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In search of sulfate-reducing consortia able to degrade acetate under acidic conditions

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Abstract

BACKGROUND: Sulfate-reducing microorganisms (SRM) can help to remediate acidic effluents containing metals. One drawback of sulfate reduction is that some SRM do not completely oxidize the substrate to CO_2 and acetic acid may remain as a byproduct, affecting the process efficiency. Acidic environments are a potential source of sulfate-reducers able to thrive in acidic conditions. This work aimed to develop cultivable consortia of sulfate-reducing microorganisms able to consume acetate at acidic pHs and analyze their community composition.

RESULTS: Starting from sediment enrichments from a natural acidic source, by successive transfers and combinations of electron donors and pHs we obtained seven sulfate-reducing consortia. All of the consortia consumed the acetate produced from the incomplete oxidation of the substrate (lactate or glycerol) and used 53–75% of the reducing equivalents for sulfate reduction. The sulfide production rate of the consortia was between 0.22 and 0.26 mmol L^{-1} day^{-1} in the pH range 3–6, being slightly higher at acidic conditions (pH 4–5). The microbial diversity of the consortia was dominated by 21 operational taxonomic units, including taxa of acetotrophic sulfate reducers (i.e. *Desulfotomaculum* and *Desulfatirhabdium*) and fermenting bacteria.

CONCLUSION: The consortia reported here have the potential to serve as inoculum for sulfate-reducing bioreactors and could help to overcome acetate accumulation at low pHs.
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Supporting information may be found in the online version of this article.

Keywords: acetate; acidic pH; acidophilic; consortia; community; sulfate reduction

INTRODUCTION

The biological sulfate reduction process is based on the oxidation of an electron donor, which can be an organic substrate or molecular hydrogen, coupled to the reduction of sulfate (terminal electron acceptor) to produce sulfide. Sulfate-reducing microorganisms (SRM) are responsible for sulfate reduction and are a group of prokaryotes, remarkably adaptable, that can be found in terrestrial and aquatic environments, mainly in sulfate-rich anoxic environments in very diverse natural environments such as saline, alkaline, acidic or thermal habitats.[1, 2]

Recently, sulfate reduction at low pH has raised interest for the treatment of metal-containing effluents, such as acid mine drainage (AMD),[3, 4] whereby the biologically produced sulfide can react with heavy metal ions such as iron (Fe^{+2}), zinc (Zn^{+2}), copper (Cu^{+2}) or cadmium (Cd^{+2}) and precipitate them as insoluble metal sulfides.[5–8] Such effluents are somewhat acidic (pH < 5) owing to the acidification of the waste generated from the exploitation of minerals, either by chemical or biological processes, and generally contain low amounts of organic carbon (<10 mg L^{-1}), these characteristics diminish the efficiency of the sulfate reduction process.[9]

The activity of SRM retrieved from environmental samples (i.e. sediments or streams) has been observed under extremely (pH 1–3) and moderately (pH 4–5) acidic conditions and many efforts have been made to enrich, cultivate and eventually isolate

SRM at those conditions.[10] The development of several types of reactors for the treatment of AMD became possible by using communities from this kind of acidic environment. For instance, Ñancucheo and Johnson[11] treated synthetic AMD successfully in a continuous reactor inoculated with an enrichment obtained from the stream of an abandoned copper mine, and bioaugmented with pure cultures of *Desulfosporosinus* M1 and *Desulfobacillus acidavidus*. The community developed on glass beads was the key to the successful operation of the reactor at pH as low as 2.1. In another work, sulfate-reducing consortia and four isolates of SRM eventually were retrieved from the extremely acidic environment of Rio Tinto in Spain.[10] The isolates were cultivated at pH 5.5–4.0 using

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glycerol, methanol and lactate as substrates, but glycerol and lactate were incompletely oxidized to acetate. To date, only a few isolates of the genera *Desulfovibrio*, *Desulfosporosinus*, *Desulfobacillus* and *Desulfurella* have been identified as acid-tolerant or acidophilic; none of these isolates can oxidize acetate.[12]

Lactate and ethanol are the substrates typically used to promote the activity and growth of SRM at neutral pH.[13, 14] However, a challenging area in the field of sulfate reduction at acidic pH is that when incomplete oxidation of these substrates occurs, the efficiency of substrate oxidation via sulfate reduction is lower because acetate remains as a by-product.[15] The acidic pH adds another constraint to the use of acetate by SRM because at pH < 4.76 (i.e. the pKa of acetic acid), undissociated acetic acid is the predominant form, and this nonionized molecule will cross the cell membrane and inhibit cellular respiration.[12, 16] By contrast, glycerol has been used successfully as a substrate for the enrichment, cultivation and even isolation of SRM, at acidic conditions (pH ≤ 4.0).[10, 17] Glycerol does not ionize at acidic pH, avoiding the harmful effects that ionizable substrates such as organic acids may cause, but acetate is still a common by-product of glycerol oxidation.[11] Therefore, to efficiently apply sulfate-reduction for AMD treatment, it is critical to count on acetate consuming sulfate-reducing communities thriving at acidic pH. This work aimed to expand the scope of SRM at acidic pH by developing and characterizing sulfate-reducing consortia. Using the acclimation approach, we obtained seven sulfate-reducing communities cultivated at low pH (3 or 4) that can consume acetic acid.

