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A new bacterial strain mediating As oxidation in the Fe-rich biofilm naturally growing in a groundwater Fe treatment pilot unit

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Abstract

A bacterial strain B2 that oxidizes arsenite into arsenate was isolated from the biofilm growing in a biological groundwater treatment process used for Fe removal. This strain is phylogenetically and morphologically different from the genus *Leptothrix* commonly encountered in biological iron oxidation processes. T-RFLP fingerprint of the biofilm revealed that this isolated strain B2 corresponds to the major population of the bacterial community in the biofilm. Therefore, it is probably one of the major contributors to arsenic removal in the treatment process.

Keywords: Arsenic; Oxidation; Bacteria; Iron treatment process

1. Introduction

Arsenic which is widespread in groundwater, in relation with geology or anthropogenic sources (mining, agriculture), is a very toxic element. Considering that concentration close to $50 \mu\text{g l}^{-1}$ in drinking water significantly increases the risk of cancer (Ravault et al., 2003), the European Commission has lowered the maximum concentration limit for arsenic in drinking water from 50 to $10 \mu\text{g l}^{-1}$.

Some water treatment processes such as reverse osmosis (Ning, 2002) or nanofiltration (Sato et al., 2002) allow to reach these legal requirements; however, these treatments are expensive for small drinking water production units. Adsorption on alumina (Singh and Pant, 2003), Fe or Mn oxi-hydroxides (Meng et al., 2001; Roberts et al., 2004; Zhang et al., 2004; Driehaus et al., 1995) or zero-

valent iron corrosion products (Manning et al., 2002; Su and Puls, 2003) appears to be the method of choice; however, As(III) is slightly retained and must at first be oxidized chemically. Katsoyiannis and Zouboulis (2004) showed that As(III) was efficiently removed by biological filtration process used for Fe treatment. They proposed a mechanism based on the oxidation of As(III) by the bacteria involved in Fe oxidation, i.e. *Leptothrix* and *Gallionella* and subsequent coprecipitation of As(V) and Fe-oxides.

This study confirms the occurrence of biological As(III) oxidation in Fe removal plant. Traditional isolation and culturing techniques were used in combination with advanced molecular methods in order to find out the bacteria responsible for this oxidation.

2. Materials and methods

The experiments were performed using groundwater from a well that supplies the village of Ambacourt (Vosges,

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France) with drinking water. This slightly oxygenated groundwater ($0.4\text{--}1.4\text{ mg O}_2\text{ l}^{-1}$) exhibits a chemical composition within the range of drinking water standards except regarding iron ($700\text{--}900\text{ }\mu\text{g l}^{-1}$) and arsenic ($10\text{--}40\text{ }\mu\text{g l}^{-1}$). Fe is present as Fe(II) while As(III) is the predominant As species. The predominance of reduced species together with the presence of hydrogen sulphide gives evidence for reducing conditions in this groundwater.

Both elements are removed efficiently (100% for Fe, 70–90% for As) through a fixed-bed biological filtration unit using a flow rate of $50\text{ m}^3\text{ h}^{-1}$ and an average residence time of 6 min. The later, designed by IRH Environnement, is similar to that used by Katsoyiannis and Zouboulis (2004). The iron oxide-rich biofilm that grows naturally when well water is allowed to pass through the fixed-bed was sampled for bacterial analysis and laboratory bioassay.

The structure of the bacterial population in the biofilm was analyzed using terminal-restriction fragment length polymorphism (T-RFLP) as described previously (Fourçans et al., 2004). Predictive digestions were made according to RDP web site [TAP:T-RFLP Analysis Program]. Genomic DNA was extracted from the biofilm and from the isolated strain using UltraClean Soil DNA Isolation Kit according to manufacturer's recommendations (MoBio Laboratories Inc., USA) and stored at $-20\text{ }^\circ\text{C}$ until further processing. 16S rRNA encoding sequences and phylogenetic analysis were carried out according to the protocols described by Guyoneaud et al. (2002).

The bacteria were cultured at $30\text{ }^\circ\text{C}$ on R2A medium (Difco Laboratory) which has been shown to allow the isolation of organo-heterotrophic bacteria from potable water (Reasoner and Geldreich, 1985). Morphologically different colonies were isolated by three successive spreadings on the R2A medium. Some characters of the strain metabolism were investigated using "API 20 NE" gallery (API système Biomérieux, France).

