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Microbial Diversity in a Pyrite-Rich Tailings Impoundment (Carnoulès, France)

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The microbial communities have been investigated in the sub-surface waters of the Carnoulès pyrite-rich tailings impoundment (France) for two hydrological situations characterized by the presence of oxygenated waters during winter and suboxic conditions in early autumn. In these acidic waters (2–5) characterized by elevated concentrations of Fe ($1608\text{--}3354\text{ mg}\cdot\text{l}^{-1}$), As ($130\text{--}434\text{ mg}\cdot\text{l}^{-1}$) and sulfates ($5796\text{--}14318\text{ mg}\cdot\text{l}^{-1}$) and variable dissolved oxygen content, the cultivable bacteria found in these system are *Thiomonas* and *Acidithiobacillus ferrooxidans*. Molecular methods, Terminal-Restriction Fragment Length Polymorphism (T-RFLP), and 16S rRNA encoding gene library analysis indicate low diversity. The environment is dominated by only a few types of microorganisms, with 70–80% of the whole bacterial population assigned to two or three Terminal-Restriction Fragments (T-RFs). Most of these organisms are uncultured, newly described, or recently associated with acid mine drainage. Modifications of the community structure are observed as a function of the sampling period and seem to be related to the aqueous chemistry of the tailings water. At low Dissolved Oxygen ($\text{DO} = 1\text{ mg}\cdot\text{l}^{-1}$) concentrations and moderately acidic conditions ($\text{pH} = 5.7$), the dominant organisms are related to

the uncultured clone BA31 affiliated with *Desulfosarcina variabilis*, a sulfate-reducing bacteria (SRB), *Acidithiobacillus ferrooxidans* and the uncultured clone BVB20, closely related to *Thiobacillus*. At high ($12\text{ mg}\cdot\text{l}^{-1}$) DO concentrations and low (<2) pH values, the microbial diversity is less important and 65% of the population is assigned to the uncultured bacterium clone AS6 related to *Desulfosarcina variabilis*.

Keywords microbial diversity, arsenic, mine tailings, acid mine drainage, terminal restriction fragment length polymorphism

INTRODUCTION

The mining of metallic sulfide ores produces pyrite-rich mine wastes which generate Acid Mine Drainage (AMD) when exposed to oxygenated water. The formation of AMD, which contains high concentrations of sulfates, iron, arsenic, and metals (Pb, Cd, Zn), depends on the availability of oxygen and Fe(III), both acting as oxidants (Langmuir 1997). The reaction is mediated by acidophilic iron-oxidizing microorganisms (Silverman and Ehrlich 1964) which play a major role in maintaining the Fe(III) availability, especially at low pH, where the abiotic oxidation rate of Fe(II) by dissolved oxygen (DO) is slow (Edwards et al. 1999). In spite of their importance in the generation of AMD, the populations of naturally occurring prokaryotic microorganisms in AMD are poorly known. Until a decade ago, relatively few acidophiles were recognized,

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and enrichment/cultivation based studies have probably overestimated the importance and the role of *Acidithiobacillus* ("A.") *ferrooxidans* and *A. thiooxidans* in AMD.

The use of molecular-based analysis has highlighted the significant variability of microbial diversity in AMD (Johnson and Hallberg 2003). To date, bacteria from several divisions (proteobacteria, nitrospira, firmicutes, actinobacteria, and acidobacteria) have been detected in AMD environments (Baker and Banfield 2003). Among these organisms, the most extensively studied group (but possibly the least relevant under AMD-generating conditions) are the proteobacteria, specifically *Acidithiobacillus* spp. (Kelly and Wood 2000) and *Thiobacillus* spp.

The objective of this study was to characterize the microbial communities structures in As-rich AMD and their variations as a function of dissolved oxygen concentration and pH. The study was carried out in the aquifer of the tailings stock of the Carnoulès mine (France). The water is acidic (pH = 2–5) and can contain occasionally up to $12000 \text{ mg} \cdot \text{l}^{-1}$ of As (Leblanc et al. 2002). The water chemistry and especially its oxygen content drastically varies as a function of hydrological conditions (Koffi et al. 2003). The variations of the community structure for contrasting dissolved oxygen concentrations were investigated using two complementary molecular methods (T-RFLP, and 16S rRNA encoding gene library analysis) and usual culturing techniques (isolation, counting, and identification of culturable bacteria).

MATERIALS AND METHODS

Site Description

The Pb-Zn Carnoulès mine is located in southern France (Figure 1). The ore extraction, which stopped in 1962 has left 1.5 Mt of wastes containing quartz (75 wt.%), and arsenic-rich pyrite (5–15 wt.%) (Casiot et al. 2003). The mean annual rainfall is 1320 mm of rain with a distribution typical of the Mediterranean climate characterized by long drought periods and heavy rains occurring mainly in autumn and spring. The aquifer is not fed by the vertical percolation of rainwater through the tailings, but rather originates from former natural springs that were buried under the tailings (Koffi et al. 2003). The water table occurs at 1 to 10 m below the surface of the tailings stock according to season and location. With the exception of the temperature, which is almost constant at about 15°C , the other physicochemical parameters of ground water change in relation to the hydrological conditions as shown by Koffi et al. (2003) and Casiot et al. (2003). The highest DO concentrations (up to $12 \text{ mg} \cdot \text{l}^{-1}$) are observed during the rise of the water table, the water is saturated with regards to oxygen. When the water level stabilizes, the DO concentrations drop suddenly to microaerobic conditions ($1 \text{ mg} \cdot \text{l}^{-1}$). The pH varies inversely to DO with strongly acidic values (≤ 1.8) at high DO concentrations. It increases to 5–6 at low DO values.