MATERIALS AND METHODS

Source of microorganisms
Enrichments previously cultured were used as inocula to develop the acetotrophic sulfate-reducing consortia reported here; these enrichments originated from the sediments of the acidic leachates from an abandoned sulfur mine and were cultivated with different carbon sources (acetate, lactate or glycerol) at different pHs (3, 4 or 5) as reported elsewhere.[18] To start the cultures of the consortia, we screened 45 enrichments and selected a total of 38 to be used as inoculum, based on the sulfide production and acetate consumption capacity of each enrichment (Fig. S1). In this work, we aimed to obtain consortia free of sediment.

Culture medium and cultivation conditions
The following minimal anaerobic medium was used to develop the consortia (mmol L^{-1}): 50 NH$_4$Cl, 30 NaCl, 40 MgCl$_2$·6H$_2$O, 75 CaCl$_2$·H$_2$O; 1 mL L^{-1} trace element solution [(mmol L^{-1}) 50 HCl, 1 H$_3$BO$_3$, 0.5 MnCl$_2$, 7.5 FeCl$_2$, 0.5 CoCl$_2$, 0.1 NiCl$_2$ and 0.5 ZnCl$_2$) and 0.1 g L^{-1} of yeast extract, modified from Stams *et al.*[19] The medium was supplemented with 10 mmol L^{-1} Na$_2$SO$_4$ as the electron acceptor and the stoichiometric amounts of electron donor (mmol L^{-1}): 10 acetate, 6.6 lactate or 5.71 glycerol. All cultures were developed in 120-mL serum bottles, containing 80 mL of minimal anaerobic medium supplemented with the corresponding substrate, sodium sulfate, and anaerobic atmosphere (N$_2$/CO$_2$; 80%:20%). All the bottles were incubated at 30 °C in the dark without agitation.

Development of the consortia by successive transfers
In order to develop the consortia by successive transfers, we started from the 38 initial enrichments selected as inoculum. These enrichments were divided into two groups. Group 1: those initial enrichments incubated at initial pH 4.0 and fed with lactate,

acetate or glycerol, six bottles each. The successive transfers of this group were inoculated with 20% of slurry from the enrichment or 20% of the previous transfer (see Fig. S1). Group 2: 20 bottles in total, enriched at initial pH 3 with lactate or acetate (six bottles each) or glycerol (eight bottles); for starting up the successive transfers from this group, we assayed two ways of inoculation: (i) inoculation with 10% supernatant (liquid fraction after sedimentation) and (ii) inoculation with 20% slurry (the mixture of liquid media and sediment after vigorous agitation). The development of the cultures was monitored periodically through the concentration of substrates (acetate, lactate, sulfate), the concentration of sulfide, and the pH until the sulfide concentration was constant and almost complete consumption of acetate was observed (around 30 days). At this point, the cultures that showed sulfide production and acetate consumption were transferred again to new media with the corresponding substrate and initial pH; in this way, another transfer was obtained. In total, five successive transfers were needed to obtain each one of the seven consortia presented here; all of the consortia were devoid of the original sediment. Those cultures that did not produce sulfide and did not consume acetate were discarded (Fig. S1). During the successive transfers, the pH of the cultures was not controlled.

Characterization of the final consortia
Each final consortium (fifth transfer) was characterized by sulfide production, sulfate consumption, acetate production, pH and optical density (600 nm; OD600) in triplicate. The time profiles obtained in this assay were used to calculate the maximum rates of lactate and acetate consumption and sulfide production to verify the reproducibility of the activity of the consortia.

Favorable pH interval
The final consortia were cultivated (in duplicate) with their corresponding substrate but varying the initial pH of the culture medium with the addition of 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH. For the consortia originally cultivated at pH 4.0, we screened the following initial pH values: 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 7.0. For the consortia originally cultivated at pH 3.0, the initial pH was adjusted to 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 7.0. The concentration of sulfide, pH and optical density were determined every seven days; the sulfate reduction rate was indirectly obtained from the slope of the sulfide production curve (in mmol L^{-1}) *versus* time. Subsequently, the rates obtained were plotted at each pH value to obtain the interval of favorable pH of each consortium.

