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Mediation of arsenic oxidation by *Thiomonas* sp. in acid-mine drainage (Carnoulès, France)

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ABSTRACT

Aims: To isolate, identify, and characterize heterotrophic bacteria in acid-mine drainage that mediate oxidation of As(III).

Methods and Results: Samples of acid-mine drainage were collected over a period of 14 months. Heterotrophic and non-obligatory acidophilic bacteria in the samples were cultured on a solid medium (pH 7.0–7.2), and three strains were isolated. The three different strains belong to the genus *Thiomonas*, and have more than 99% homology with the group Ynys1. Culturing in mineral media demonstrated that the isolated strains used thiosulphate as an energy source, and oxidized iron in the presence of thiosulphate. However, none of the strains were able to oxidize arsenic in the presence of thiosulphate, nor could they use iron or arsenic alone as an energy source. *In vitro* experiments demonstrated that two of the *Thiomonas* strains were able to oxidize more than 90% of the As(III) present in the acid-mine drainage, whereas no abiotic oxidation of arsenic occurred.

Conclusions: Two strains of newly identified *Thiomonas* sp. found in acid-mine drainage are capable of oxidizing arsenic.

Significance and Impact of Study: These results represent the first reported oxidation of arsenic by *Thiomonas* sp. Biologically mediated oxidation and subsequent immobilization of arsenic is of great interest for the remediation of contaminated mine sites.

Keywords: acid-mine drainage, arsenic oxidation, bioremediation, eco-type, *Thiomonas*.

INTRODUCTION

Arsenic is relatively abundant in the earth's crust with an average concentration of about 3 mg kg⁻¹, and is mainly associated with sulphur minerals (Amran *et al.* 1995). Anthropogenic dispersion of arsenic dates back to the industrial revolution, resulting mostly from mining and metallurgy (North *et al.* 1997). The milling and processing of sulphide-rich ores often produces large quantities of

wastes that may contain significant amounts of arsenic-rich pyrite. When exposed to meteoric water, this material generates acidic water (acid-mine drainage; AMD) containing high concentrations of sulphate, iron, and arsenic. Arsenic is a recognized carcinogen present worldwide in surface and ground water (Nordstrom 2002), and the World Health Organization has recently recommended lowering drinking water standards from 40–50 µg l⁻¹ to 10–20 µg l⁻¹ because of human health effects related to arsenic (Smith *et al.* 1999, 2002).

Changes in the oxidation state of arsenic have implications for its mobility and toxicity in aquatic systems. In surface waters, arsenic occurs principally as As(III) (arsenite) and

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As(V) (arsenate) (Cullen and Reimer 1989). The mobility of arsenic in fluvial systems is limited because of its ability to bind to solid phases such as Fe- and Al-oxides (Oscarson *et al.* 1981). The affinity of arsenic for these surfaces depends on its oxidation state: at low pH, As(V) adsorbs much more strongly than As(III) to Fe(III) hydroxides (Bowell 1994). Furthermore, As(III) is more toxic than As(V) (Lett *et al.* 2001), by a factor of as much as 60 under some conditions (Squibb and Fowler 1983). Thus, the oxidation and subsequent precipitation of arsenic is an effective way to mitigate its toxicity and mobility in AMD.

Arsenic oxidation is slow in the absence of microbiological mediation (Eary and Schramke 1990), but several autotrophic and heterotrophic bacteria catalyzing arsenic oxidation have been identified, principally in neutral waters (Ilyaletdinov and Abdrashitova 1979; Santini *et al.* 2000) and in hydrothermal waters (Gihring *et al.* 2001; Langner *et al.* 2001). The presence of As-oxidizing bacteria in AMD has been recognized (Wakao *et al.* 1988; Leblanc *et al.* 2002), and one species has recently been identified (Battaglia-Brunet *et al.* 2002). To date, no *Thiomonas* bacteria have been implicated in As oxidation.