Bioassays were carried out using 50 ml of filter-sterilized groundwater inoculated with the biofilm (2.5×10^5 CFU) or 0.1 ml of a pure bacterial strain suspension (10^6 cells ml^{-1}) previously isolated from the fresh biofilm. The assays were incubated at $30\text{ }^\circ\text{C}$ for 90 h under continuous shaking. A control sample with sterilized biofilm (by addition of formaldehyde 0.5% v/v) was run in the same conditions. The concentrations of As(III, V) species in the solution were monitored using coupled HPLC-hydride generation-atomic fluorescence spectrometry according to the protocol described previously (Casiot et al., 2003).

3. Results

In the groundwater spiked with fresh unsterilized biofilm, As(III) removal reached completion within 16.5 h (Fig. 1). In the presence of sterilized biofilm, the rate of As(III) removal was six times lower and some As(III) was still present in solution after 89 h.

Among the six bacterial strains isolated from the biofilm, the strain B2 significantly increased the rate of As(III)

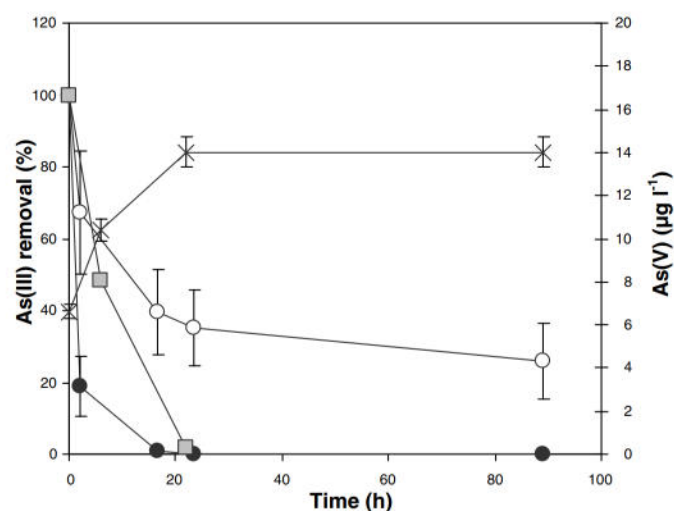


Fig. 1. Evolution of dissolved As(III) as a function of time in groundwater inoculated with sterilized (open circle) and unsterilized (full circle) biofilm or pure bacterial strain B2 (grey square) and the corresponding As(V) concentration in the experiment with isolated strain B2 (cross). Original As(III) concentration in groundwater was $6.4\text{ }\mu\text{g l}^{-1}$, pH 7.4, $T = 30\text{ }^\circ\text{C}$. The error bar corresponds to the standard deviation obtained for four replicates.

disappearance compared to the control sample (Fig. 1). This rate, calculated from experiments with original As(III) concentrations of 6.4, 23 and $41\text{ }\mu\text{g l}^{-1}$, averaged $0.33 \pm 0.09\text{ }\mu\text{g l}^{-1}\text{ h}^{-1}$. In addition, B2 strain clearly promoted the oxidation of As as indicated by the increase of As(V) in solution (Fig. 1).

Table 1
Major metabolic properties of the strain B2 according to API 20 NE™ gallery testing

Substrates	Reactions/enzymes	Results
Potassium nitrate	Reduction of nitrates to nitrites	Negative
	Reduction of nitrates to nitrogen	Negative
Tryptophane	Indole production	Negative
Glucose	Acidification	Negative
Arginine	Arginine dihydrolase	Negative
Urea	Urease	Negative
Esculin	Hydrolysis (β -glucosidase)	Negative
Gelatine	Hydrolysis (protease)	Positive
<i>p</i> -Nitrophenyl- β -D-galactopyranoside	β -Galactosidase	Negative
Glucose	Assimilation	Negative
Arabinose	Assimilation	Positive
Mannose	Assimilation	Negative
Mannitol	Assimilation	Negative
<i>N</i> -acetyl-glucosamine	Assimilation	Negative
Maltose	Assimilation	Negative
Gluconate	Assimilation	Negative
Caprate	Assimilation	Negative
Adipate	Assimilation	Positive
Malate	Assimilation	Positive
Citrate	Assimilation	Negative
Phenyl-acetate	Assimilation	Negative
Tetramethyl- <i>p</i> -phenylene diamine	Cytochrome oxidase	Positive

The strain B2 is a Gram-negative rod that forms white rounded opaque colonies on R2A medium, reaching 0.1 mm diameter after 5 d of incubation. It is motile but tends to aggregate and adhere to solid phases. It grows rap-

Table 2
Comparison of B2 DNA sequence with reference sequences using the BLAST program

16S rDNA sequences	EMBL accession numbers	Number of base pairs and similarity (%)	Characteristics
<i>Leptothrix</i> sp. MBIC3364	AB015048	1330 (95%)	Isolated from a hot spring
Beta proteobacterium HS5/S24542	AY337603	1330 (95%)	Isolated from rice paddy soil
<i>Rubrivivax gelatinosus</i> strain OK303	AF487435	1335 (95%)	New thermotolerant strains of the purple non-sulfur bacterium <i>Rubrivivax gelatinosus</i>
Phenanthrene-degrading bacterium 90	AY177371	1328 (95%)	

