aligned using ClustalW [55]. Alignments were checked by hand and positions with more than 1% of gaps were automatically removed. Neighbor-joining trees were constructed with 185 amino acids in the case of the GroEL sequences and with 310 nt in that of the 16S rRNA encoding sequences. Trees were drawn up using the iTOL website (<http://itol.embl.de/>) [44].

Results and Discussion

Physical and Chemical Characteristics of Samples

The physicochemistry of the Reigous Creek water at the time of sampling was typical of that revealed during the long-term monitoring study [23]. The water sample was acid (pH=3.28) and moderately oxygenated (dissolved oxygen concentration=3.5±0.5 mg L⁻¹); it contained extremely high concentrations of SO₄²⁻ (2700±300 mg L⁻¹), Fe (620±30 mg L⁻¹), and As (140±4 mg L⁻¹), with a large predominance of Fe(II) (90±10% of total Fe concentration) and equal proportion of As(III) and As(V). The removal of As during the course of the Reigous Creek from its source to the sampling station

COWG reached 38%, corroborating the typical removal rates measured during the long-term monitoring study [23]. The pale-yellow loosely packed sediments previously observed during the dry season at the sampling point chosen in this study (COWG ~30 m downstream of the spring), consisted of an amorphous Fe(III)–As(V) hydroxysulfate mineral with an As/Fe molar ratio of 0.5 to 0.6 [48]. However, various other biominerals may be formed from dissolved Fe(II) and As(III) [47, 48]. The exact nature and structure of the sediment sample studied was, therefore, further investigated. X-ray powder diffraction (data not shown) and X-ray absorption spectroscopy data obtained at the As–K edge (Fig. 1) showed that arsenic was present in these samples in an amorphous Fe(III)–As(V) hydroxysulfate phase as previously observed [48]. These analyses showed that despite the presence of a minor As(III) impurity, the oxidized arsenic form As(V) predominates in this sediment (Fig. 1a). In a previous study, it has been shown that the catalytic oxidation of As(III) by *Thiomonas* sp. strains accelerates such As–Fe precipitation process [48]. Therefore, using extended X-ray absorption fine structure analysis at the As–K edge (Fig. 1b), the structure observed in our samples was compared with that of the minerals obtained after As(III) oxidation by the *Thiomonas* sp. strain B2 in bioassays in which sterilized Carnoulès Creek water was used [48]. These comparisons (Fig. 1b, c) showed that the molecular structure of the amorphous Fe(III)–As(V) hydroxysulfate phase observed in these sediments was similar to that of the Fe(III)–As(V) hydroxysulfate obtained in the presence of *Thiomonas*, further supporting its role in situ.

Composition of Bacterial Communities in Reigous Creek Water and Sediments

Two 16S rRNA encoding gene libraries were constructed (Table 2), containing 229 clones in the sediment library and 221 in that of the water library. The Shannon index (see “Methods”) and equitability values were greater in the water library than in the sediment library (Table 1), which suggests that the bacterial diversity was lower in the sediment than in the water samples. Eleven different species were identified in the sediments and 13 in the water (Table 2, Fig. 2).

Several of the bacteria identified in the present study in both the waters and the sediments have been previously detected in the Reigous waters [12, 14, 15, 18, 21, 22]. Classified first by abundance order, several sequences were affiliated to *Thiobacillus* sp. ML2-16. This bacterium has been frequently reported to occur in AMD [5]. The presence of several strains affiliated to this *proteobacteria* is in agreement with the results of previous studies and shows that these bacteria persist in this

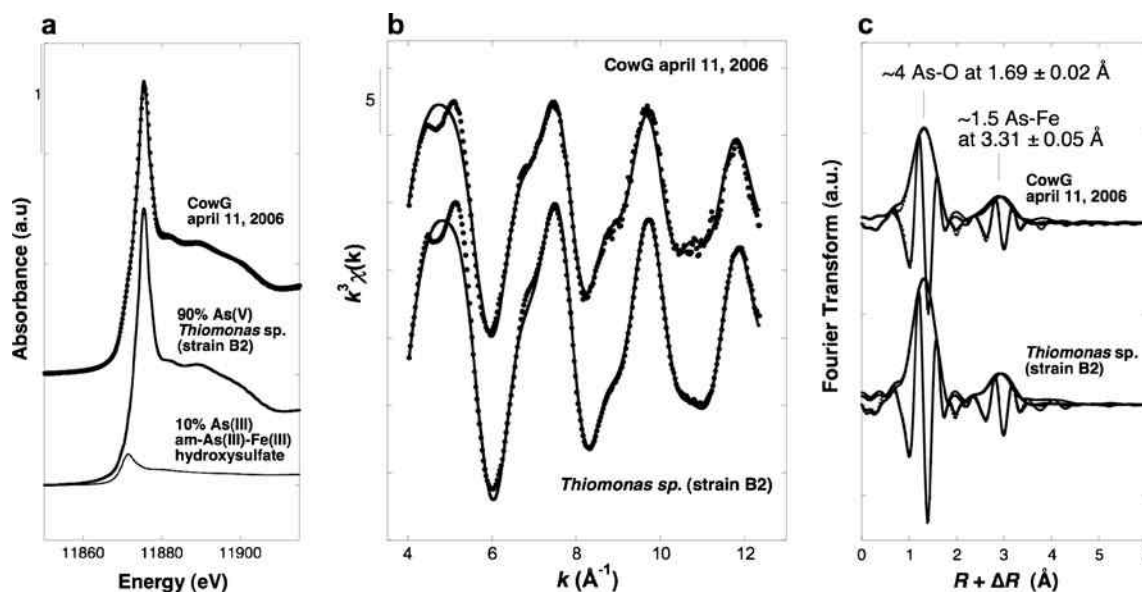


Figure 1 X-ray absorption spectra at the As-K edge of the sample, (COWG April 11, 2006,) showing similarities with the X-ray amorphous Fe(III)–As(V) hydroxysulfate phases obtained after incubating sterilized Carnoulès water with the *Thiomonas* sp. strain B2 isolated at the site [48]. These phases reached a molar As/Fe ratio of 0.8, as described in [48]. **a** Linear least-squares fitting of XANES data showed that the largest arsenic fraction ($90 \pm 2\%$) was in the As(V) oxidation state. A small arsenic fraction ($10 \pm 2\%$) was in the As(III) oxidation state, which resulted in a slight decrease in the amplitude in the EXAFS spectrum. **b** Shell by shell fit of the EXAFS spectra in k -

space for the COWG sediment sample and the *Thiomonas* sp. strain B2 precipitate sample. **c** Corresponding Fourier transforms of the experimental and fit curves. Dotted lines experimental; solid lines fitting curves. The local structure of both the COWG and the laboratory *Thiomonas* sp. samples includes bidentate arsenate–oxygen–iron complexes characterized by ~ 1.5 – 2.0 Fe atoms at an As–Fe distance of 3.31 ± 0.02 Å. A small arsenic fraction ($10 \pm 2\%$) was in the As(III) oxidation state, which resulted in a slight decrease in the amplitude in **b** the EXAFS spectrum and in **c** the corresponding Fourier transform

