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Research article

Characterization of a hyperthermophilic sulphur-oxidizing biofilm produced by *archaea* isolated from a hot spring

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ABSTRACT

Background: Sulphur-oxidizing microorganisms are widely used in the biofiltration of total reduced sulphur compounds (odorous and neurotoxic) produced by industries such as the cellulose and petrochemical industries, which include high-temperature process steps. Some hyperthermophilic microorganisms have the capability to oxidize these compounds at high temperatures (>60°C), and archaea of this group, for example, *Sulfolobus metallicus*, are commonly used in biofiltration technology.
Results: In this study, a hyperthermophilic sulphur-oxidizing strain of archaea was isolated from a hot spring (Chillán, Chile) and designated as M1. It was identified as archaea of the genus *Sulfolobus* (99% homology with *S. solfataricus* 16S rDNA). Biofilms of this culture grown on polyethylene rings showed an elemental sulphur oxidation rate of 95.15 ± 15.39 mg S l^{-1} d^{-1}, higher than the rate exhibited by the biofilm of the sulphur-oxidizing archaea *S. metallicus* (56.8 ± 10.91 mg l^{-1} d^{-1}).
Conclusions: The results suggest that the culture M1 is useful for the biofiltration of total reduced sulphur gases at high temperatures and for other biotechnological applications.

1. Introduction

The use of sulphur-oxidizing bacteria (SOB) has been proposed to oxidize total reduced sulphur compounds (TRS) present in industrial gas emissions [1,2,3]. These gaseous compounds, emitted by several industrial processes, are toxic [4] and odorous [5]. The use of biofilters inoculated with SOB has been shown to be a good solution for treating these emissions at moderate temperatures, but there are many gaseous emissions from industrial processes containing these compounds at high temperatures (>50°C), especially in boiler combustion, petroleum refinery, smelting and composting facilities [6,7] because most biochemical transformations occur more rapidly at high temperature [8].

To date, most of the studies reporting TRS oxidation using SOB biofilms involve mesophilic or moderate thermophilic conditions [9,10,11,12]. The immobilization of thermophilic desulphurisation prokaryotes on a packing support material in a bioreactor operating under thermophilic conditions produces more rapid and economical treatment processes [13]. The advantages of using a thermophilic bioreactor are high degradation kinetics and lower cost than a mesophilic reactor because no additional cooling equipment is necessary [8]. Few reports have been published about biofiltration under thermophilic conditions [14], and only four of them describe TRS biodegradation [7,12,15,16]. However, there are several reports that describe microbial communities and enriched consortia from hot springs composed mainly of chemolithotrophic archaea from the genera *Sulfolobus*, *Acidianus*, and *Metallosphaera* [17,18]. In fact, all known sulphur-oxidizing extreme hyperthermophiles (optimum temperature > 60°C) are crenarchaeotes [19]. Some hyperthermophilic sulphur-oxidizing prokaryote cultures have been isolated, predominated by archaea, for example, the VS2 culture found in hot spring sediments from underground mines at Hokkaido, Japan [18], which can be used in bioleaching. Another example is found in the hyperthermophilic microorganisms characterized in geysers in the Yellowstone National Park, USA [17]. In these reports, the main archaea genera were *Sulfolobus*, *Metalosphaera*, and *Thermoplasma*; however, the latter is mainly heterotrophic. In the first study mentioned above, the optimum temperature for S^0 (elemental sulphur) oxidation was 70°C in a culture dominated by *Sulfolobus metallicus* and *Thermoplasma acidophilum*, with S^0 oxidation rate of 99 mg S^0 L^{-1} d^{-1}. One of the most frequently studied genera of hyperthermophilic sulphur-oxidizing archaea is *Sulfolobus*, which contains widely described species like *S. metallicus* and *Sulfolobus solfataricus.*

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To date, no S^0-oxidizing microorganisms from volcanic thermal environments have been described; therefore, the study of the S^0 oxidizing efficiency of a biofilm of hyperthermophilic prokaryotes from such environment is of great interest for the application in TRS biofiltration under thermophilic conditions.