Because As-oxidizing bacteria can play an important role in the oxidation and immobilization of arsenic in AMD, and thus represent a potentially useful tool for bioremediation, the aim of this study was to isolate, identify, and characterize some of the bacteria that mediate As(III) oxidation in As- and Fe-rich AMD. Most studies of acidophilic bacteria in AMD have focused on two iron-oxidizing bacteria: *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (Schrenk *et al.* 1998). However, while *A. ferrooxidans* is able to metabolize sulphur (Kelly and Wood 2000), it is not capable of oxidizing arsenic (Wakao *et al.* 1988) and the arsenite-oxidizing ability of *Leptospirillum* has yet to be demonstrated. For this reason, we focus here on the heterotrophic and non-obligatory acidophilic bacteria in AMD that may play a role in As oxidation and immobilization. The study was carried out at the Carnoulès mine (France) (Fig. 1), where oxidation of arsenic and precipitation of ferric arsenate occurs within a few metres downstream of the spring draining the mine tailings (Leblanc *et al.* 1996).

Site description

The Carnoulès mine, located near the city of Alès in southern France (Fig. 1), has been inactive since 1962. As a result of the mining activity, about 1.5 Mt of tailings containing 0.7% Pb, 10% FeS₂, and 0.2% As remain at the site (Leblanc *et al.* 1996). The tailings are contained behind a dam. Water percolating through the tailings emerges as an acidic spring (pH 2.2–4.0) at the base of the dam. This spring is the headwaters of Reigous Creek, a tributary of the Rhône River. Reigous Spring contains up to 350 mg l⁻¹ of

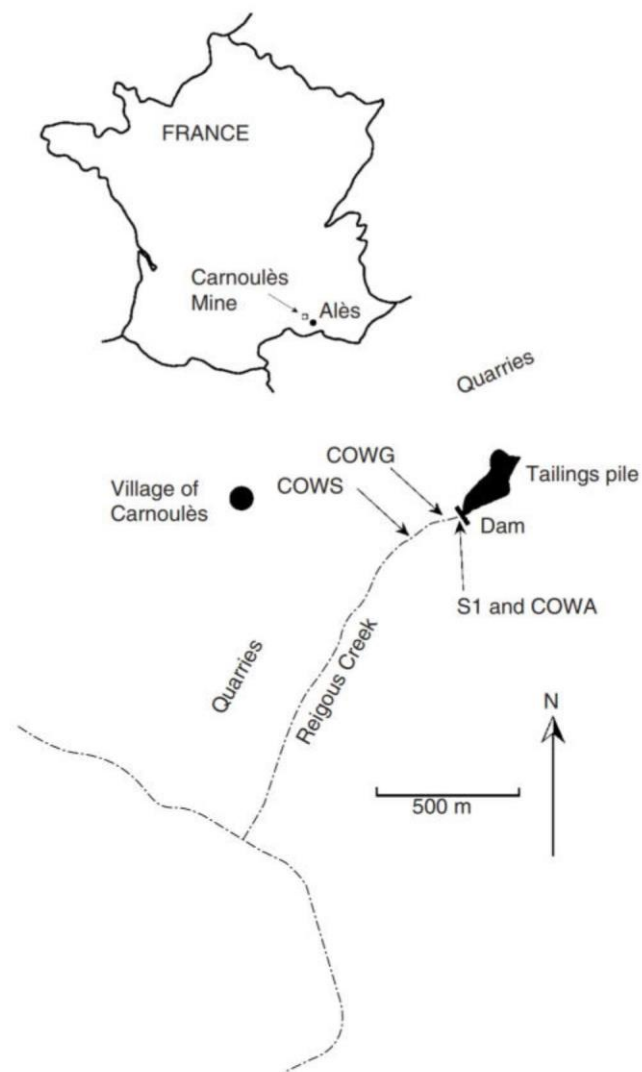


Fig. 1 Location of the Carnoulès acid-mine drainage study site

As, 750–2700 mg l⁻¹ of Fe, and 2000–7500 mg l⁻¹ of SO₄²⁻ (Leblanc *et al.*, 1996).