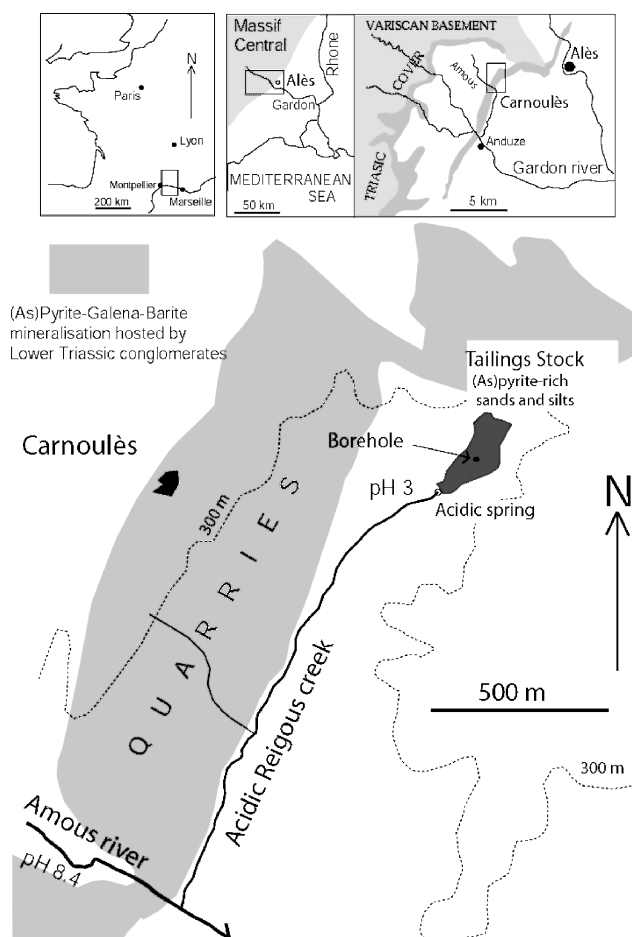


FIG. 1. Location and map of the Carnoulès mining site.

Sampling Procedure and Physicochemical Determinations In Situ

Water samples were collected from a borehole located in the center of the tailing where the water chemistry varies in a particularly large range according to hydrology (Koffi et al. 2003). Samples for usual cultural techniques were collected in the end of 2001 until the beginning of 2003. The corresponding pH are presented in Figure 7. Sampling for molecular microbial determinations was carried out in October 2002 and January 2003. The two periods correspond to contrasting water chemistry as shown in Table 1. At each time, 200 ml of water were filtered through a $0.22 \mu\text{m}$ sterile Nucleopore filter, introduced into a tube, frozen in liquid nitrogen, and stored at -20°C until further analysis. Samples for counting of viable bacteria and for bacterial isolation were collected in sterile vials and processed for microbiological studies within 5 h of sampling.

Culture Media and Bacterial Identification

Two kinds of solid media were used for counting of viable bacteria from the tailings. Heterotrophic and nonobligatory acidophilic bacteria in the samples were cultured on a solid

TABLE 1
Physicochemical characteristics of the water in the tailings during the sampling in October 2002 and January 2003

Sampling period	pH	T (°C)	Eh	Do (mg · l ⁻¹)	As(III) (mg · l ⁻¹)	As(V) (mg · l ⁻¹)	Fe T (mg · l ⁻¹)	Fe(II) (mg · l ⁻¹)	SO ₄ ²⁻ (mg · l ⁻¹)
October	5.7	13.5	20	1	104	24	1667	1608	5796
January	1.88	13.3	257	12	413	21	3468	3354	14318

medium R2A (pH 7.0) (Difco laboratory) and medium 100:10 (pH 3.5) (Schrader and Holmes 1988), developed for the growth of acidophilic lithotrophic bacteria (containing a mixture of both ferrous iron and thiosulfate as available energy sources). 0.1 ml of sample was spread on the media (three replicates) and incubated 21 days at 30°C. Each colony type was identified by 16S rDNA sequencing (Bruneel et al. 2003).

Bacterial Enumeration

The number of Colony Forming Units (CFU) of each type was counted on the three replicates. The total bacterial number in the tailings samples was estimated by epifluorescent microscopy after DAPI staining.

DNA Isolation

Genomic DNA was extracted from filtered water using the UltraClean Soil DNA Isolation Kit according to the recommendation of the manufacturer (MoBio Laboratories Inc., USA). All extracted genomic DNA samples were stored at -20°C until further processing.

T-RFLP Analysis

Primers 8F and 1489R (Lane 1991; Weisburg et al. 1991) were used for T-RFLP analysis to assess the bacterial community structures. Forward (8F) and reverse (1489R) primers were fluorescently labeled with TET and HEX (E.S.G.S. Cybergene group, France), respectively. The PCR amplification mixture contained 12.5 µl Hot Start Taq polymerase master mix (Qiagen, Netherlands), 0.5 µl of each primer (20 µM) and 10 ng of DNA template. A final volume of 50 µl was adjusted with distilled water. 16S rDNA gene amplification reactions were cycled in a PTC200 thermocycler (MJ Research, USA) with a hot start step at 94°C for 15 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1.5 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. The amount of PCR product was determined by comparison to known concentrations by the "dots method" (Smartlader, Eurogentec, Belgium) after migration on agarose gel. PCR products were purified with the GFX PCR DNA purification kit (Amersham-Pharmacia, U.K.).