ecosystem [12]. Secondly, bacteria affiliated to *Gallionella capsiferiformans* were detected in both the water and sediments. *G. capsiferiformans* is an oxygen-dependent ferrous iron-oxidizing bacterium that grows at circum-neutral pH [59]. Relatives of *Gallionella*, such as *G. ferruginea* in particular, have often been detected, sometimes as the dominant group in microbial mine water communities, including Carnoulès [12, 33–35]. Bacteria related to *At. ferrooxidans* as well as *Thiomonas* strains have been previously isolated from this site [14, 18, 21, 22]. *At. ferrooxidans*, which was the first microorganism to be isolated from an acidic leaching environment, occurs ubiquitously in AMD, as does *Thiomonas* [32]. In addition to these groups, other species that have not previously been described at this site were detected in this study. Some of these species were found to occur in both sediments and water. For example, bacteria affiliated to the *Firmicutes Alicyclobacillus* sp. BRG 73 were identified. This genus, found in AMD [5], is characterized by moderately thermophilic, acidophilic, strictly aerobic, and endospore-forming bacilli [30]. Likewise, bacteria related to “*Ferrovum myxofaciens*” PSTR were detected in both the waters and sediments. “*Ferrovum myxofaciens*” is an autotrophic iron-oxidizer which predominates in some AMD and is able to grow litho-autotrophically, using ferrous iron as an electron

donor [32]. Lastly, bacteria affiliated to *Leptospirillum ferrooxidans*, an iron-oxidizing member of the *Nitrospirae* [36], were also present. This bacterium has been found to occur in several acidic environments and in biofilms originating from AMD [5, 9, 32].

Other newly characterized groups were identified only in sediments (Table 2). The presence in acidic mine waters of *Acidocella* sp., a non-iron-oxidizing heterotrophic acidophile is quite common in AMD [33, 35]. *Ferrimicrobium* is an iron-oxidizing heterotroph that can also use iron as an electron acceptor [20]. Other prokaryotes detected in sediments were affiliated to *Acidiphilium* sp. CCP3, a non-iron-oxidizing heterotrophic acidophile that is also quite common in AMD [33] and *Dokdonella koreensis*, a γ -proteobacteria.

Six newly characterized groups were identified only in water. Some of these bacteria were related to *Sideroxydans lithotrophicus* LD-1, an oxygen-dependent ferrous iron-oxidizing bacterium that grows at circumneutral pH [59]; *Rhodoferrax ferrireducens*, a psychrotolerant, facultative anaerobic bacterium which is able to oxidize acetate with the reduction of Fe(III) [28]; and an *Acidobacteriaceae* bacterium, CH1. Members of *Acidobacteria* have previously been reported in AMD [5, 32]. Finally, three sulfate-reducing bacteria were related to strain JHA1, *Desulfomonile limimaris*,

Table 2 Bacterial clones detected at the Carnoulès mine drainage with their phylogenetic group, the closest isolated relative and the relative abundance of each group versus the total number of clones (100%)

Sampling	Clones	Phylogenetic group	Closest isolated relative (accession number)	Percentage of similarity	Relative abundance of clones (%)
Sediment	CGA6Sd1a, 5a, 10b, 13c, 23c, 34c, 59b, 89c, 92c	<i>β-Proteobacteria</i>	<i>Thiobacillus</i> sp. ML2-16 (DQ145970)	95–96	31
	CGA6Sd4b, 36c, 48c	<i>β-Proteobacteria</i>	<i>Gallionella capsiferriformans</i> ES-2 (DQ386262)	96	21
	CGA6Sd13a	<i>γ-Proteobacteria</i>	<i>Acidithiobacillus ferrooxidans</i> DX-1 (EU084695)	99	10
	CGA6Sd10a, 37a	<i>α-Proteobacteria</i>	<i>Acidocella</i> sp. M21 (AY765998)	99–100	8
	CGA6Sd31c, 36a	<i>β-Proteobacteria</i>	<i>Thiomonas</i> sp. PK44 (AY455806)	99	6
	CGA6Sd6b, 18a, 27b	<i>γ-Proteobacteria</i>	<i>Dokdonella korensis</i> NML 01–0233 (EF589679)	92	5
	CGA6Sd32a	<i>Actinobacteria</i>	<i>Ferrimicrobium</i> sp. BGR 49 (GU167992)	99	3
	CGA6Sd38c	<i>α-Proteobacteria</i>	<i>Acidiphilium</i> sp. CCP3 (AY766000)	99	3
	CGA6Sd20a, 76c	<i>Firmicutes</i>	<i>Alicyclobacillus</i> sp. BGR 73 (GU167996)	92–99	3
	CGA6Sd58b	<i>Nitrospirae</i>	<i>Leptospirillum ferrooxidans</i> Sy (AF356839)	99	2
	CGA6Sd51c	<i>β-Proteobacteria</i>	“ <i>Ferrovum myxofaciens</i> ” PSTR (EF133508)	100	2
	CGA6Wt4c, 7a, 15c, 20a, 21b, 22b, 23a, 31b, 32a, 33c, 45b, 54c, 63b, 73c	<i>β-Proteobacteria</i>	<i>Thiobacillus</i> sp. ML2-16 (DQ145970)	94–96	26
	CGA6Wt7c, 17b, 21a, 23c, 27b, 35b, 36b, 67c, 80c, 86c	<i>β-Proteobacteria</i>	<i>Gallionella capsiferriformans</i> ES-2 (DQ386262)	89–97	18
	CGA6Wt25c, 56b	<i>γ-Proteobacteria</i>	<i>Acidithiobacillus ferrooxidans</i> BGR-110 (GU168011)	100	10
Water	CGA6Wt5a, 48c, 79b	<i>Firmicutes</i>	<i>Acidithiobacillus ferrooxidans</i> DSM 2392 (AJ459800)	91–92	8
	CGA6Wt9a, 19a, 27a, 9b, 61c	<i>β-Proteobacteria</i>	<i>Alicyclobacillus</i> sp. BGR 73 (GU167996)	91–99	8
	CGA6Wt11a, 29c, 78c	<i>β-Proteobacteria</i>	<i>Sideroxydans lithotrophicus</i> LD-1 (DQ386859)	94–97	8
	CGA6Wt3a, 8c	<i>β-Proteobacteria</i>	“ <i>Ferrovum myxofaciens</i> ” PSTR (EF133508)	98–99	4
	CGA6Wt86b	<i>β-Proteobacteria</i>	<i>Thiomonas</i> sp. PK44 (AY455806)	94	3
	CGA6Wt15a	<i>Nitrospirae</i>	<i>Leptospirillum ferrooxidans</i> (AB510912)	94	3
	CGA6Wt51b	<i>β-Proteobacteria</i>	<i>Rhodoferrax ferrireducens</i> T118 (CP000267)	99	3
	CGA6Wt61b	<i>Acidobacteria</i>	<i>Acidobacteriaceae</i> bacterium CH1 (DQ355184)	96	3
	CGA6Wt42c		Sulfate-reducing bacterium JHA1 (EF442984)	82	3
	CGA6Wt30a	<i>δ-Proteobacteria</i>	<i>Desulfomonile limimaris</i> (NR_025079)	87	3
	CGA6Wt10a	<i>δ-Proteobacteria</i>	<i>Desulfuromonas svalbardensis</i> 60 (AY835390)	82	1

Sequences closely related to 16S rRNA genes from *Euglena* spp. chloroplast were also detected (data not shown). The 16S rRNA encoding gene of chloroplasts is closely related to the bacterial 16S rRNA encoding gene and can therefore be amplified by primers 8F and 1489R

an anaerobic dehalogenating bacterium from marine sediments [54] and *Desulfuromonas svalbardensis* 60, a psychrophilic, Fe(III)-reducing bacterium isolated from Arctic sediments [56] (Table 2).