Reigous Spring and Reigous Creek, in addition to having very high concentrations of arsenic, are characterized by the presence of As-rich sediments (up to 22% As). The dissolved arsenic in the spring water and in Reigous Creek downstream occurs mostly as As(III) (60–100%). Fe(II) is the dominant form of iron in the creek, and its oxidation results in the precipitation of Fe(III) along the first 40 m of the creek (about 1 h residence time). Twenty to 60% of the dissolved arsenic in the spring water is removed as it moves downstream and co-precipitates with iron. The precipitate, which forms around stromatolitic bacterial structures (Leblanc *et al.* 1996), is mainly composed of As(V), Fe(III), and minor amounts of SO₄²⁻. Thus, the oxidation of soluble As(III) to the less soluble As(V) and its subsequent

precipitation with Fe(III) results in a rapid decrease in the concentration of dissolved arsenic in Reigous Creek.

MATERIALS AND METHODS

Sampling

Samples were collected every 3–4 weeks from June 2001 to August 2002 at four locations along the first 150 m of Reigous Creek (Fig. 1). The sample sites were Reigous Spring (S1); 3 m downstream of the spring (COWA); 30 m downstream (COWG); and 150 m downstream (COWS). Samples for enumeration of viable bacteria and bacterial isolation were collected in sterile vials and processed for microbiological studies within 5 h of sampling.

Culturing media and bacterial isolation

Demineralized water (MILLIQ™, Millipore) was used for the preparation of all media, and pH was adjusted with H₂SO₄. The media were sterilized by autoclaving at 120°C for 15 min except where otherwise indicated.

Because this study focused on identification of heterotrophic and non-obligatory acidophilic bacteria, the isolation of the different types of heterotrophic bacteria present in Reigous Creek was carried out on medium R2A (Difco Laboratory). This medium is recommended for the culture of environmental organoheterotrophic bacteria, and its neutral pH (7.0–7.2) allows for the selection of bacteria not necessarily acidophilic (Reasoner and Geldreich 1985).

Because R2A was relatively inefficient for the subculturing of isolated bacteria, we synthesized a new liquid organic medium for this purpose. This medium was also used to conserve bacteria at room temperature and to prepare inocula of pure strains. It was made by dissolving 2 g of (NH₄)₂SO₄, 0.1 g of KCl, 0.5 g of K₂HPO₄, 1 g of Na₂S₂O₃, and 0.5 g of MgSO₄·7H₂O in 480 ml of H₂O. The pH was then adjusted to 3.0 and the solution was autoclaved. One hundred milligrams of As were added, using a solution of As(III) prepared from NaAsO₂ and filtered through 0.2-µm pore size Nucleopore™ membranes. After addition of 10 ml of a glucose solution (10%) and of yeast extract (1%), separately autoclaved, the volume was adjusted to 1000 ml with sterile water.

To test the lithotrophic growth ability of the isolated strains, five mineral media were synthesized. These media used the same base, to which thiosulfate, Fe(II), As(III), Fe(II) and thiosulphate together, or As(III) and thiosulphate together were added. The media base was prepared by dissolving 2 g of (NH₄)₂SO₄, 0.1 g of KCl, 0.5 g of K₂HPO₄, and 0.5 g of MgSO₄·7H₂O in 500 ml of MILLIQ water. The pH was adjusted to 3 and the solution sterilized.

The volume was completed to 1000 ml using a solution of either 1 g l⁻¹ of sodium thiosulphate, 2.2 g l⁻¹ of FeSO₄·7H₂O (plus 1 g l⁻¹ of sodium thiosulphate for the Fe-thiosulphate combination), or 173.2 mg l⁻¹ of NaAsO₂ (plus 1 g l⁻¹ of sodium thiosulphate for the As-thiosulphate combination). The solutions were sterilized by filtration through a 0.2-µm pore size membrane.