The present study aimed to characterize a microbial community obtained from a hot spring located in the Andes Mountains near Chillán, Chile, by determining the oxidation rate of S^0 in comparison with that achieved by a biofilm of *S. metallicus*. The microbial cultures were characterized using 16S rDNA profile, sequencing and phylogenetic analysis.

2. Materials and methods

2.1. Samples

Three samples of sediment (5-g wet weight, taken using a sterile steel spoon), and water (40 mL, taken using a sterile plastic 50-mL tube) were taken from a hot spring located in the Andes Mountains near Chillán (36° 54′ 35″S/71° 25′ 4″W, 80°C and pH = 3), as described by Coram-Uliana et al. [20], and transported to the laboratory where they were stored at 4°C in darkness.

2.2. Standard bacterial strains

S. metallicus DSMZ 6482 was used as standard positive control in S^0 degradation and for molecular characterization experiments.

2.3. Microbial enrichment

Cultures were performed in 500-mL Erlenmeyer flasks with hermetic stopper, using a 9 K modified culture medium (Table 1), with 10 g L^{-1} of S^0 as energy source, and incubated on a shaker (170 rpm) at 60–80°C [7,18,21]. The cultures were transferred to new flasks with fresh medium and incubated in the same conditions three times to avoid carry-over. The pH drop was measured as growth indicator, as described by Salo-Zieman et al. [18]. Ten millilitre of samples with positive growth was transferred to hermetically closed 250-mL glass flasks and made up to 100 mL with a 9 K culture medium. The flasks were incubated on a rotary shaker at 170 rpm at 60–80°C. This transfer was carried out at least three times. Growth was monitored as described above.

2.4. Biofilm development

Ten millilitre (10^7 cell mL^{-1}) of the enrichment culture M1 or the standard strain were transferred to 250 mL flasks containing 4 g of polyethylene rings, size 7 × 5 × 1 mm (490 mm^2 total surface per ring), made up to 100 mL with the 9 K liquid culture medium using 10 g L^{-1} of S^0 as energy source. The flasks were incubated on a rotary shaker at 170 rpm at 70°C for 30 d using 10 g L^{-1} S^0 as energy source. The biofilm growth was monitored by counting the attached cells by epifluorescence microscopy with DAPI (4′,6-diamidino-2-phenylindole) stain.

Table 1
9 K modified medium composition.*

Compound	Concentration (g l^{-1})
$(NH_4)SO_4$	1.5
$K_2PO_4 \cdot 3H_20$	0.66
$MgSO_4 \cdot 7H_2O$	0.5
KCl	0.1
$Ca(NO_3)_2 \cdot 4H_2O$	0.014
Double distilled water	1
S^0	10
Yeast extract	0.2

* Adjust pH 2.5 with conc. H_2SO_4.

2.5. Sulphur oxidation rate

At the end of the 30 d incubation period, the culture medium was discarded and made up with fresh 9 K liquid culture medium, with the addition of 10 g L^{-1} S^0 as energy source. The S^0 oxidation rate was monitored for 30 additional days to measure the sulphate concentration as the final product of the S^0 bio-oxidation process, assuming that 71 mg S^0 L^{-1} d^{-1} is equivalent to 214 mg L^{-1} d^{-1} de SO_4^{-2} [18]. The SO_4^{-2} concentration was measured by $BaSO_4$ spectrophotometry at 420 nm using a SulfaVer 4 kit and following the manufacturer's instructions (HACH LANGE, Dusseldorf, Germany). The measurements were performed every 7 d, and a sulphate (mg L^{-1}) vs, time (d) plot was made. The sulphate oxidation rate was calculated by linear regression using the software Prim 6.0 (GraphPad Software Inc.). In this phase, growth was controlled as described in Section 2.5. All the experiments were conducted with the M1 strain and the standard strain, including a negative control, in triplicate.

2.6. Analysis of results

For biofilm development and sulphur oxidation rates, the values are expressed as the mean of three replicates. Growth differences and oxidation rate comparisons were made by measuring the significance of the difference between the averages of the parameters mentioned, using one-way ANOVA ($\alpha \leq 0.05$).