Purified PCR products (600 to 700 ng) were digested with 12 units of enzyme HaeIII or HinfI (New England Biolabs, USA). The length of T-RFs from the digested PCR products was determined by capillary electrophoresis on ABI prism 310, (Applied Biosystems, USA). About 50 ng of the digested DNA from

each sample was mixed with 10 µl of deionized formamide and 0.25 µl of TAMRA size standard and then denatured at 94°C for 2 min and immediately chilled on ice prior to electrophoresis. After an injection step of 10 s, electrophoresis was carried out for up to 30 min applying a voltage of 15 KV. T-RFLP profiles were performed using GeneScan software (ABI).

Dominant Operational Taxonomic Units (OTUs) represent T-RFs whose fluorescence was higher than 100 Fluorescence Units for at least one sample. Predictive digestions were made on the RDP web site (<http://rdp.cme.msu.edu/html/index.html>) using the T-RFLP Analysis Program (TAP).

Cloning and Restriction Analysis

To further characterize the bacterial populations inhabiting the borehole in each sampling period, the bacterial diversity was analyzed by cloning PCR amplified 16S rRNA encoding genes, obtaining a clone library for each sampling period. Sixty clones from each library were analyzed according to their RFLP patterns (HaeIII and HinfI digestion). The clones were divided into four dominant groups for October and three groups for January.

Bacterial 16S rRNA encoding gene (16S rDNA) was amplified with unlabelled 8F and 1489R primers. These PCR products were cloned in *E. coli* TOP 10 using the pCR2.1 Topo TA cloning kit (Invitrogen, Inc.). Cloned 16S rDNA fragments were amplified using the primers TOP1 and TOP2 located on the vector, and then digested with the enzymes HaeIII or HinfI. Restriction profiles were analyzed on 2.5% agarose gel electrophoresis (small fragments resolution agarose; QA agarose, QBiogene, Inc.).

16S rDNA Sequencing

Partial sequences of the 16S rDNA gene (from 8 to 336 according to *E. coli* numbering) were determined by the dideoxy nucleotide chain-termination method using the BigDye cycle sequencing kit (Applied Biosystems) on an ABI PRISM 310 Genetic analyzer (Applied Biosystems). DNA sequence analyses were performed via the infobiogen server (<http://www.infobiogen.fr>) by using the FASTA, BLAST, ALIGNN, and CLUSTALW programs (Altschul et al. 1990; Baneyx 1999; Felsenstein 1993; Thompson et al. 1994). Phylogenetic trees were constructed by using the PHYLIP computer package (Felsenstein 1993). The confidence level of the phylogenetic tree topology was evaluated by performing 100 bootstrap replications with the programs SEQBOOK and CONSENSE.

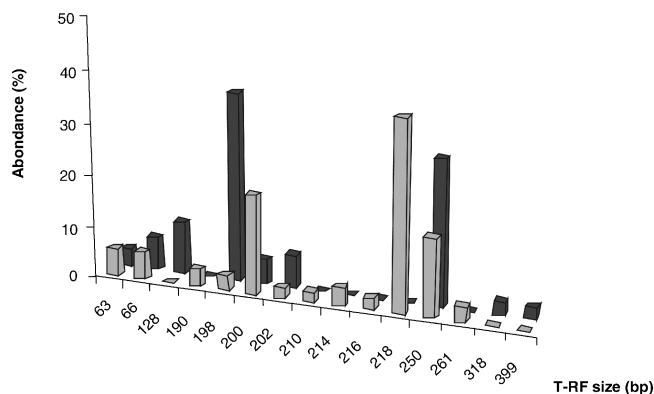


FIG. 2. Comparison of bacterial community T-RFLP fingerprints in tailings water at the Carnoulès Mine. October (□) and January (■) samples.

RESULTS

Communities Structures

The results of T-RFLP analysis are presented in Figure 2. The number and distribution of major OTUs, indicates a generally low bacterial diversity (12 OTUs in October and 9 in January). The bacterial populations characterized by OTUs 63, 66, 198, 200, 202, and 250 bp are found in both sampling periods but the importance of each OTU vary greatly between the 2 sampling periods. Differences occur for other bacterial population, OTUs 190, 210, 214, 216, 218, and 261 bp are found only in October whereas the OTUs 128, 318, and 399 bp are present only in January.

Composition of Bacterial Communities

The results are presented in Table 2. Most of the clone sequences are related to sequences already found in AMD,

TABLE 2

Bacterial clones from each sampling period with closest match organism or clone name, percent similarity, phylogenetic group, closest relative and postulated metabolism and percent number of each group compared to the total number of clones.