Bacterial enumeration and characterization

After culturing on the R2A medium, the number of colony forming units (CFUs) was counted on three replicates. In addition, individual colony types were characterized by their morphology on solid R2A medium and the isolated strains were observed after Gram staining and by epifluorescent microscopy after DAPI staining. The isolates were purified by repeated single colony isolation. The number of total bacteria in the creek samples was counted after DAPI staining.

DNA isolation and amplification of the *rrs* gene encoding 16S rRNA

DNA was extracted and purified with the IsoQuick Nucleic Acid Extraction kit (ORCA Research, Bothell, WA). Extracted DNA was amplified using universal 16S rRNA primers A, corresponding to positions 8–28 of *Escherichia coli* 16S rRNA (Edwards *et al.* 1989), and rJ, corresponding to the complement of positions 1510–1492 (Janvier and Grimont 1995). The amplification reaction was carried out in a final volume of 100 µl, with 1 µl DNA template, 20 pmol l⁻¹ of each primer, 200 µmol l⁻¹ of dATP, dTTP, dGTP, and dCTP, 50 mmol l⁻¹ MgCl₂, 2.5 units of *Taq* polymerase, and reaction buffer ·10 (Amersham International, Amersham, UK). Initial denaturation was carried out for 4 min at 94°C. Thirty-five cycles of amplification were performed in a DNA Thermal Cycler 9700 (PE Applied Biosystems, Foster City, CA). Each cycle consisted of three steps: denaturation at 94°C for 1 min, annealing at 49°C for 1 min, and extension at 72°C for 2 min. The final extension was performed at 72°C for 5 min.

Amplification products were detected by electrophoresis on 0.8% w/v agarose gels in Tris-borate EDTA buffer (0.089 mol l⁻¹ Tris-borate, 0.018 mol l⁻¹ EDTA), with a 1 kb DNA ladder (Gibco BRL, Gaithersburg, MD) as a molecular size marker. The amplified product was sequenced by Genome Express (Montreuil, France) with three primers, E, rE, and D, in conserved regions of *E. coli* 16S rRNA. Primer E corresponded to positions 787–806, rE corresponded to the complement of the E primer, and D corresponded to positions 519–536 (numbering according to Brosius *et al.* 1978).

Phylogenetic analysis

Modules of the Lasergene software (DNASTar, London, UK) were used in all computations on a Macintosh G4 (Apple Computer, Cupertino, CA). Modules EditSeq and SeqMan were used to assemble about 1400 bp of the *rrs* gene. GenBank was used to search for sequences related to that of the three isolates using GLAST (NCBI). The closest sequences were imported from the Ribosomal Database Project II database (<http://rdp.cme.msu.edu/html/>) and aligned with that of the three isolates using module MegAlign. A neighbour-joining tree (Saitou and Nei 1987) was drawn.

Bioassays

The action of the three isolated strains of bacteria on arsenic and iron in the AMD was investigated in the laboratory using samples of Reigous Spring water. Immediately after sampling, aliquots of 100 ml of water from Reigous Spring were filtered through 0.2 μm pore-sized membranes and placed in pre-sterilized (160°C) flasks. Each flask was inoculated with 0.1 ml of a suspension containing one of the three bacterial strains adjusted to 10^7 cells per ml. A sample in which the bacterial suspension was replaced by sterile MILLIQ water served as a control (sample T). The cultures were incubated for eight days at 30°C while agitating. After incubation, they were filtered through a 0.45 μm pore size membrane prior to analysis of As and Fe. Environmental samples (no added bacteria or incubation) for analysis of total As and Fe were acidified immediately after collection to a pH of 1 with HNO_3 (14.5 mol l^{-1}) and stored at 4°C in polyethylene bottles. Environmental samples for determination of Fe and As species were analysed within 24 h of collection.