All in all, 17 species of bacteria were identified in the water and sediments sampled at the Reigous creek. Only seven genera were found to be present in both phases, six were found only in water, and four only in the sediments (Table 2). Most of these species are common residents of AMD [5, 32]. This quite low bacterial diversity was probably due to the high concentration of toxic compounds in this AMD and was consistent with previous observations showing that the biodiversity of acidic, metal-rich mine waters is mainly restricted to specialized prokaryotes and some eukaryotes such as *Euglena* [15, 52], which has been detected in this study (data not shown). In the Reigous system, in both sediments and water, the populations observed were mainly involved in the Fe, As, and S cycles. The populations involved in Fe(II) oxidation were related to *Gallionella*, *At. ferrooxidans*, *Ferrimicrobium*, *Leptospirillum*, *Sideroxydans lithotrophicus*, or “*Ferrovum myxofaciens*” [32, 59], whereas ferric iron reduction has been described for populations like *Acidiphilium* spp., *Acidocella*, *Desulfuromonas svalbardensis*, *Rhodoferrax ferrireducens*, or even *At. ferrooxidans* and *Ferrimicrobium acidiphilum* [20, 28, 56]. Since the Carnoulès creek spring contains mainly Fe(II) and As(III) in the form of dissolved species [18], the Fe(III) and As(V) may be formed as the result of microbial oxidation processes via the activity of acidophilic iron- and arsenite-oxidizing bacteria [24, 48]. In other words, the large amounts of soft pale-yellow As(V)–Fe(III) hydroxysulfate sediments analyzed here (Fig. 1) were probably formed by the joint activities of iron-oxidizing (e.g., *At. ferrooxidans* or *Gallionella*) and arsenic-oxidizing (e.g., *Thiomonas* sp.) microorganisms. Concerning S cycling, we found populations able to oxidize the reduced inorganic sulfur compounds, like *Thiobacillus*, *Thiomonas*, or *At. ferrooxidans* [32, 42, 46]. Sulfate-reducing bacteria such as *Desulfomonile limimaris* or *Desulfuromonas svalbardensis* were also found to be present in sediments and may be involved in sulfate consumption [54, 56].

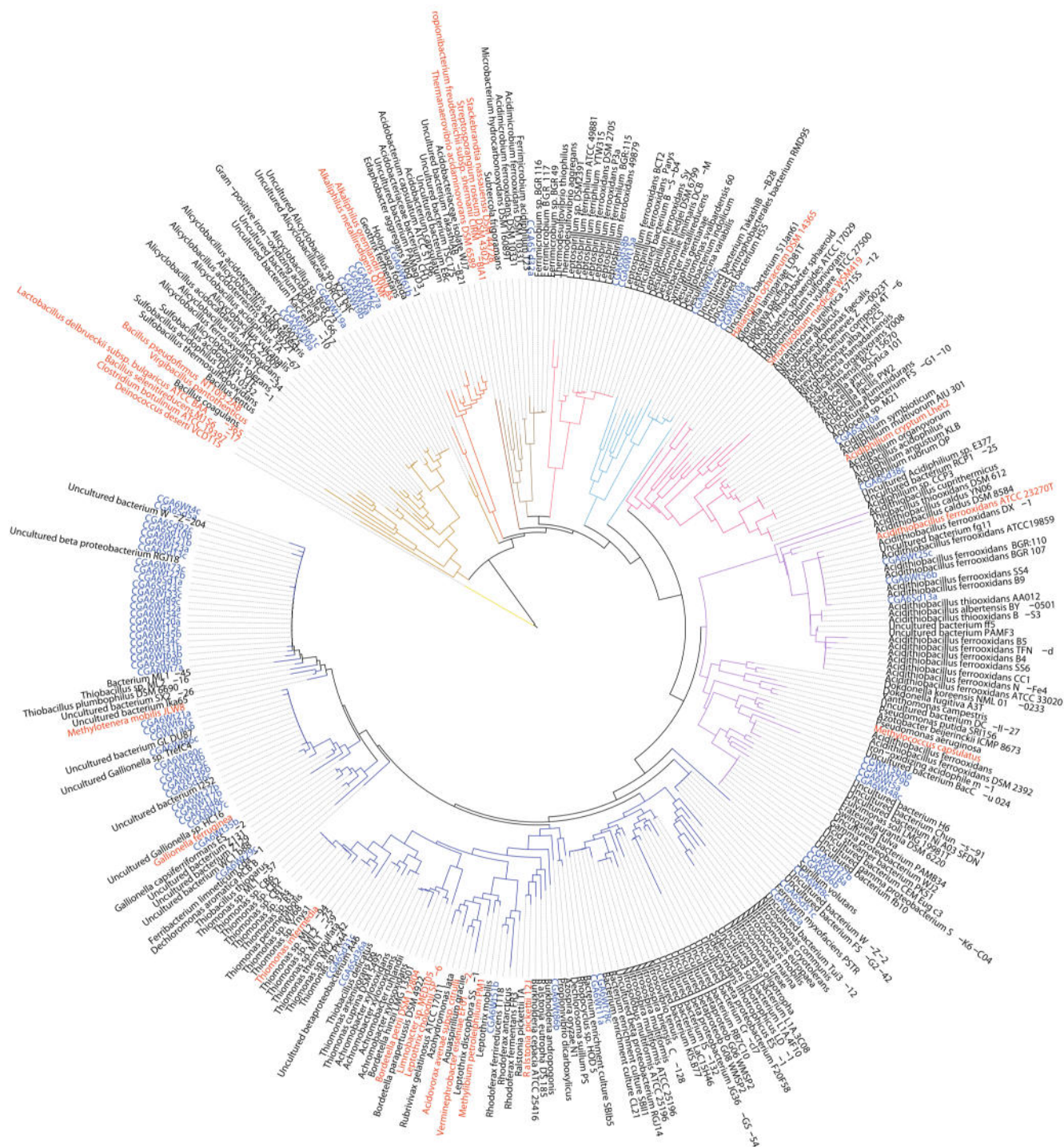
Among these prokaryotes, some bacteria may be present but not functionally active, and it was, therefore, crucial to differentiate between dead or inactive cells and functional cells. To determine which organisms play a significant role in the natural remediation processes such as the Fe(II) oxidation processes observed at the study site, a metaproteomic approach was used to list the bacterial population expressing proteins, i.e., those which were active. The metaproteomic approach was possible with sediments but failed with the water samples because larger numbers of bacterial cells were recovered from sediments than from water (data not shown).