Clone	Closest match organism or clone name and accession number	% similarity	Phylogenetic group	Closest relative and postulated metabolism	% number of clone
S5Oct30	Uncultured bacterium BA31	93	δ -proteobacteria	<i>Desulfosarcina variabilis</i> —Sulfate/iron	27
S5Oct38	(AF543508)	94		reduction	
S5Oct42		87			
S5Oct70		94			
S5Oct51		95			
S5Oct80		94			
S5Oct73		94			
S5Oct11		95			
S5Oct27	<i>Acidithiobacillus ferrooxidans</i> SS6(AJ278721)	99	γ -proteobacteria	<i>Acidithiobacillus ferrooxidans</i> —Sulfide/iron oxidation and iron reduction	14
S5Oct75	Uncultured bacterium RA13C4 (AF407404)	99			
S5Oct74	Uncultured Banisveld landfill	98	β -proteobacteria	<i>Thiobacillus</i> —Sulfide/iron oxidation	12
S5Oct85	bacterium BVB20 (AY013650)	99			
S5Oct17	Unknown <i>Actinomyces</i> MC87 (X68455)	87	Actinobacteria	<i>Acidimicrobium</i> —Sulfide/iron oxidation	2
S5Jan41	Uncultured bacterium AS6	98	δ -proteobacteria	<i>Desulfosarcina variabilis</i> —Sulfate/iron	65
S5Jan43	(AF543496)	98		reduction	
S5Jan59		98			
S5Jan36		95			
S5Jan20	Uncultured <i>Acidithiobacillus</i> <i>ferrooxidans</i> KF/GS-JG36-22 (AJ295655)	99	γ -proteobacteria	<i>Acidithiobacillus ferrooxidans</i> —Sulfide/iron oxidation and iron reduction	8
S5Jan27	<i>Acidithiobacillus ferrooxidans</i> strain SS6 (AJ278721)	99			
S5Jan28	Uncultured <i>Sulfobacillus</i> sp.	93	Firmicutes	<i>Sulfobacillus</i> —Sulfide/iron oxidation and iron	8
S5Jan35	P3-5(AF460985)	94		reduction	

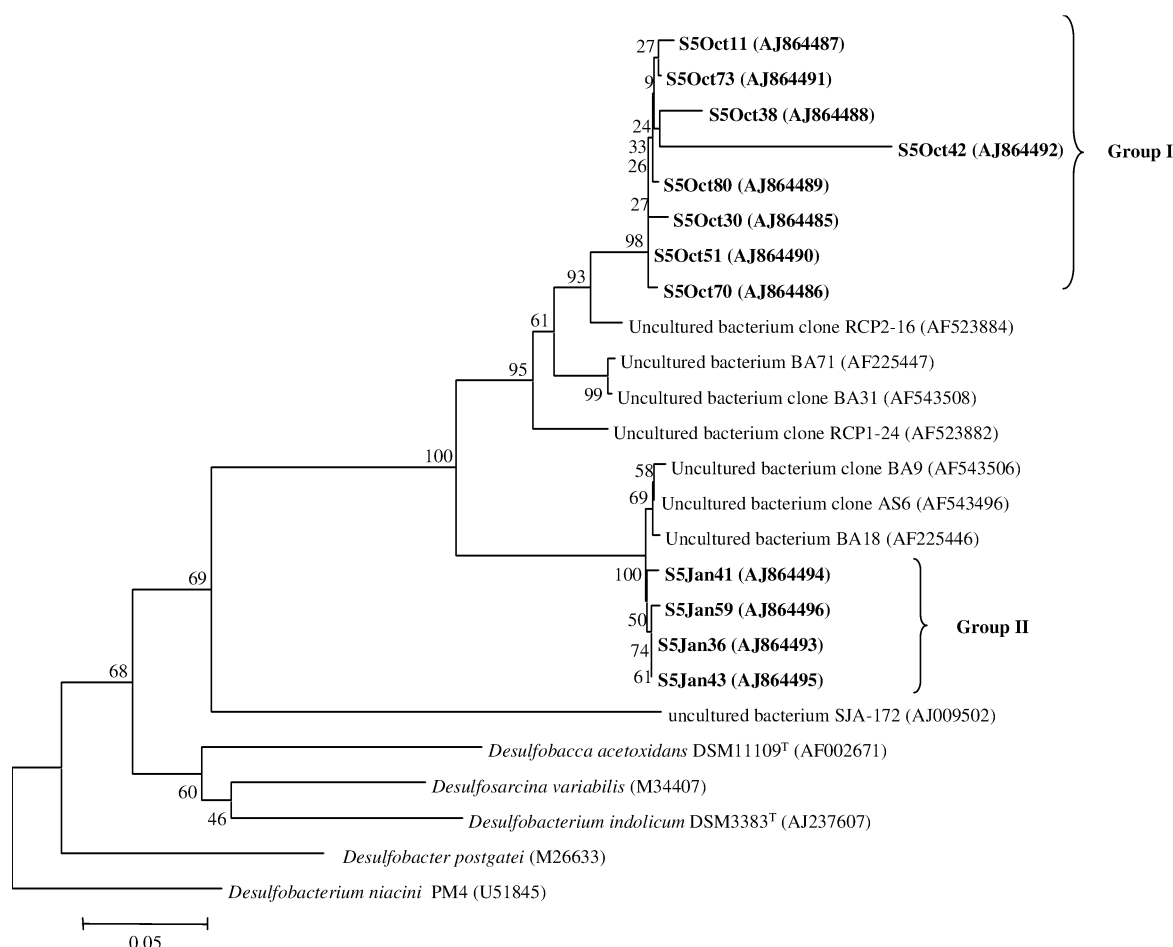


FIG. 3. Phylogenetic analysis of 16S rRNA encoding gene sequences affiliated with the *Desulfobacter* division in tailings water at the Carnoulès mine (France). Clone names in bold correspond to sequences found in October (Oct) and January (Jan).