Chemical analysis

Total arsenic concentrations were determined by ICP-MS (VG-PlasmaQuad PQ2+). The elevated As concentrations necessitated a dilution factor of 1000, and no interference due to AsCl was detected. The concentration of dissolved As(III) was determined by hydride generation coupled with ICP-MS (Elbaz-Poulichet *et al.* 2000). The detection limit was 75 ng l^{-1} and the precision better than 5%. The concentration of As(V) was computed as the difference between total arsenic and As(III). For Fe(II) determination, samples were buffered to pH 4.5 with an ammonium acetate/acetic acid buffer in the field, and Fe(II) was complexed by adding 1 ml of a 0.5% (w/v) 1,10-phenanthroline chloride solution to 10 ml of sample (Rodier *et al.* 1996). Analyses were carried out by colorimetry at 510 nm with a precision better than 5%.

RESULTS

The number of autochthonous bacteria that could be cultured on the R2A medium varied little over the 11 months during which the four creek sites were repeatedly sampled (Fig. 2). Over this period, the total number of bacteria ranged from 5 to 6 log fluorescence unit per millilitre (FU ml^{-1}) after DAPI staining (data not shown). The number of bacteria culturable on the solid R2A medium ranged from 3 to 3.5 log CFU ml^{-1} , and thus represents

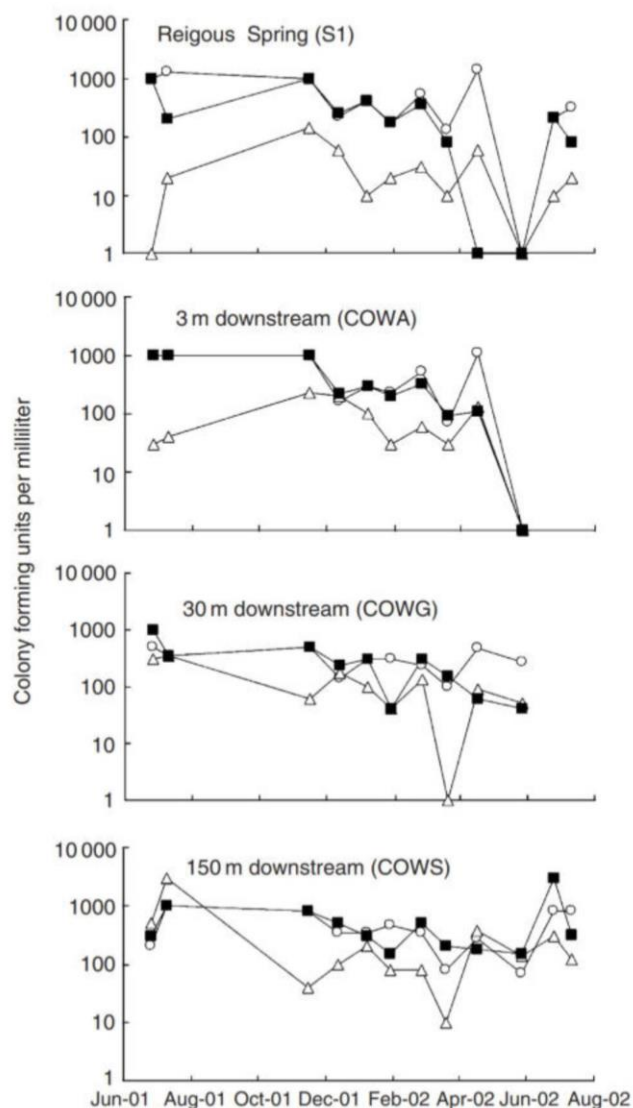


Fig. 2 The number of CFU of bacterial strains B1 (○), B2 (△), and B3 (■) cultured on medium R2A from samples collected from the four sampling sites over an 11-month period. Sampling sites COWA, COWG, and COWS are along Reigous Creek 3, 15, and 150 m downstream of Reigous Spring (S1), respectively