2.7. DNA extraction

Pellets were collected from 1.6 mL of an active culture or an original sample diluted by centrifugation in Eppendorf tubes at 8000 rpm for 3 min. The supernatant was eliminated, and each pellet was re-suspended in 310 μL of HTE buffer (HTE: 50 mM Tris–HCl, 20 mM EDTA, pH = 8), after which 350 μL of SDS 2% in HTE buffer was added. Five microlitre RNase A was added, and the mixture was incubated at 37°C for 15 min. It was then incubated with 35-μL Proteinase K at 50°C for 60 min and shaken for 2 min in vortex. A 700 μL of phenol:chloroform:isoamilic alcohol was added (25:24:1), mixed briefly, and centrifuged at 13,000 rpm for 3 min. The upper aqueous phase was transferred into new tubes. The phenol:chloroform:isoamilic alcohol step was performed twice. Sodium acetate 3 M was added at 1/10 of the final volume and mixed. The mixture was refrigerated overnight at -20°C. Each tube was then centrifuged at 11,000 rpm for 10 min. The supernatant was eliminated, and the DNA pellet was washed with ethanol 70%. Finally, the latest extract was centrifuged at 10,000 rpm for 10 min, the supernatant was eliminated, and the washed DNA pellet was re-suspended in 30–50 μL of deionized water. The water used in all the extraction procedures was treated with diethylpyrocarbonate (DEPC) for nuclease inhibition.

2.8. Polymerase chain reaction for bacteria and archaea detection

The DNA extracted was used as a template in polymerase chain reaction (PCR) for detecting bacteria and archaea in the original samples with the 341Fgc and 907R 16S rDNA universal bacteria primers and 344Fgc and 915R 16S rDNA universal archaea primers (Table 2) [7,22,23]. Each PCR reaction contained 1-μL DNA template mixed with 23-μL PCR mix composed of 5 μL Go Taq Buffer (Madison Wisconsin, Promega, USA), 1.5 μL $MgCl_2$ (25 mM), 0.5 μL dNTP (10 mM), 1.25 μL of each primer (10 μM), and 0.125 μL of Gotaq. For bacteria, the reactions were performed as follows: one step of 94°C for 2 min; 30 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min; and a final extension step of 72°C for 10 min. For archaea, the reactions were performed as follows: one step of 95°C for 2 min; 25 cycles of

Table 2
Primer used in this study.

Primer	Especificity	Sequence	Reference
9/27F*	Universal	5′ AGAGTTTGATCMTGGCTCAG 3′	[39]
1492R*	Universal	5′ GGTTACCTTGTTACGACTT 3′	[39]
341Fgc**	Bacteria	5′ CCTACGGGAGGCAGCAG 3′	[40]
907R**	Universal	5′CCGTCAATTCCTTTGAGTTT3′	[41]
21F*	Universal	5′ TTCCGGTTGATCCYGCCGGA 3′	[39]
915R**	Universal	5′GTGCTCCCCCGCCAATTCCT3′	[22]
344Fgc**	Archaea	5′ACGGGGCGCAGCAGGCGCGA3′	[24]

GC fragment: 5′CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCC3′.
* first PCR
** second PCR

95°C for 30 s, 55°C for 45 s, 72°C for 1 min; and an extension step of 72°C for 10 min. PCR products were visualized by 1.2% agarose electrophoresis gel (in 0.5× TAE buffer).