indicating that the clone libraries have not been contaminated. In accordance with the T-RFLP results, this analysis indicates major differences in the bacterial populations between the sampling periods even if it's the same functional group. About 90% of the sequences are closely related to sequences of uncultured bacterial clones, and only 10% to 16S rDNA sequences of identified species. The most abundant sequence type, representing 27% of the clones in October and 65% in January, are positioned within the delta subdivision of the *Proteobacteria* (Table 2, Figure 3). Clones from October form a distinctive group (Group I), showing about 94% similarity with the sequence of the clone BA31 found in AMD at Iron Mountain (unpublished data, AF543508) and about 85% similarity with the sequences from clones found in January (Group II). The Group II sequences are closely related (99%) to the sequence of the clone AS6, also found in AMD at Iron Mountain (unpublished data, AF543496). The phylogenetic analysis, does not relate the clone sequences to any representative of the subdivision. The closest relative is *Desulfosarcina variabilis*, a sulfate-reducing bacteria.

The sequences representing the second most abundant type (22% of the clones) are firmly positioned in the *Acidithiobacillus ferrooxidans* group (Figure 4). These sequences represent 14% of the clones in October and 8% in January (Table 2). The sequences are distributed within three genomovars. Sequences S5Jan27 and S5Oct27 are found in the genomovar II, having more than 99% similarity with the sequence of *A. ferrooxidans* strain SS6, isolated in an uranium mining waste pile (Selenska-Pobell et al. 2001). Sequence S5Oct75 falls in the genomovar IV, showing 99% similarity with the sequence of the clone RA13C4, found in monochlorobenzene contaminated groundwater (Alfreider et al. 2002). Sequence S5Jan20 belongs to the genomovar I having 99% similarity with *A. ferrooxidans* KF/GS-JG36-22 isolated in uranium mining waste piles (Selenska-Pobell et al. 2001).

The next most abundant sequence type, representing 12% of the clones in October, is positioned within the beta subdivision of the *Proteobacteria* (Table 2, Figure 5), having >98% similarity to the sequence of the clone BVB20, obtained from a landfill leachate-polluted aquifer (Röling et al. 2001). These