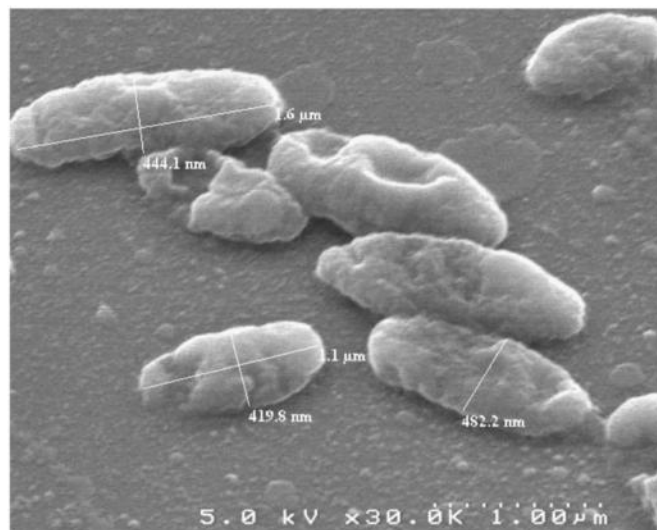


Fig. 3. Microphotograph of B2 strain in R2A culture medium analyzed by SEM. 1.9 cm represents 1 μm.

idly with malate and more slowly on arabinose and adipate (Table 1). Supplementation of the R2A medium with As(III) does not increase growth.

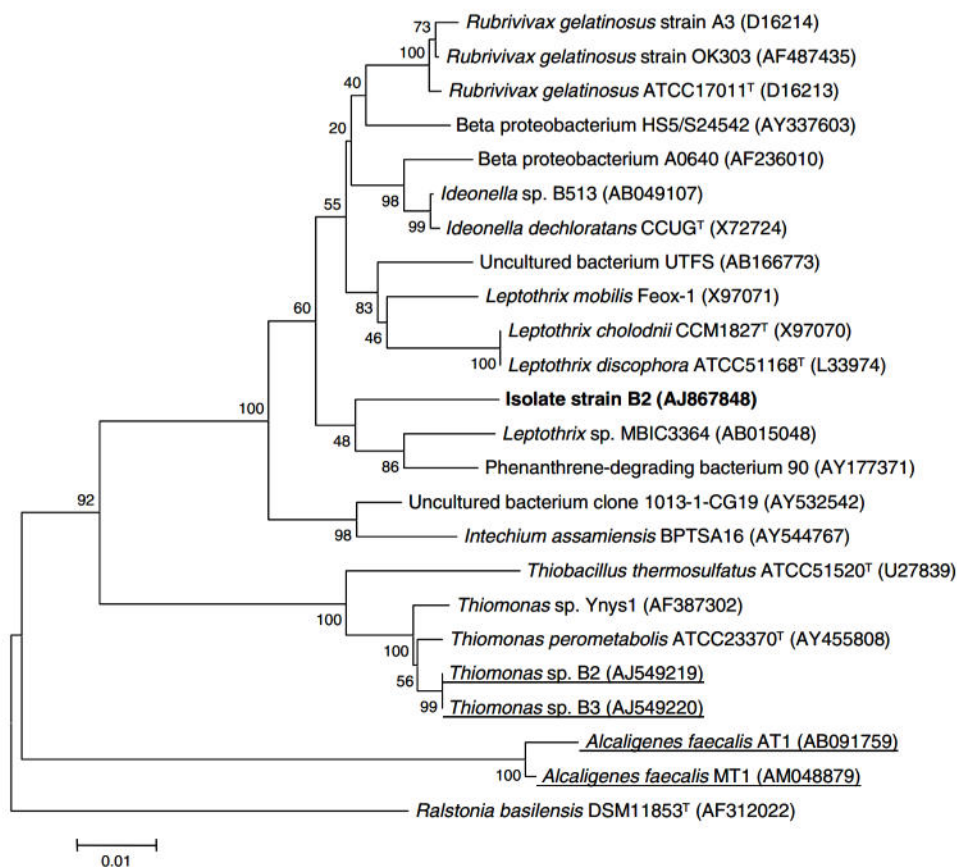


Fig. 2. Phylogenetic relationships between 16S rDNA sequence of the B2 strain and related sequences. The tree was rooted with the *Ralstonia basilensis* sequence. Bar indicates 1% difference in nucleotide sequence. Bootstrap values are marked at the junctions. Strain numbers and EMBL sequence accession numbers are indicated. Bacteria able to perform arsenite oxidation are underlined.