2.9. Denaturing gradient gel electrophoresis

The PCR mixtures for denaturing gradient gel electrophoresis (DGGE) were prepared in the same way as described in Section 2.7. Nested PCR was prepared for bacteria and archaea. For bacteria, the primers 9/27F and 1492R were used in the first PCR and the primers 341Fgc and 907R were used in the second PCR (Table 2). For archaea, the primers 21F and 907R were used in the first PCR and the primers 344Fgc and 907R were used in the second PCR (Table 2). For bacteria, the first PCR reactions were performed as follows: one step of 95°C for 2 min; 30 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 1 min; and a final extension step of 72°C for 2 min. For archaea, the first PCR reactions were performed as follows: one step of 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 1 min; and an extension step of 72°C for 7 min. The temperature programmes for the second PCR reactions were the same as described in Subsection 2.7. The amplified fragments were separated and analysed by DGGE. DNA from the archaea *S. metallicus* was used as internal control. *Thiobacillus thioparus* DNA was used as bacterial control. Each PCR product was visualized by agarose gel electrophoresis (1.2% in 0.5× TAE buffer) before running DGGE.

For DGGE, a 30–65% urea-formamide denaturing gradient was used, in 8% *w*/*v* polyacrylamide gel. Twenty-five microlitre of each sample was loaded, and electrophoresis was run for 12 h at 120 V and 60°C, in a DCode system (BioRad Laboratories, USA). Ethidium bromide staining (0.5 mg L^{-1}) and UV visualization were used.

DNA samples for sequencing were obtained by cutting bands from DGGE and resuspending DNA in 20 μL of 0.1% *v*/*v* H_2O/DEPC. This DNA was amplified again with the same primers described before, but without GC clamp; for archaea, internal PCR primers were used.

Table 3
Total S^0 oxidation rate (mg L d^{-1}) by culture.

Culture	S^0 oxidation rate (mg L^{-1} d^{-1})	
	Suspension	Biofilm
S. metallicus DSM 6482 (SM)	86.80 ± 6.40	56.8 ± 10.91
M1 (Chillán, Chile)	84.30 ± 5.77	95.15 ± 15.39

The resulting PCR products were purified and sequenced (Macrogen Inc., Korea).

2.10. Phylogenetic analysis

Separate BLAST searches were carried out to relate the DNA sequences obtained for the M1a and M1b fragments (query sequences) by using Megablast on the non-redundant database. The parameters for the DNA sequence search were set by default. Nine sequences that presented a match higher than 95% with the query sequences were selected and subjected to multiple alignment using ClustalW [24]. The default match/mismatch scoring system was used. The affine gap penalization scheme was used with parameters 15/6.66, while the delay divergent cut-off was set to 50 (default) [24]. The evolutionary history was inferred using the neighbour-joining method, which was validated by utilizing the bootstrap consensus tree inferred from 1000 replications and displaying a scale of 0.01 nucleotide substitution per sequence position. The evolutionary distances were computed using the maximum composite likelihood method [25]. All positions containing gaps and missing data were eliminated from the dataset (by using the "complete deletion" option). Phylogenetic analyses were conducted using the MEGA7 software [26]. *T. thioparus* 16S rDNA sequence was used as root. The nucleotide sequences obtained from the DGGE of the M1 strain were deposited in the GenBank database.

3. Results

3.1. Microbial enrichment

Sulphur-oxidizing hyperthermophilic organisms were enriched from hot spring samples obtained from the Andes Mountains (Chillán, Chile) on sulphur in 9 K medium using a high-temperature incubator and shake flasks. A hyperthermophilic enrichment culture, designated M1, was obtained in this medium at 70°C using S^0 as the only energy source. In the planktonic phase, sulphate production did not differ significantly between M1 and *S. metallicus* 6482 ($P > 0.05$, Fig. 1a). The sulphate production rate for M1 at 70°C was 254.1 ± 17.38 mg SO_4^{-2} L^{-1} d^{-1}, equivalent to 84.30 ± 5.77 mg S^0 L^{-1} d^{-1} (Table 3). The pH dropped from 2.5 to 0.82 in 30 d. *S. metallicus* DSMZ 6482 showed a