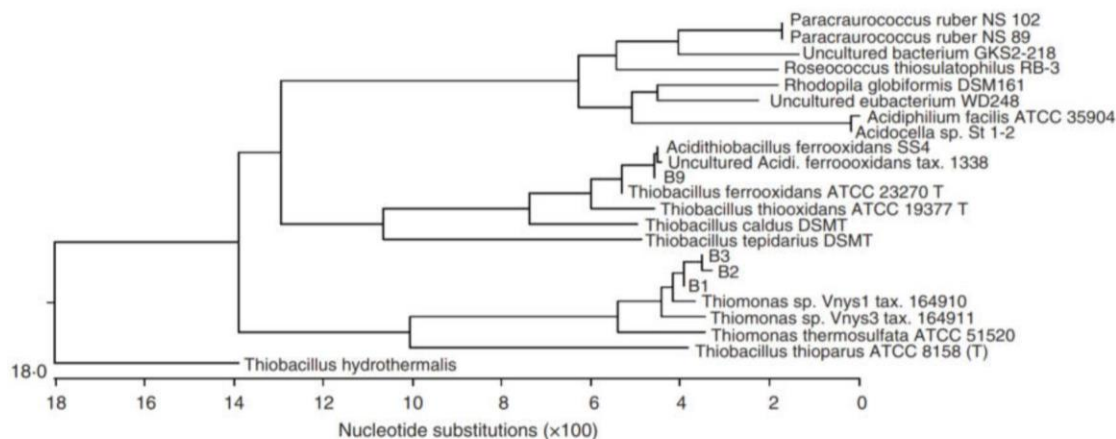


Fig. 3 Phylogenetic tree showing the relationship between the three *Thiomonas* strains B1, B2, and B3 isolated from Reigous Spring and Reigous Creek and other closely related bacteria

only about 0.5% of the total bacteria in Reigous Creek. An exception to the relatively constant number of culturable bacteria occurred in late May 2001. This sample was collected shortly after a period of intense rainfall: in 1 week, the area around the Carnoulès mine received as much rainfall as normally falls during a 6-month period. It is probable that the large quantity of infiltration temporarily affected the ground-water geochemistry and the autochthonous bacterial population.

Of the bacteria that grew on the R2A medium, three strains represented 95–100% of the CFU in the spring water, and more than 80% of the CFU in the creek water. Based on their DNA sequences, the three strains (B1, B2, and B3) belong to the genus *Thiomonas* (Moreira and Amils 1997), taxon 164910, and have more than 99% homology with group Ynys1 described by Dennison *et al.* (2001). The phylogenetic tree of the three strains is shown in Fig. 3. The 16S rDNA sequences were submitted to the EMBL/GenBank/DBJ databases. The accession numbers are AJ549218 (B1); AJ549219 (B2); AJ549220 (B3).

The colonies of B1, as observed on the R2A medium, were pale straw yellow, opaque, circular, rounded, and smooth, with a diameter of about 1 mm after 7 days of incubation. Colonies of B2 were almost colourless and translucent, circular, flat, and about 1 mm in diameter after 10 days. Colonies of B3 appeared only after 15 days of incubation; they were white, opaque, and very small (less than 0.25 mm), circular, and rounded after 20 days. The other colonies present generally grew rapidly, were large and coloured, and were different in each sample.

The three *Thiomonas* sp. Ynys1 strains were Gram-negative rods. B1 and B3 were non-motile, and B2 was motile. All three strains tended to aggregate and to adhere to solid phases, and formed clusters in the organic

medium. When cultured in water from Reigous Spring, B1 and B3 often became elongated up to 20 μm long, while B2 retained its original length. After DAPI coloration, B1 and B3 were observed as chains of fluorescent spots, suggesting that the long bacterial forms were in fact bacterial chains that had not divided (Figs 4a and c), whereas B2 had a more typical bacterial morphology (Fig. 4b). The DNA tended to concentrate in one part of the cell in an unusual way. A similar phenomenon was observed repeatedly in other types of bacteria in untreated Reigous Creek samples (Fig. 4d).