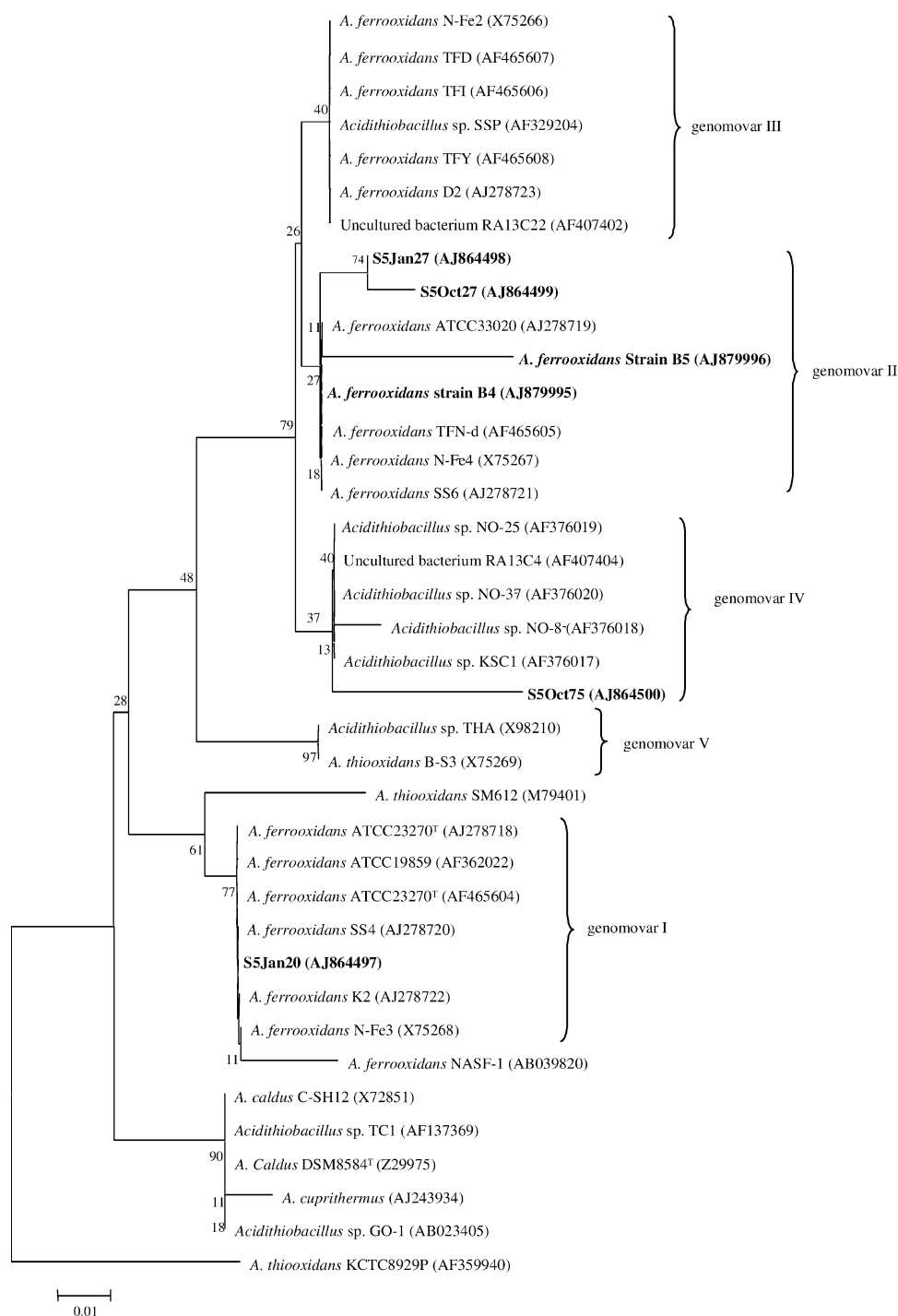


FIG. 4. Phylogenetic analysis of 16S rRNA encoding gene sequences affiliated with the *Acidithiobacillus* division in tailings water at the Carnoulès mine (France). Clone names in bold correspond to sequences found in October (Oct) and January (Jan).

sequences are distantly related (>92% similarity) to the sequence of *Thiobacillus* sp.; therefore, the phylogenetic position within the group cannot be further resolved.

The other sequence type detected only in January, representing 8% of the clones, is affiliated with the *Sulfobacillus*

group, with >93% similarity with the *Sulfobacillus* sp. P3-5 strain, isolated from a pyrite-oxidizing bacterial population (Figure 6). Finally, the last sequence type clustering within the *Actinobacteria* group (Table 2) is the least abundant clone. The sequence is distantly related to sequences of environmental

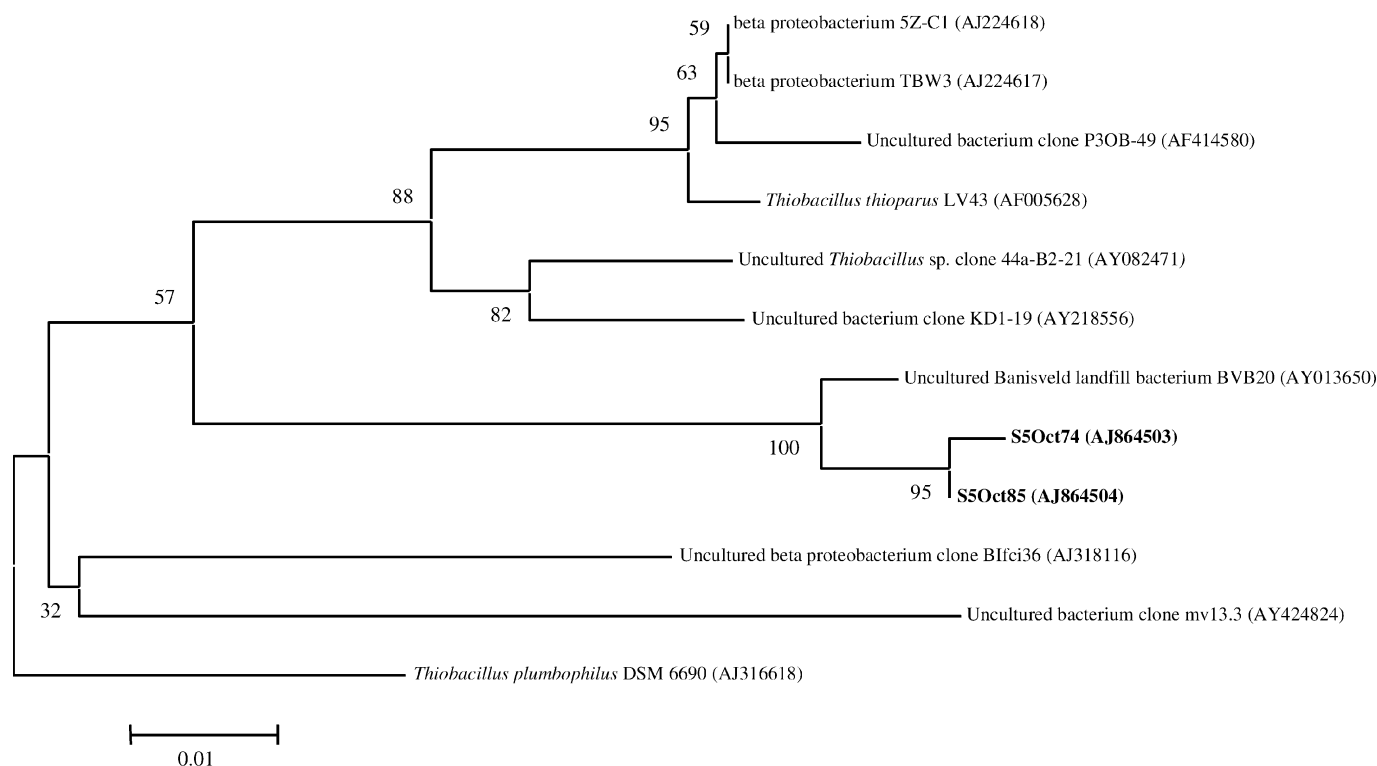


FIG. 5. Phylogenetic analysis of 16S rRNA encoding gene sequences affiliated with the *Thiobacillus* division in tailings water at the Carnoulès mine (France). Clone names in bold correspond to sequences found in October (Oct) and January (Jan).

clones (92%), especially to that of MC87 obtained from Iron Mountain (Stackebrandt et al. 1993) and related to *Acidimicrobium* (formerly *Actinomycete*, (Stackebrandt et al. 1997)).