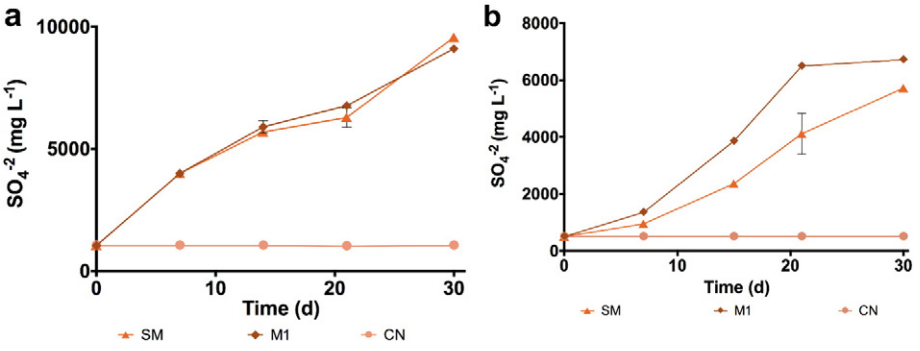

Fig. 1. M1 and *S. metallicus* DSM 6482 sulphate production at 70°C in (a): planktonic phase and (b): biofilms developed on polyethylene rings. Error bars shows standard error media. CN: negative control (uninoculated medium), M1:M1 culture and SM: *S. metallicus* DSMZ 6482. The assays were performed in triplicate and measured independently.

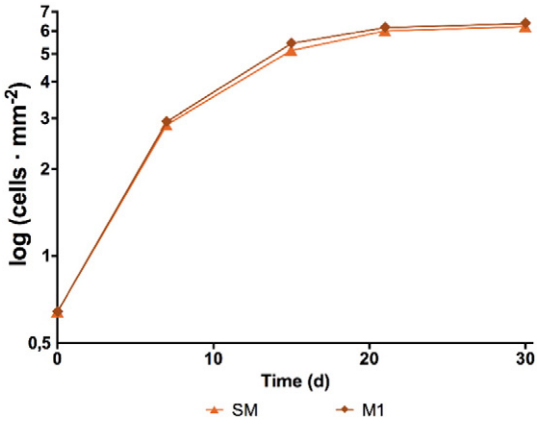

Fig. 2. Cell adhesion per mm^2 of polyethylene: M1:M1 culture; SM: *Sulfolobus metallicus* DSMZ 6482; negative control: no growth observed (uninoculated medium). Error bars shows standard error media. The assays were performed in triplicate and measured independently.

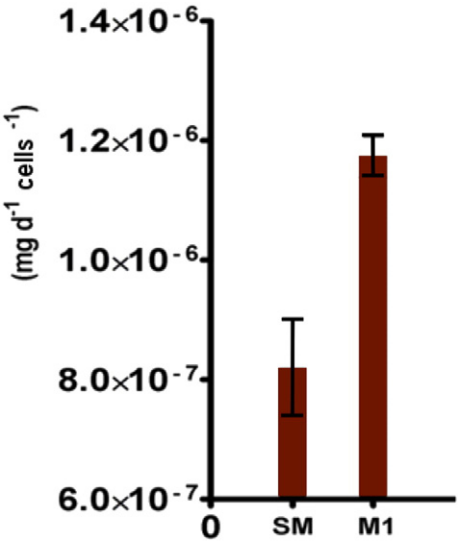

Fig. 4. S^0 oxidation efficiency per cell attached to polyethylene (mg d^{-1} $cell^{-1}$). SM: *Sulfolobus metallicus* DSMZ 6482, M1: Culture M1 from Andes hot spring, Chillán, Chile. Error bars shows standard error media.

sulphate production rate of 261.7 ± 19.3 mg SO_4^{-2} L^{-1} d^{-1}, equivalent to 86.80 ± 6.40 mg S^0 L^{-1} d^{-1} (Table 3). In *S. metallicus* the pH dropped from 2.5 to 0.86 in 30 d.

3.2. Biofilm development

The growth curves on polyethylene rings at 70°C (attached cells) are shown in Fig. 2. At 70°C, there is no significant difference in the growth rate between M1 and *S. metallicus* DSMZ 6482 ($P > 0.05$, Fig. 2). The specific growth rate was 0.031 ± 0.004 h^{-1} for *S. metallicus* DSMZ 6482 and 0.034 ± 0.005 h^{-1} for the M1 culture. SEM of the M1 culture biofilms are shown in Fig. 3.