All three *Thiomonas* strains were able to grow in the mineral medium containing only thiosulphate as an energy source. None of the bacteria grew in the media containing solely Fe or As as an energy source. In the medium containing both thiosulphate and Fe(II), the Fe(II) was oxidized to Fe(III), indicating the ferro-oxidizing ability of all three strains. However, in the medium containing both thiosulphate and As(III), the As(III) was not oxidized to As(V) by either B2 or B3, indicating that additional species other than thiosulphate must be required for the oxidation of arsenic by these strains.

In contrast to what was observed in the mineral media, when incubated in Reigous Creek water, none of the three *Thiomonas* sp. Ynys1 strains oxidized Fe(II) (Table 1). However, two of the three strains, B2 and B3, oxidized more than 90% of the As(III) initially present in solution, while B1 had no effect on arsenic speciation. Comparison of the analyses of untreated Reigous Creek water to those of the abiotic control (T) after incubation indicated that a small amount of abiotic oxidation of Fe(II) and precipitation of Fe(III) occurred over the 8 days of incubation. The co-precipitation of As resulted in a slight decrease in the concentration of As(III) in the control.

10 μm

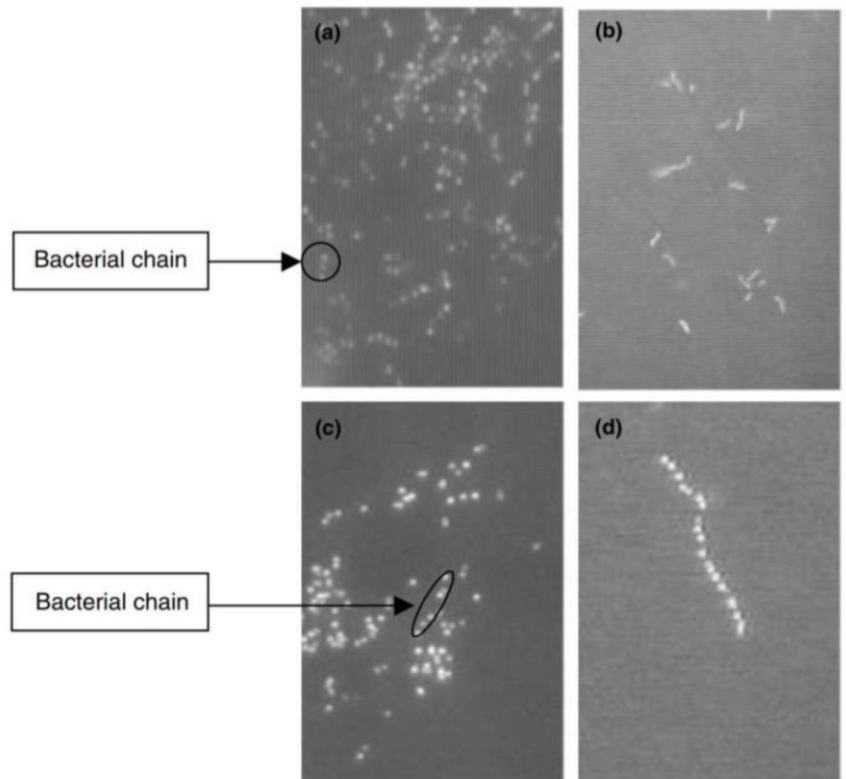


Fig. 4 Microphotographs of bacteria after DAPI staining. Photographs (a)–(c) show *Thiomonas* strains B1, B2, and B3, respectively, after culturing in water from Reigous Spring for 8 days. Photograph (d) shows an example of the long-chain morphology often seen in a variety of different bacteria in untreated samples from Reigous Creek