Figure 2 Phylogenetic tree based on 16S rRNA-encoding sequences. Sequences were aligned using ClustalW. Alignments were checked by hand and positions with more than 1% of gaps were automatically removed. Neighbor-joining trees were drawn up with 310 nt using ITOL (<http://itol.embl.de/>) [44]. Accession numbers: see [supplementary data](#). In *red*: bacteria identified based on the metaproteomic (GroEL identifications) approach; in *blue*: bacteria identified using the 16S rRNA encoding gene library; in *black*: sequences detected in NCBI databases which are closely related to the bacteria present in the Reigous sediment

Characterization of the Main Proteins Expressed by the Sediment Community

Proteins expressed by this community were liable to correspond to orthologs originating from diverse prokaryotes and to have similar amino acid sequences. For those reasons and to improve their characterization, proteins were separated by performing 2-D gel electrophoresis (supplementary Fig. 1). A total number of 89 proteins were identified, 44% of which (39 proteins, Table 3) originated from bacteria and 39% (35 proteins) from protists, while 15 proteins originated from higher plants, probably from decomposed plant debris present at the Reigous creek. One third of the proteins originated from protists, mainly consisting of *E. mutabilis* detected in our samples (data not shown) and could not be completely removed using the Nycodenz Gradient. *E. mutabilis* is a common inhabitant of AMD [2, 11, 15, 39] and was also present at the surface of sediments. These *Euglena* proteins are involved in various metabolic processes, suggesting that this eukaryote may play a relevant role in this ecosystem, in agreement with recent results (Gouhlen-Chollet and Bertin, unpublished data). Only proteins originating from bacteria were further analyzed in this study. The 2-D gel electrophoresis approach used here allowed identifying from a single sample only the most abundant cytoplasmic proteins. Therefore, some relevant proteins that might be expressed in this environment, such as rusticyanin from *At. ferrooxidans*, which is known to be involved in Fe(II) oxidation and was previously thought to possibly play a functional role at this site [21, 24, 48], were not detected in this study. This membrane protein may not be resolved in the 2-D gel, or it may not be abundant at the sampling point. In addition, the majority of the bacteria forming the Carnoulès community have never been grown and studied in vitro so far. Their genome sequences, and hence their protein sequences, which are required for MS identification purposes, may not be available in public databases, except for those of *At. ferrooxidans*, *Thiomonas*, and *Gallionella* strains. All these hypotheses may explain why so few bacterial proteins were identified in this study.

The bacterial proteins identified originated from *Aquificae* were *Actinobacteria*, *Deinococcus*, *Synergistetes*, *Firmicutes*, *Bacteroidetes*, and α -, β -, and γ -*proteobacteria*. Among the



- | | |
|---|---|
| — γ -proteobacteria | — Actinobacteria |
| — β -proteobacteria | — Synergistetes |
| — α -proteobacteria | — Firmicutes |
| — δ -proteobacteria | — Acidobacteria |
| — Nitrospirae | — Deinococcus/Thermus |

Table 3 Bacterial proteins identified in the Reigous sediment's microbial community

Phylum	Class, Family, Genus	Organism	Level of discrimination	Protein name	Protein accession numbers	Spot number ^b	Peptide sequence
<i>Aquificae</i>	<i>Aquificae</i> (class); <i>Aquificales</i> ; <i>Aquificaceae</i> ; <i>Hydrogenobaculum</i>	<i>Hydrogenobaculum</i> sp. Y04AAS1	Species	Putative uncharacterized protein	A7WFJ8	25	IGAAVIGR
<i>Deinococcus-Thermus</i>	<i>Deinococci</i> ; <i>Deinococcales</i> ; <i>Deinococcaceae</i> ; <i>Deinococcus</i>	<i>Deinococcus deserti</i>	Species	60 kDa chaperonins ^a	C1CZP1	4, 6	APFGGDR QLVFDEAAR AVLVAIEEK
<i>Synergistetes</i>	<i>Deinococci</i> ; <i>Deinococcales</i> ; <i>Deinococcaceae</i> ; <i>Deinococcus</i>	<i>Deinococcus</i> sp. A62	Species	60 kDa chaperonins ^a	A1YUK7	2	IAQVASISANDK
<i>Synergistetes</i>	<i>Synergistia</i> ; <i>Synergistales</i> ; <i>Synergistaceae</i> ; <i>Thermanaerovibrio</i>	<i>Thermanaerovibrio acidaminovorans</i>	Genus or below	60 kDa chaperonins ^a	D1B621	4	FGSPTITNDGVTTIAK
<i>Firmicutes</i>	<i>Bacilli</i> ; <i>Lactobacillales</i> ; <i>Lactobacillaceae</i>	<i>Lactobacillus delbrueckii</i> subsp. <i>Indicus</i> or <i>delbrueckii</i>	Subspecies	60 kDa chaperonins ^a	Q70BV2, Q70BV5	4, 5	APFGGDR YGAPTITNDGVTTIAK
	<i>Bacilli</i> ; <i>Bacillales</i> ; <i>Bacillaceae</i>	<i>Virgibacillus pantothenicus</i>	Family or below	60 kDa chaperonins ^a	A9LHR1	4, 5, 6	AVEVAVK
	<i>Bacilli</i> ; <i>Bacillales</i> ; <i>Bacillaceae</i> ; <i>Bacillus</i>	<i>Bacillus halodurans</i> , <i>pseudofirmus</i> or <i>selenitireducens</i>	Species	60 kDa chaperonins ^a	O50305, A8VUQ6, D3FSF9	2	NVTSGANPMVIR
	<i>Clostridia</i> ; <i>Clostridiales</i> ; <i>Clostridiaceae</i> ; <i>Alkaliphilus</i>	<i>Alkaliphilus metalliredigens</i> or <i>orlandii</i>	Genus or below	60 kDa chaperonins ^a	A6TLJ1, A8HJ57	6	LSGGVAVIQVGAATETELK
	<i>Clostridia</i> ; <i>Clostridiales</i> ; <i>Clostridiaceae</i> ; <i>Clostridium</i>	<i>Clostridium botulinum</i>	Species	60 kDa chaperonins ^a	A5I723, A7FYP3, A7GIN3, B1IFD4, B1L1K0, C1FLV5, C3KUC8, B1Q9U6, B1Q157 B6FW06	4	APFGGDR LGIDIIR
	<i>Clostridia</i> ; <i>Clostridiales</i> ; <i>Clostridiaceae</i> ; <i>Clostridium</i>	<i>Clostridium hiranonis</i>	Species	60 kDa chaperonins ^a		4, 5, 6	APFGGDR KALEEPLR VGAATEVEMK
	<i>Clostridia</i> ; <i>Clostridiales</i> ; <i>Clostridiaceae</i> ; <i>Clostridium</i>	<i>Clostridium</i> <i>papyrosolvens</i>	Species or sub-species	60 kDa chaperonins ^a	C7IKN8	4, 6	TNDIAGDGTITATVLAQAIR APFGGDR
<i>Bacteroidetes</i>	<i>Flavobacteria</i> ; <i>Flavobacteriales</i> ; <i>Flavobacteriaceae</i> ; <i>Gramella</i>	<i>Gramella forsetii</i> (strain KT0803)	Species	ATP synthase; beta subunit Phosphoglycerate kinase	A0M791 A0M6J2 A5JUG8	11 13, 20 4, 5, 6	FGSPTITNDGVTTIAK MPSAVGYQPTLATEMGAMQER LGDIYVNDAFGTAHR NVTAGANPIELK
<i>Actinobacteria</i>	<i>Actinobacteria</i> (class); <i>Actinobacteridae</i> ; <i>Actinomycetales</i> ; <i>Propionibacterineae</i> ; <i>Propionibacteriaceae</i>	<i>Propionibacterium freudenreichii</i>	Species	60 kDa chaperonins ^a			
	<i>Actinobacteria</i> (class); <i>Actinobacteridae</i> ; <i>Actinomycetales</i> ; <i>Glycomycineae</i> ; <i>Glycomycetaceae</i> ; <i>Stackebrandtia</i>	<i>Stackebrandtia nassauensis</i>	Suborder or below	60 kDa chaperonins ^a	C4DUC7	6	GMNALADAVK
	<i>Actinobacteria</i> (class); <i>Actinobacteridae</i> ; <i>Actinomycetales</i> ; <i>Sreptosporangineae</i> ; <i>Sreptosporangiaceae</i> ;	<i>Sreptosporangium roseum</i>	Family	60 kDa chaperonins ^a	D2BBD1	4	APFGGDR GTFTSVAVK
	<i>Actinobacteria</i> (class); <i>Actinobacteridae</i> ; <i>Actinomycetales</i> ; <i>Sreptosporangium</i>	<i>Arthrobacter, Janibacter, Clavibacter</i> or <i>Kineococcus</i>	Order	ATP synthase; beta subunit	A0JY64, A1R7V3, A3TGD9, A5CQ60, A6W7G9	10, 31, 32, 33, 38	DVQNQDVLLFDINIFR VALSALTMAEYFR IGLFGGAGVGK
<i>Proteobacteria</i>	<i>α-proteobacteria</i> , <i>Rhizobiales</i> ;	<i>Sinorhizobium medicae</i>	Genus or	60 kDa chaperonins ^a	A6UH06	2, 4	LVAAGMNPMDLK