3.3. Sulphur oxidation rate in biofilms

In the biofilms developed on polyethylene rings, the behaviour was different to the planktonic phase (suspension). The sulphate production curve was significantly higher than that for *S. metallicus* DSMZ 6482 ($P < 0.05$ at all points, Fig. 1b). The sulphate production rate was 286.1 ± 46.39 mg SO_4^{-2} L^{-1} d^{-1} for the M1 enrichment and 171.2 ± 32.88 mg SO_4^{-2} L^{-1} d^{-1} for the *S. metallicus* DSMZ 6482 cultures, equivalent to 95.15 ± 15.39 mg S^0 L^{-1} d^{-1} and 56.8 ± 10.91 mg S^0 L^{-1} d^{-1} respectively (Table 3). Similarly, the M1 sulphur oxidation efficiency by attached cells of the M1 enrichment was higher than that of the standard strain (Fig. 4).

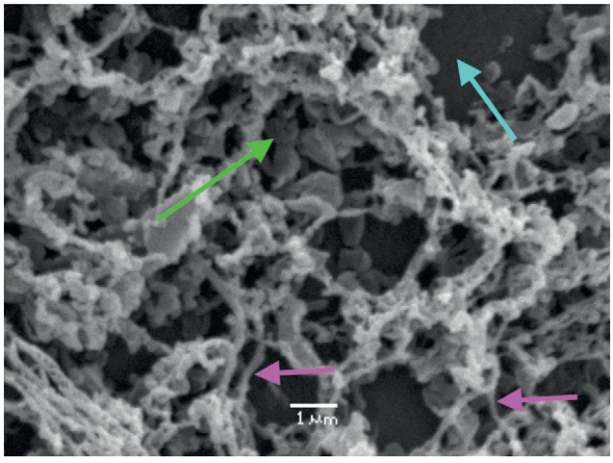

Fig. 3. Electronic micrograph showing biofilms of the M1 culture cells at 10,000X. All cells are indicated by *green arrows*. Polyethylene surface is indicated by *blue arrows*. The micrographs show cells attached to the polyethylene (blue arrows). Exopolysaccharide net (EPS) is indicated by *purple arrows*.

3.4. PCR for bacteria and archaea detection

The gel electrophoresis for bacterial and archaeal detection by PCR with universal primers in the original samples is shown in Fig. 5a and b respectively. M1 contained bacteria and archaea, whereas *S. metallicus* DSMZ 6482 contained only archaea. After culturing at 70°C in the laboratory, only archaea were detected in M1 (Fig. 5b).

3.5. DGGE and phylogenetic profile

In DGGE, the M1 culture showed two 16S rDNA fragments, called M1a and M1b. The latter had a similar denaturation point to the *S. metallicus* strain (Fig. 6). This is consistent with BLAST identity

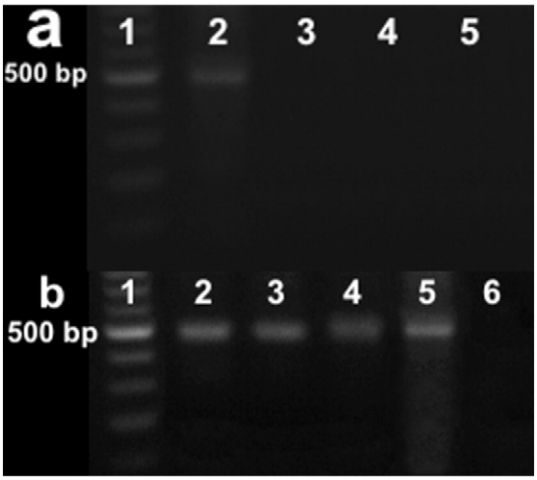

Fig. 5. Gel electrophoresis for PCR-based detection of bacteria (a) and archaea (b) in the original sample of M1 culture at environmental temperature (line 2), *S. metallicus* DSMZ 6482 at environmental temperature (line 3), M1 culture at 70°C (line 4), *S. metallicus* DSMZ 6482 at 70°C (line 5) and negative control (line 6). The expected PCR product was 500 bp. Line 1: 100 bp ladder.