Bacterial Isolation, Enumeration, and Identification

The total number of bacteria, ranged from 6 to 7 log FU (Fluorescence Units per milliliters) (Figure 7). The solid R2A and 100:10 media result in the growth of only two groups of bacteria belonging respectively to the genus *Thiomonas* and to the species *Acidithiobacillus ferrooxidans* (Bruneel et al. 2003; Morin et al. 2003). For the *A. ferrooxidans* group, two strains were isolated, B4 and B5 but these strains are not detected in the 16S rRNA encoding gene libraries (Figure 4). The number of *Thiomonas* and *Acidithiobacillus ferrooxidans* ranged respectively from 1 to 4 logs CFU · ml⁻¹ and from 0 to 3 logs CFU · ml⁻¹, representing only 0.1% of the total bacteria (Figure 7). The evolution of these two groups exhibits strong variation. The group of *Thiomonas* is always present, whereas *A. ferrooxidans* is absent in April and May during the period of high water level. The number of *Thiomonas* increases with the pH (Figure 7), reflecting a neutrophilic capability of growth. It always exceeds the number of *A. ferrooxidans*, excepted in December and January during the low pH period.

DISCUSSION

The results show the predominance of only three populations representing about 70% of the populations whatever the sam-

pling date. This low bacterial diversity revealed by molecular-based methods is probably due to the small number of metabolically beneficial reactions available in AMD generated by pyrite oxidation as previously reported (Baker and Banfield 2003). In this kind of water, the number of available electron donors and acceptors is restricted, shaping the microbial ecology.

Most of the clone sequences (up to 90%) although related to sequences already found in AMD, can not be closely related to cultured organisms, suggesting that they may constitute new taxa. Only clones from *Acidithiobacillus ferrooxidans* can be closely related to cultured organisms. However, although bacterial strains have not been isolated, it is possible to predict their physiological role, as it has been demonstrated that phylogenetically-related microorganisms share metabolic and physiological characteristics (Bond et al. 2000).

The dominant bacterial populations are related to the delta subdivision of the *Proteobacteria*, with closest affiliation to the sulfate-reducing bacteria *Desulfosarcina variabilis*. This population is found during both seasons. The anaerobic respiration is the dominant physiological trait of this group. In this respect, the occurrence of this bacterial population, observed in January, where the water is saturated with regards to oxygen may be surprising. However, this is in agreement with several recent studies indicating the presence of SRB and iron-reducing bacteria in the sediment of mining lakes and in mine tailings, even under oxic and acidic conditions where they participate to Fe and S cycling (Friese et al. 1998; Fortin et al. 2000). SRB have also