<i>Rhizobiaceae; Sinorhizobium/Ensifergroup; Sinorhizobium</i>	<i>Acidiphilium cryptum</i>	below	60 kDa chaperonins ^a	ASG1G2	5, 6	AAVEEGIVAGGVALLR
<i>α-proteobacteria, Rhodospirillales; Acetobacteraceae; Acidiphilium</i>		Genus or below				APGFGDR
						AAVEEGIVPGGVALAR
						AVAAAGMNPMDLK
						AGIIDPTK
						ENTTIVEGAGK
<i>α-proteobacteria, Rhodobacterales; unclassified Rhodobacterales</i>	Rhodobacterales bacterium HTCC2083	Family or below	60 kDa chaperonins ^a	B6AWC8	4, 5	APGFGDR
						EIELADPFENMGAQLVK
<i>β-proteobacteria, Burkholderiales; Comamonadaceae; Acidovorax</i>	<i>Acidovorax avenae</i>	Species	60 kDa chaperonins ^a	A1TKQ5, DISTJ1	4	SVAAGMNPMDLK
						APGFGDR
						VGAATEVEMK
						AVTALVAELKK
						VTLADLGQAK
<i>β-proteobacteria, Burkholderiales; Alcaligenaceae; Bordetella</i>	<i>Bordetella petrii</i>	Species	60 kDa chaperonins ^a	A9f685	2, 4, 5	AAVEEGIVAGGVALLR
						APGFGDR
						AVEEPLR
						VGAATEVEMK
						EGVITVEDGK
						VQIEEATSDYDREK
						DLLPVLEQVAK
						VEDALHATR
						VQIEEATSDYDR
<i>β-proteobacteria</i>	<i>Bordetella avium</i> (strain 197 N)	Species	50S ribosomal protein L7/L12	Q2L2M6	NC14	AEILDALAGMTVLELSELIK
						DLVDGAPKPVK
<i>β-proteobacteria; Burkholderiales; Oxalobacteraceae; Herminiimonas</i>	<i>Herminiimonas arsenicoxydans</i>	Species	Glutathione-dependent formaldehyde dehydrogenase (alcohol dehydrogenase class III), HEAR2039	A4G6P6	NC12	IIAIDTNPVK
						TNLCVAVR
						LEDAPAAYK
						VIDYVGDCR
						GMTMGHEMTGEVIEVGSDVEVVK
						FPELITPQVK
						SFGAPTGTK
<i>β-proteobacteria, Burkholderiales; unclassified Burkholderiales; Burkholderiales Genera incertae sedis</i>	<i>Leptothrix cholodnii</i> or <i>Thiomonas intermedia</i>	Genus	60 kDa chaperonins ^a	B1XXY9, C7HZY6	2, 3, 4, 5, 36, 60	YVAAAGMNPMDLKR
						APGFGDR
						LQNMGAQMVK
						EGVITVEDGK
						VGAATEVEMK
						VIAEEVGLTLEK
						YVAAAGMNPMDLK
						VTLADLGQAK
						IQIEEATSDYDREK
						VEDALHATR
						IQIEEATSDYDR

Table 3 (continued)

<i>β-proteobacteria</i> , <i>Gallionellales</i> ; <i>Gallionellaceae</i> ; <i>Gallionella</i>	<i>Gallionella ferruginea</i>	Genus or below	60 kDa chaperonins ^a	C5V7N1	2	TNDIAGDGGTTTATVLAQAIIR VGAATEVEMK GYLSPYFINNQDR DLLPVLEQVAK VEDALHATR APFGDR HALEGFK AVIAGMNPMDLK GVNVLADAVK VVSEEIGMK VEDALHATR AMLEDMAILTGGR LESTTLADLGQAK APFGDR VGAATEVEMK VEDALHATR
<i>γ-proteobacteria</i> , <i>Acidithiobacillales</i> ; <i>Acidithiobacillaceae</i> ; <i>Acidithiobacillus</i>	<i>Acidithiobacillus ferrooxidans</i>	Species	60 kDa chaperonins ^a	B5EN19, B7J561	2, 3	QIVANAGDEPSVVLNK LTATEDAAAR KADEALAAQK ADEALAAQK ITATEDAAAR SVAAGMNPMDLK AQIEDTSSDYDR APFGDR VGAATEVEMK GYLSPYFVTDSE LEGNNALGAK
<i>γ-proteobacteria</i> , <i>Methylococcales</i> ; <i>Methylococcaceae</i> ; <i>Methylococcus</i>	<i>Methylococcus capsulatus</i>	Order or below	60 kDa chaperonins ^a	Q60AY0	2, 3, 4	
<i>γ-proteobacteria</i> ; <i>Pseudomonadales</i> ; <i>Pseudomonadaceae</i>	<i>Pseudomonas</i>	Genus	Outer membrane lipoprotein OprI	A2VC34, A4XVE5, O85409-085430-O85432-O85437, O85439-O85444, Q3K906, Q48K14, Q883S8	4, 15, 17, 19, 22, 26-27, 40, 44, 49, 55, 60, 62, 64-65, NC7-NC10	
<i>γ-proteobacteria</i> , unclassified <i>Gammaproteobacteria</i> ; OMG group; SAR92 clade <i>δ-proteobacteria</i> , <i>Mycococcales</i> ; <i>Namtocystineae</i> ; <i>Haliangiaceae</i> ; <i>Haliangium</i>	<i>Gammaproteobacterium HTCC2207</i> <i>Haliangium ochraceum</i>	Group or clade Order or below	60 kDa chaperonins ^a	Q1YSA6 D0LRR3	5 4, 6	
<i>Proteobacteria</i>	Several <i>Proteobacteria</i>	Phylum	Succinyl-CoA synthetase; beta subunit	Q3KFU6 ^c	NC1, NC3, NC11	