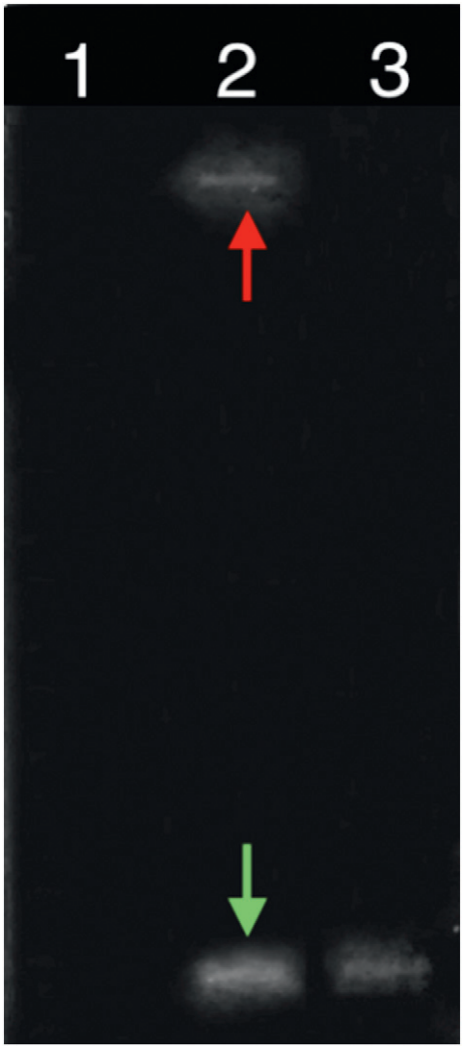

Fig. 6. Denaturing gradient gel electrophoresis (DGGE) of PCR products obtained with archaea primers, showing the two M1 culture DNA fragments designated M1a and M1b (line 2, red and green arrows respectively), *S. metallicus* DSMZ 6482 (line 3) and negative control (line 1). A 65% gradient was used. Ethidium bromide was used for staining.

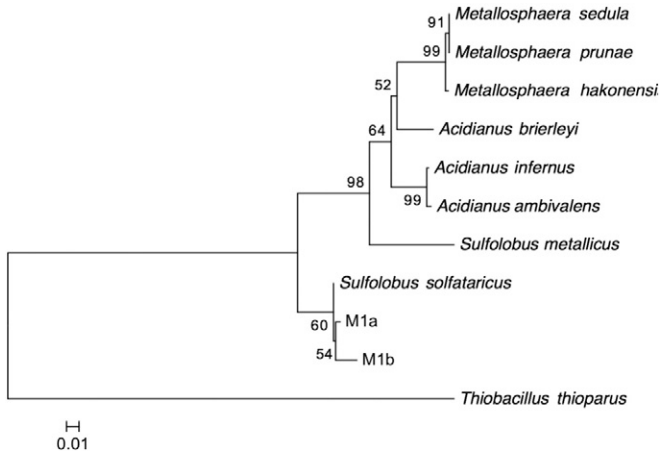

Fig. 7. Phylogenetic tree based on the 16S rDNA sequences of *Sulfolobales* members, showing the taxonomic position of the DNA sequences of the M1 culture fragments obtained by DGGE. The neighbour-joining method was used (1000 repetitions). *T. thioparus* was used as root. Scale bar represents 1% of difference between the sequences.

searches made with the 16S rDNA sequences obtained from DGGE, where both M1a and M1b fragments showed a 99% match with *S. solfataricus*. The 16S rDNA phylogenetic analysis of the two sequences showed that the M1-enriched culture was composed of *S. solfataricus*-related archaea. A phylogenetic tree (Fig. 7) shows that the M1-enriched culture sequences (both M1a and M1b) fit with the *S. solfataricus* branch. The sequences obtained for M1 are indexed in GenBank under accession numbers KX507185 (M1a fragment) and KX507186 (M1b fragment). Bacteria PCR not amplified.