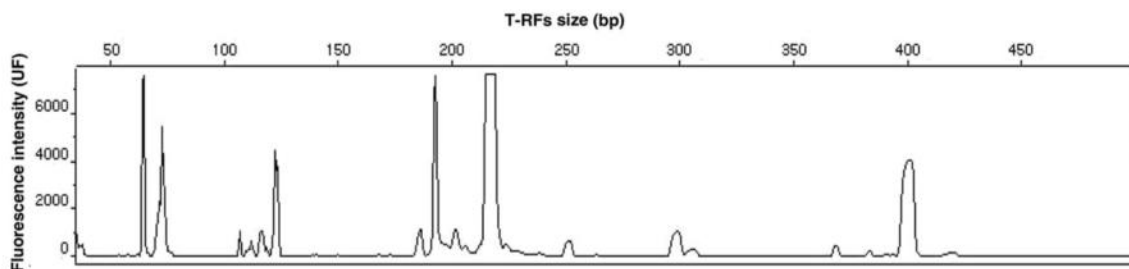


Fig. 4. Bacterial community structure of the biofilm according to T-RFLP fingerprint analysis; electropherogram obtained according to Fourçans et al. (2004).

Based on BLAST DNA sequence analysis (1370 bp), the strain B2 has 95% of similarity with the genus *Leptothrix* (AB015048) and 95% of similarity with the Beta proteobacterium HS5/S24542 (AY337603) (Table 2 and Fig. 2). The phylogenetic analysis shows that B2 strain is distant of the *Leptothrix* group (Fig. 2). Furthermore, its morphology (Fig. 3) differs widely from that of *Leptothrix* which is generally long sheathed.

The structure of the bacterial community of the biofilm, assessed using the number and distribution of major Operational Taxonomic Units (OTU), is characterized by a low bacterial diversity with 14 OTUs (Fig. 4). The bacterial community is dominated by one OTU corresponding to T-RF 219 bp, representing 42% of the entire bacterial population. The predictive digestion ascribes the OTU with T-RF 219 bp to the strain B2, suggesting that the strain B2 is the dominant bacterial population in the biofilm. The OTU with T-RF 192 bp corresponds to the sulfate-reducing bacteria *Desulforhopalus vacuolatus* str. LtK10. The occurrence of this strain may be related to the presence of hydrogen sulphide in the groundwater.

4. Discussion

Our bioassays confirm that the biofilm originating from the treatment of As- and Fe-rich groundwater through biological iron oxidation process increases the rate and efficiency of As(III) removal, in agreement with Katsoyiannis and Zouboulis (2004). In addition, our results suggest that the removal of As involves its oxidation. It cannot be solely imputed to the co-precipitation of Fe and As(III) in relation to the high affinity of As(III) for the ferrihydrite formed biologically (Raven et al., 1998). Abiotic As(III) oxidation by reactive intermediate of Fe(II) oxidation described in Leupin and Hug (2005) might be partly involved in the removal of As; however, difference in the removal rates between sterilized and unsterilized biofilm or pure bacterial strain demonstrates that biological As(III) oxidation is more efficient.

Several bacterial strains which promote As(III) oxidation have been isolated from As-rich geothermal springs (Gihring et al., 2001), soils (Macur et al., 2004), mine waters (Battaglia-Brunet et al., 2002; Bruneel et al., 2003), and arsenic contaminated waters (Weeger et al.,

1999); the latest strain, named ULPAs1, was used to oxidize As(III) in water treatment process in batch reactors with chabazite or kutnahorite as mineral phases for As(V) sorption (Lièvreumont et al., 2003). The rate of As(III) oxidation with the strain B2 in laboratory experiments ($4 \times 10^{-6} \text{ mM h}^{-1}$) is drastically slower than that obtained with the strain ULPAs1 (0.11 mM h^{-1}); however, it is the dominant bacterial population in the biofilm, which probably explains the efficiency of the pilot plant in removing As(III).

In Fe biological treatment unit, Katsoyiannis and Zouboulis (2004) have suggested that the active As-oxidizing strain might be the Fe-oxidizing bacteria *Leptothrix*. The strain B2 which promotes As oxidation and represents the major group of bacteria in the biofilm in the studied biological Fe treatment unit, is phylogenetically and morphologically different from the genus *Leptothrix* and therefore can be considered as a new genus. Thus, the results obtained by application of the 16S rDNA-based molecular technique indicate that contrary to expectation, the major population of this Fe-removal plant is not a member of *Leptothrix* or *Gallionella* group.

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