^a MS/MS data were searched against an in-house database including all the bacterial and archaeal GroEL sequences obtained from Uniprot

^b Spots from 1 to 65 originated from a pH 4 to 7 gradient gel (Supplementary Fig. 1), spots from NC1 to NC14 originated from a pH 3 to 10 gradient gel (data not shown)

^c Several proteins may correspond to this identification: Q3KFU6, A1A8Y0, A1FGM7, A1JRB6, A1KTM6, A2UEB0, A3HHN5, A3M887, A4TNT8, A4W879, A4XV90, A5W114, A5WC33, A6BTC9, A6T6F6, A6V7K5, A7FKR4, A7MQX5, A7ZJA8, A8AJ84, A8GB83, P0A836, P0A837, P0A838, P0A839, P53593, P66869, P66870, Q02K73, Q0TJW6, Q0TJW6, Q1CAG1, Q1CFM0, Q1I7L3, Q1REJ8, Q2IIW6, Q2NUM2, Q2SD35, Q324I4, Q32IK3, Q3Z476, Q4KFY6, Q4ZUW7, Q57RL3, Q5F878, Q5PCM7, Q66DA0, Q6D7G2, Q6F8L4, Q7N6V5, Q7NZ47, Q883Z4, Q88FB2, Q8ZH00, Q9JUT0, Q9IZP4

39 bacterial proteins detected, there were two distinct ATP synthases, two distinct 50 S ribosomal proteins, one phosphoglycerate kinase, and one succinyl-CoA synthetase, which are involved in energy metabolism, translation, glycolysis, and TCA cycle, respectively (Table 3). These proteins may not play a specific role in this environment since they are known to be housekeeping proteins in bacteria. Among the other proteins, one outer protein OrpI, one uncharacterized protein with an unknown function, one glutathione-dependent, and one glutathione-independent formaldehyde dehydrogenases were identified. The latter two proteins belong to the formaldehyde detoxification pathway. Although no correlation with the environmental conditions might explain the functional specificities of these proteins, it has been reported that the arsenite-oxidizing bacteria *H. arsenicoxydans* synthesizes alcohol dehydrogenase and glutathione-dependent formaldehyde dehydrogenase when grown in the presence of arsenic [58].

It is worth noting that more than half of the identified bacterial proteins were similar to the 60-kDa GroEL chaperonin. These data suggest that multiple chaperonins of various genetic origins are expressed by the Reigous creek community. GroEL is known to be ubiquitously present in Bacteria and Archaea. These proteins are generally abundantly expressed in bacterial cells, especially under stress conditions such as those occurring in this particularly toxic environment [3]. The *groEL* gene is conserved in prokaryotes, and has been found to be present in one copy in the majority of sequenced genomes, except in the case of some pathogens [45, 60]. Because of its conservation properties (supplementary Fig. 2), this gene is often used as a phylogenetic marker [31]. In addition, it has been previously established that some of the amino acids stretch occurring in GroEL are specific to one genus or family of bacteria. These peptide sequences can therefore be used as the signature of a specific phylogenetic group. These GroEL identifications (Table 3, Fig. 3) were, therefore, considered for use as a possible taxonomic tool in addition to the 16S rRNA-based taxonomic approach. To determine which bacteria in the whole community were active, i.e., able to express proteins, the organisms identified using the 16S rRNA encoding gene library- and GroEL-based approaches (Fig. 2) were compared.

Most of the bacteria identified based on GroEL belonged to five phyla divisions. Bacteria belonging to *Deinococcus* and *Synergistetes* did not feature among those identified based on the 16S rRNA encoding gene. One possible explanation for this discrepancy may be that a PCR or cloning bias may have prevented those bacteria from being detected with this method. These findings suggest that metaproteomic methods used as taxonomic tools can provide a useful complementary tool in addition to the 16S rRNA encoding gene approach. Interestingly, *Thiomonas*, *At. ferrooxidans*, *Acidiphilium*, and *Gallionella*, expressed proteins and were, therefore, active.

16S rRNA-encoding gene analysis showed that these bacteria abound in this ecosystem. Bacteria affiliated to *At. ferrooxidans* and *Gallionella* are able to oxidize iron. In addition, many strains of the *Thiomonas* genus are able to oxidize As(III) into As(V) under laboratory conditions [6, 14, 22]. The fact that their proteins were detected shows that these bacteria were viable and metabolically active. This finding supports the hypothesis that the oxidation of Fe(II) to Fe(III) catalyzed by iron-oxidizing microorganism such as *At. ferrooxidans* and *Gallionella* and oxidation of As(III) into As(V) by As(III) oxidizers such as *Thiomonas*, probably leads to the precipitation of the more or less ordered iron oxy-hydroxides (Fe(III)–As(V) hydroxysulfate) detected in this study (Fig. 1) [48]. This finding is in agreement with previous data showing that *Gallionella ferruginea* efficiently remove Fe, As(III), and As(V) in water [41]. The present data show for the first time that this bacterium is active and probably plays a functional role in the sediments of the Reigous creek. Some heterotrophic bacteria such as *Acidiphilium* were also found to be active in this AMD, suggesting that they could cope with the low amount of organic carbon (dissolved organic carbon concentration 1.7 ± 0.4 mg/L [16]) of Reigous creek water. It has previously been suggested that these acidophilic heterotrophic bacteria may be involved in organic carbon turnover processes [32]. Interestingly, these four bacteria (*At. ferrooxidans*, *Thiomonas*, *Gallionella*, and *Acidiphilium*) found to be active members of this AMD community have been previously identified in AMD, but some of them were thought to have different optimum pH levels. Indeed, *G. ferruginea* is a neutrophilic bacterium which oxidizes Fe, but relatives of *Gallionella*, have often been detected in AMD [12, 33–35]. The strain occurring at Carnoulès showed less than 97% homology with *G. capsiferriformans* and its physiological characteristics are probably different. It seems probable that an acid-tolerant relative of this bacterium is able to oxidize iron under acid pH conditions.