4. Discussion

A culture of sulphur-oxidizing archaea was obtained from a hot spring in the Andes Mountains (Chillán, Chile). This culture was designated M1. All the cultures in this study were grown at temperatures higher than 60°C (optimum T = 70°C) and pH < 1. Therefore, they are considered as hyperthermophile and acidophile [27,28,29,30]. In suspension, the sulphur bio-oxidation of M1 did not differ significantly from that of *S. metallicus* DSMZ 6482 ($P > 0.05$; 84.30 ± 5.77 mg S^0 L^{-1} d^{-1} and 86.80 ± 6.40 mg S^0 L^{-1} d^{-1}). These values are higher than those reported for acidothermophilic cultures [17,18].

The biofilm growth curves on polyethylene rings at 70°C (attached cells) for M1 and *S. metallicus* DSMZ 6482 showed no significant difference ($P > 0.05$, Fig. 2), and the growth rate showed the same pattern (0.031 ± 0.004 h^{-1} for *S. metallicus* DSMZ 6482 and 0.034 ± 0,005 h^{-1} for M1). The growth curves (Fig. 2) and the SEM micrograph (Fig. 3) demonstrated that M1 and *S. metallicus* both form biofilms on the packing material used in the experiment. The biofilm SEM micrographs in this study are similar to those described by Koerdt et al. [31]. The sulphur oxidation rates in these biofilms were completely different from those in suspension. Biofilms of the M1 culture had a higher sulphur oxidation rate than those of *S. metallicus* DSMZ 6448 (95.15 ± 15.39 mg S^0 L^{-1} d^{-1} for M1 and 56.8 ± 10.91 mg S^0 L^{-1} d^{-1} for *S. metallicus*, Table 3). This result is consistent with the sulphur oxidation efficiency of M1, which was higher than that of *S. metallicus* (Fig. 4). These rates are in the range reported previously, between 29 and 51 mg S^0 L^{-1} d^{-1} for *S. metallicus* pure cultures and 99 mg S^0 L^{-1} d^{-1} at 70°C for a hyperthermophilic culture obtained from an underground mine at Hokkaido, Japan [18]. These facts not only suggest that M1 is as efficient as other sulphur-oxidizing acidothermophilic archae described previously, but also suggest that the biofilm state confers oxidation efficiency advantages on the M1 culture. It is believed that this is because of the dissolution of the sulphur in the exopolysaccharide (EPS) layer, a common component of biofilms. The higher quantity of iron ions and glucuronic acids within the EPS result in higher mineral oxidation activity than in cultures with low amounts of these components [32].

DGGE analysis was performed, and two fragments were found for 16S rDNA of culture M1, termed M1a and M1b. The latter presented a similar denaturation point to *S. metallicus* (Fig. 6), suggesting that M1 was composed principally of archaea of the *Sulfolobus* branch. The 16S rDNA phylogenetic analysis of the M1a and M1b sequences showed that the M1 culture was composed of *S. solfataricus*-related archea (Fig. 7). The difference between the denaturation points of the two fragments results from a small difference in the sequence, although the two sequences are closely related. Such differences are quite common between archaea sequences from the same species, and even greater variability has been detected [33]. This archaea feature can be explained by the maturation process from circular pre-rRNA to mature rRNA occurring in the Euryarchaeota and Crenarchaeota kingdoms: a processing step conserved across the archaea [34]. The finding of this strain in a hotspring is similar to many examples reported in the literature [18,35,36,37].

In conclusion, a culture (M1) formed of sulphur-oxidizing hyperthermophilic archaea of the genus *Sulfolobus* was obtained; it

forms biofilms on polyethylene and is more efficient in S^0 oxidation than *S. metallicus*. Culture M1 is even more efficient in biofilm. These characteristics suggest that M1 has a potential use in high-temperature biofilters for TRS elimination and in other biotechnological applications [38].

Conflict of interest

The authors declare no conflict of interest.

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