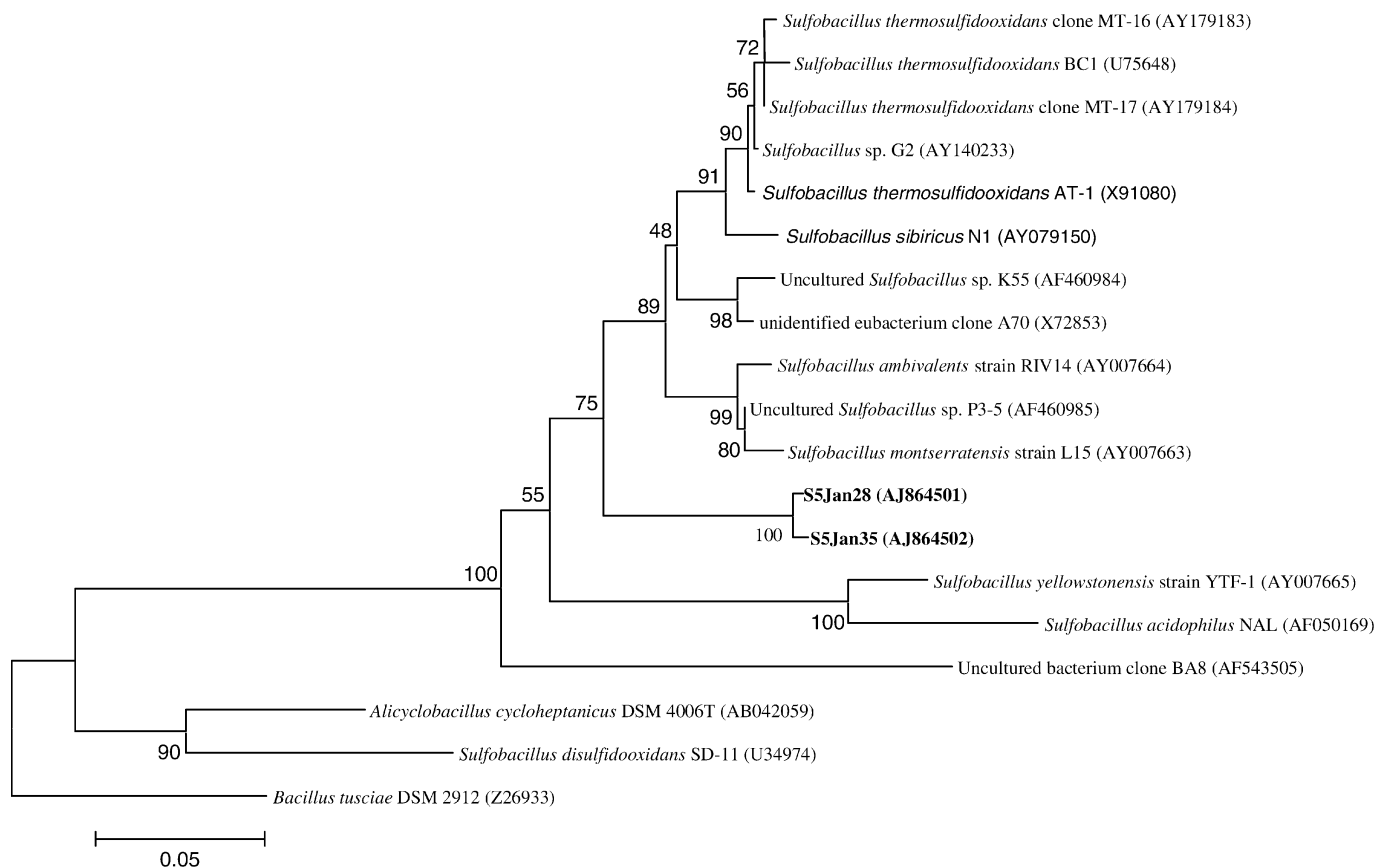


FIG. 6. Phylogenetic analysis of 16S rRNA encoding gene sequences affiliated with the *Sulfobacillus* division in tailings water at the Carnoulès mine (France). Clone names in bold correspond to sequences found in October (Oct) and January (Jan).

been reported in marine oxic sediment layers (Wieringa et al. 2000). In the Carnoulès tailing, the phylogenetic analysis reveals that clone sequences of *D. variabilis* are distributed in two clusters (Figure 3), group I (October) and group II (January), suggesting possible differences in their physiology according to the availability of oxygen. Since it is surprising that sulfate and iron reduction occurs at such O_2 concentrations it would be necessary to elucidate precisely the ecophysiological function of these bacteria. For this purpose it is needed to isolate them by using specific media and culture conditions.

Clones that could be related to oxidizing bacteria (sulfide and/or metals) are found in both seasons. *A. ferrooxidans* was also found in the two sampling period. In October, the oxidizing bacteria are positioned in the β subdivision of the *Proteobacteria* closely related to the *Thiobacillus* group, whereas in January they are related to the gram positive *Sulfobacillus* species belonging to the *Firmicutes* group. Isolates of *Sulfobacillus* spp. are chemolithotrophs that can use reduced sulfur or iron compounds as energy source; they show mixotrophic and autotrophic growth (Golovacheva and Karavaiko 1978). These bacteria oxidize ferrous iron or reduce ferric iron, according to their metabolic activity related to the availability of DO (Bridge and Johnson 1998).

According to these authors, these iron-reducing bacteria accelerate the reductive dissolution of ferric iron-containing minerals such as jarosite and goethite. Clones falling within *Actinobacteria* group can also be related to iron-oxidizing, heterotrophic, acidophilic bacteria capable of autotrophic growth, such as *Acidimicrobium*. Species of heterotrophic and

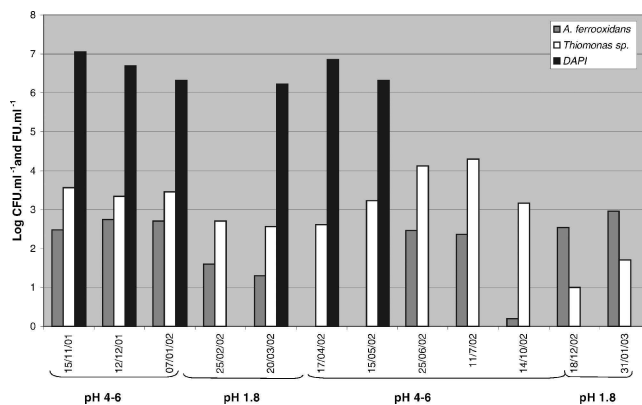


FIG. 7. Changes in *Thiomonas* sp. and *A. ferrooxidans* populations (CFU on solid media) and total number of bacteria (FU after DAPI staining) in Carnoulès tailings stock over a one-year period.

autotrophic oxidizing bacteria have been found to coexist in AMD, where they are suspected to play a synergistic role in removing organic carbon and reduced metal forms (Bond et al. 2000). *Thiomonas* which has been isolated and shown to be very active on the oxidation of As (Bruneel et al. 2003) could not be detected using molecular techniques reflecting probably their low abundance.

In conclusion, the seasonal variations of chemical parameters in the Carnoulès AMD system influence the bacterial community structure. In January, when DO is high and pH low, the system is running with populations related to *D. variabilis* group II, *A. ferrooxidans* genomovar I and II, and populations related to *Sulfobacillus*. In October, when DO is low and pH high, the system involves *D. variabilis* group I, populations related to *A. ferrooxidans* genomovar II, *Thiobacillus* and *Acidimicrobium*. However, is it noteworthy that *D. variabilis* and *A. ferrooxidans* are present whatever the physicochemical conditions. Thus, it appears that microorganisms are adapted to suit specific physical and chemical conditions conferring to the bacterial communities present in Carnoulès AMD the ability to maintain the system over the seasonal variations.

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