In addition to the bacteria belonging to these four genera, other bacteria were also found to be active, but a discrepancy was again observed between the bacteria identified based on the results of 16S rRNA encoding gene analysis and the metaproteomic approach. The GroEL protein sequences of some bacteria identified using the 16S rRNA encoding gene library were not available in the Uniprot database, which might explain this discrepancy. However, phylogenetic comparisons between the 16S rRNA and metaproteomic data obtained (Fig. 2) suggested which of the bacteria present in this ecosystem may express a GroEL identified in the metaproteomic study. Based on these comparisons, it seems likely that in addition to *Thiomonas*, *At. ferrooxidans*, *Acidiphilium*, and *Gallionella*, clones related to β -, γ -, and δ -proteobacteria, such as *Limnobacter* or *Methylococcus* (*At. ferrooxidans* DSM

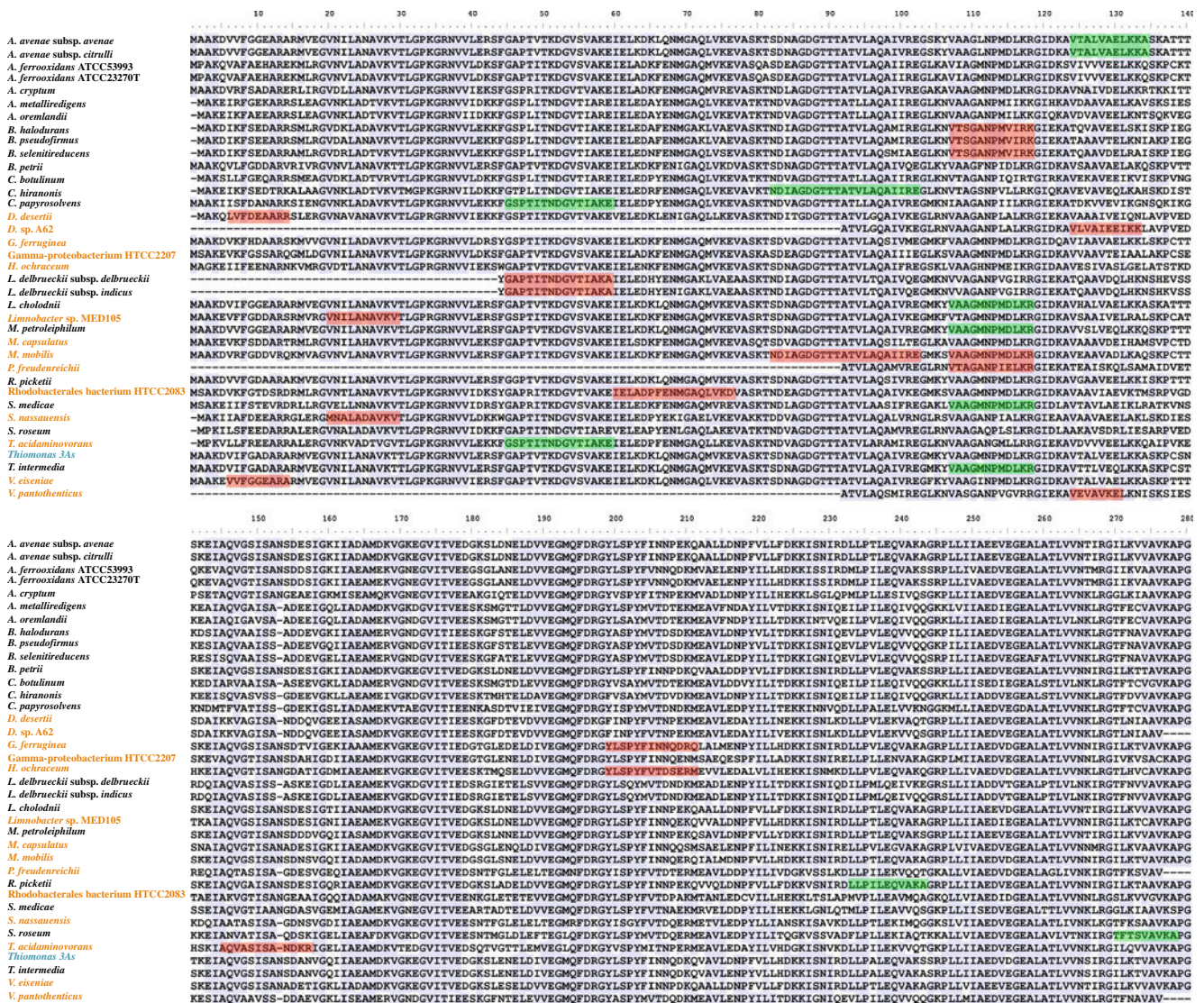


Figure 3 Alignment of the amino acid sequences of 60 kDa chaperonins. Thirty-six sequences matched the MS results: 29 Proteins were identified in Table 3: 22 of them unambiguously matched one protein (the bacterial name of which is given in *orange*), whereas fifteen identifications matched at least two proteins (22 bacterial names given in *black*). *Thiomonas* sp. 3As data were added for the sake of comparison (in *blue*). Thirty-seven sequences in all were aligned. These sequences were compared with ClustalW2 using the default parameters (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Alignment was performed using the Jalview software program [19]. The *blue* highlighted letters correspond to identical residues among the 37 orthologs (BLOSUM62 score option). *Highlighted letters* corresponding to the peptide sequences identified by MS allowed distinguishing several orthologs. First, 23 proteins were identified because at least one identified peptide matched only this GroEL: these peptides (labeled in *red*) corresponding to signature sequences specific to one species, genus, or family of bacteria are located in the same homology regions but differed from other peptides by at least one amino acid substitution. Secondly, 13 of the proteins identified were confirmed, since several identified peptides (labeled in *green*) matched this GroEL. Each of these individual peptides matched several proteins; however, only one protein detected in Uniprot contained all these amino-acid sequences, which suggests that a relative of this protein was probably present in this extract. Since the full amino-acid sequences of almost all the

chaperonins expressed by the bacteria present at the study site are unknown, the possibility cannot be ruled out that two identified peptides were erroneously assigned to two distinct chaperonins, whereas these peptides may in fact have originated from one protein, the amino acid sequence of which has not yet been included in the databases. Nevertheless, because of the high level of conservation observed in GroEL proteins (Supplementary Fig. 2), the proteins identified may be expressed by a close relative of the organism identified using the Uniprot database. GroEL originated from *Acidiphilium cryptum*; *Acidithiobacillus ferrooxidans* ATCC23270; *Acidithiobacillus ferrooxidans* ATCC53993; *Acidovorax avenae* subsp. *avenae*; *Acidovorax avenae* subsp. *citrulli*; *Alkaliphilus metalliredigens*; *Alkaliphilus oremlandii*; *Bacillus halodurans*; *Bacillus pseudofirmus*; *Bacillus selenitireducens*; *Bordetella petrii*; *Clostridium botulinum*; *Clostridium hirononis*; *Clostridium papyrosolvens*; *Deinococcus desertii*; *Deinococcus* sp. A62; *Gallionella ferruginea*; *Haliangium ochraceum*; *Lactobacillus delbrueckii* subsp. *delbrueckii*; *Lactobacillus delbrueckii* subsp. *indicus*; *Leptothrix cholodnii*; *Limnobacter* sp. MED105; *Methylobium petroleiphilum*; *Methylococcus capsulatus*; *Methylotenera mobilis*; *Propionibacterium freudenreichii*; *Ralstonia pickettii*; *Rhodobacteriales bacterium* HTCC2083; *Sinorhizobium medicae*; *Stackebrandtia nassauensis*; *Streptosporangium roseum*; *Thermanaerovibrio acidaminovorans*; *Thiomonas* 3As; *Thiomonas intermedia*; *Verminephrobacter eiseniae*; *Virgibacillus pantothenicus*; and *Gammaproteobacterium* HTCC2207