Table 1. As and Fe composition of Carnoulès Spring water (S1) used as the medium in laboratory experiments (mg l^{-1})

	As total	As(III)	As(V)	Fe total	Fe(II)	Fe(III)
Medium before incubation	232 \pm 12	232 \pm 12	$<7.5 \times 10^{-5}$	1734 \pm 90	1734 \pm 90	<0.05
T	204 \pm 10	204 \pm 10	$<7.5 \times 10^{-5}$	1678 \pm 80	1678 \pm 80	<0.05
B1	204 \pm 10	204 \pm 10	$<7.5 \times 10^{-5}$	1536 \pm 75	1536 \pm 75	<0.05
B2	174 \pm 9	5.0 \pm 0.3	169 \pm 17	1678 \pm 80	1678 \pm 80	<0.05
B3	144 \pm 7	4.0 \pm 0.2	140 \pm 14	1678 \pm 80	1678 \pm 80	<0.05

DISCUSSION

The growth of three strains of *Thiomonas* sp. Ynys1 isolated in water draining from tailings from the Carnoulès mine on R2A medium indicates that these bacteria are acidophilic but can also grow at near-neutral pH. The temporally stable numbers of the three strains at all four sampling sites over the 11 months of sampling suggests that they are autochthonous in the AMD discharging from Reigous Spring (Fig. 2). In contrast, the sporadic appearance of other bacterial colonies on the R2A medium suggests an allochthonous origin.

The three isolated *Thiomonas* strains have more than 99% homology with the group Ynys1 described by Dennison

et al. (2001). The 16S rDNA analyses showed that although these three bacterial strains are very similar, they can be considered as three eco-types (Fig. 3). The biodiversity within the *Thiomonas* genus and the Ynys1 group is comparable to that within *A. ferrooxidans* (Merroun and Selenska-Pobell 2001), in which different co-existing strains possess different capabilities to accumulate and tolerate uranium. Selenska-Pobell *et al.* (2001) suggest that this microdiversity reflects the genetic adaptation of the strains to different concentrations of heavy metals. The bacteria described here clearly have acquired a tolerance to high concentrations of arsenic and low pH, although their anomalous divisions (Fig. 4) suggest that they may be subject to some stress in relation to the water chemistry.

Additional molecular ecological investigation is needed to better determine the structure of the natural bacterial community in Carnoulès AMD.

The results of this study demonstrate, for the first time, the ability of strains of *Thiomonas* group Ynys1 to oxidize As(III) to As(V) *in vitro*. The bacterial strains B2 and B3 did not, however, oxidize As(III) when it was the only energy source present. Their capacity to oxidize As(III) is clearly dependent on the presence of one to more other chemical species present in Carnoulès AMD.

In the Reigous Spring system, the precipitates are composed primarily of As(V) and Fe(III) with small amounts of As(III) and Fe(II), whereas the species in the aqueous phase consist entirely of As(III) and Fe(II) (Leblanc *et al.* 2002). None of the *Thiomonas* strains can be directly responsible for the formation of the As(V)–Fe(III) precipitates, as this requires Fe oxidation, and none of the three strains were capable of oxidizing Fe in Reigous Creek water. The precipitation therefore is most likely mediated by iron-oxidizing bacteria such as *A. ferrooxidans* identified at the site (Leblanc *et al.* 2002), or by *L. ferrooxidans*, which is common in AMD (Schrenk *et al.* 1998). We suggest, however, that *Thiomonas* strains B2 and B3 may be responsible in part for the oxidation of arsenic so that it then co-precipitates with Fe(III) as As(V). At the Carnoulès mine site, it is likely that the co-existence of *Thiomonas* and *A. ferrooxidans* result in the precipitation of arsenic and the overall bioremediation of AMD.

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