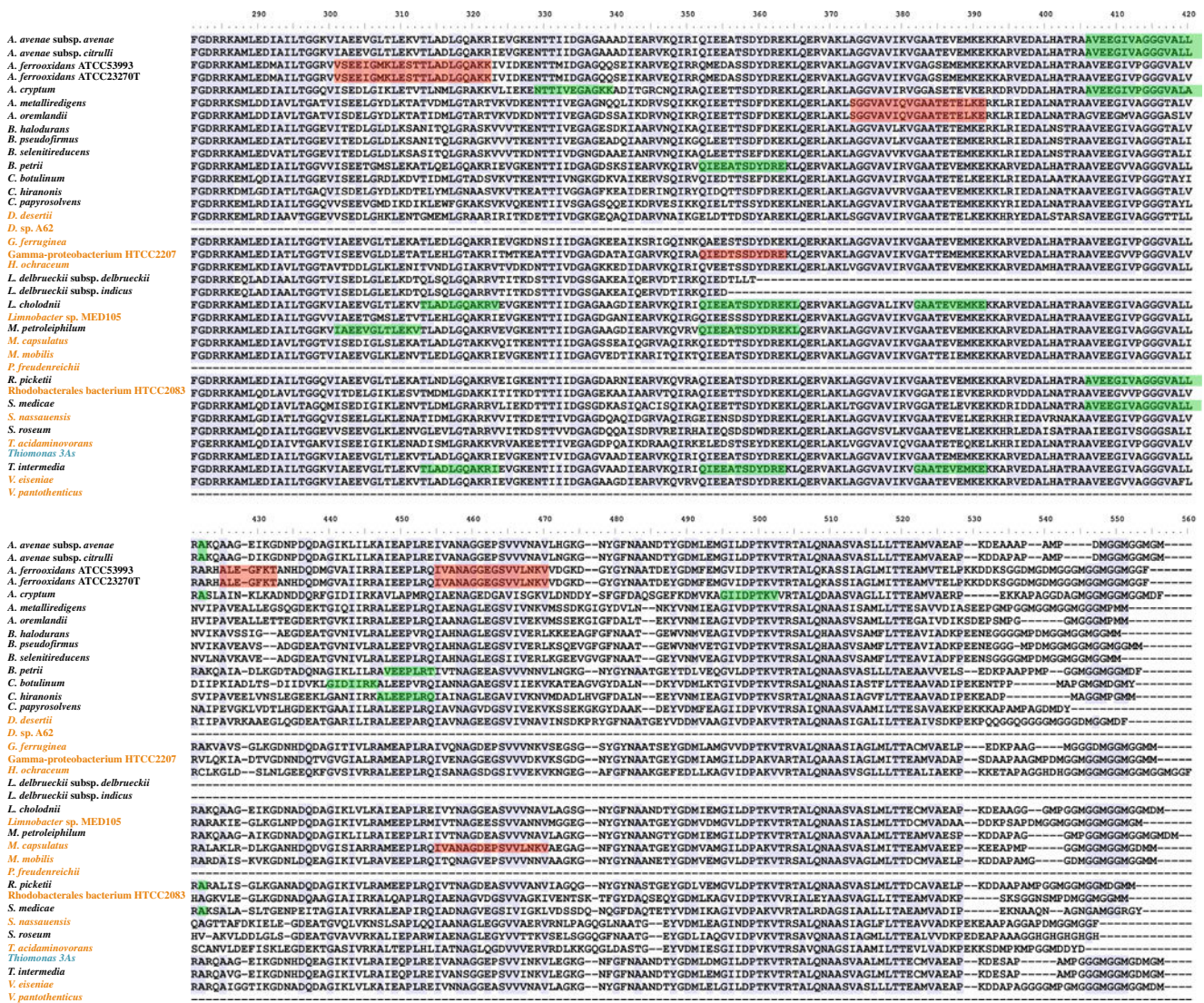


Figure 3 (continued)

2392 affiliated bacteria), *Methylobacter* (*Thiobacillus*-related clones), *D. korensis* (which is affiliated to the γ -proteobacteria HTCC2207), *Ferrimicrobium*-like bacteria (which are related to the *Actinobacteria* *S. nassauensis* and *S. roseum*), and *Haliangium* (clones affiliated to sulfate-reducing bacteria JHA1, *D. limimaris* and *D. svalbardensis*) may play a role in this ecosystem. All in all, the data obtained here show that *Firmicutes*, which could be affiliated to *Alicyclobacillus ferrooxidans*, are also active. These bacteria are able to oxidize ferrous iron [37] and may, therefore, participate in the transformation of the iron present in high concentrations in these waters. The active population as a whole was not only composed of several iron-oxidizers in addition to *At. ferrooxidans* but also contained iron reducers, one known arsenite-oxidizer, sulfate-reducing, and sulfur compound oxidizers, and both autotrophic and heterotrophic bacteria. All these bacteria

may contribute importantly to the remediation process observed in situ.

In Conclusion

The active bacterial species inhabiting Carnoulès AMD ecosystem were identified in this study using high-sensitivity nanoLC-chip-MS/MS methods combined with a 16S rRNA based phylogenetic approach. The meta-proteomic data obtained here show for the first time that *Gallionella*, *Thiomonas*, *At. ferrooxidans*, and *Acidiphilium* actively express proteins in situ. Previous hypotheses based on experiments performed under laboratory conditions may contribute to the arsenite oxidation and As immobilization occurring in the heavily contaminated AMD at the Carnoulès mine via iron oxidation processes.

Since these bacteria were found to be active and to express proteins which are among the most abundant proteins encountered at this site, it seems likely that the large amounts of pale-yellow As(V)–Fe(III) hydroxysulfate sediments forming at Carnoulès, which were characterized here, may result from the conjugate activities of iron-oxidizing microorganisms (such as *At. ferrooxidans*, *Alicyclobacillus ferrooxydans*, *Ferrimicrobium*, or *Gallionella*) and arsenic-oxidizing microorganisms (such as *Thiomonas* sp.). Several bacteria may be responsible in situ for changing the ratio between the oxidized and reduced forms of iron, arsenic, and sulfur compounds, promoting the formation of the Fe(III)–As(V) hydroxysulfate precipitates detected in this study. These bacteria are therefore of prime importance in the partial but efficient natural process of remediation undergone by the contaminated Carnoulès ecosystem. In addition, autotrophic iron, arsenic, and sulfur oxidizers may provide the organic carbon sources required by the functional heterotrophs such as *Acidiphilium* present in this ecosystem. All in all, the present findings provide evidence that the functional genomics approach provides a useful means of describing bacterial communities such as those inhabiting the Reigous creek and determining their contribution to natural attenuation processes. It is now proposed to use approaches of this kind in future studies to complete this picture of the functional processes at work in this ecosystem, as well as to investigate the role played by the less abundant active bacteria identified in this study.

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