Chemical analyses
Dissolved sulfide was quantified by the Cord–Ruwisch method[20] with the corresponding calibration curve (0–20 mmol L^{-1}, in triplicate; maximum error 5%) using Na$_2$S·9H$_2$O as standard. Volatile fatty acids (lactate and acetate) and sulfate were determined by capillary electrophoresis with a diode array detector according to the method of Soga and Ross[21] from calibration curves (50–1000 mg L^{-1}), using high purity standards, after centrifugation (10 000×*g*) and filtration (0.22-μm) of the samples. The pH was measured with a Orion™ VersaStar potentiometer (Thermo Fisher Scientific, Waltham, MA, USA). To quantify the increase of biomass, the OD600 was determined from fresh samples of the cultures.

Molecular characterization

In order to characterize the diversity of each final consortium (fifth transfer), the DNA was extracted from each bottle of the triplicate assay (see Characterization of the final consortia section above) using the SPIN FastDNA-T DNA Extraction Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. Then, the DNA was pooled into one composite sample, amplified and cloned. Amplification of the 16S rRNA gene was performed with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') to obtain a 1465-bp fragment. The PCR mix (50 µL) contained: 5X PCR Green GoTaq® reaction buffer, 0.2 mmol L^{-1} dNTPs, 0.1 µmol L^{-1} of each forward and reverse primer, GoTaq® DNA Polymerase (1.25 U), and 1 µL template DNA. The PCR program was: 97 °C for 5 min, followed by 30 cycles at 95 °C for 2 min, 52 °C for 40 s, 72 °C for 1.3 min, and a final extension at 72 °C for 10 min. The PCR products with the expected size (1465-bp) were cleaned by DNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA, USA), and ligated (overnight) using the pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer's instructions. Ligation was plated on Luria–Bertani (LB) agar with ampicillin (100 mg L^{-1}), IPTG (0.00238 mg L^{-1}) and X-gal (0.0040 mg L^{-1}) as selection media. Positive white colonies were selected (48 per sample) and grown in LB medium for 18 h at 37 °C; the grown cultures were plated into GATC plates and sent for Sanger sequencing with SP6 primer (Eurofins GATC Biotech, Konstanz, Germany). The DNA sequences were checked using Chromas (v2.32, Technelysium Pty Ltd, Brisbane, Australia), and contigs were constructed from the partial sequences using DNA-baser (v2.71.0, Heracle Software, Lilienthal, Germany) resulting in sequences of 800–1200 bp of the 16S rRNA gene. To find the phylogenetic affiliation of the clones, the bacterial 16S rRNA sequences were checked for anomalies using Pintail online software[22] and compared to the blastn GenBank (NCBI). Sequences were also aligned with SINA (v1.2.11), of the SILVA ribosomal database project, to find the phylogenetic affiliation of the clones using SILVAngs (v1.9.4/1.3.9) for Sanger sequencing analysis and to construct rarefaction curves. The sequences are deposited in the NCBI nucleotide sequence database GenBank under accession numbers MT022112-409. We used the R Studio program[23] to calculate the Euclidean distance matrix, and construct a dendrogram using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) building the tree by the upside down approach. The richness, Shannon–Wiener index, Simpson index of dominance, evenness and principal component analysis (PCA) were calculated with R Studio program using the vegan community ecology package (v2.5.6).

RESULTS

Development and performance of the consortia-successive transfers

Acetate-consuming sulfate-reducing consortia were enriched from previous incubations of sediments at acidic conditions (pH < 4.0) by successive transfers. To be transferred again, the cultures should produce sulfide and consume acetate completely. Only the first transfers inoculated with 20% of slurry and lactate showed sulfide production and acetate consumption; the cultures inoculated with 10 or 20% of the supernatant produced <2 mmol L^{-1} sulfide and consumed <80% of the substrate. Therefore, the following successive transfers with lactate as substrate were inoculated with 20% (v/v) of slurry. Interestingly,

Consortium 7, fed with glycerol, was the only one that was obtained using 10% (v/v) of the supernatant as inoculum in the first transfer; nonetheless, due to the long-time needed (68 days) to consume the acetate completely and produce sulfide, the successive transfers were inoculated using 20% (v/v) of supernatant.

Following this methodology, from a total of 365 incubations, only seven consortia were obtained after five successive transfers (Figs 1, S2 and S3). These consortia were free of sediment, able to produce sulfide in acidic conditions (pH 3 or 4) and consume acetate using lactate (consortia 1–6) or glycerol (Consortium 7) as the substrates. It is worth noting that, at this stage of the experiment, each consortium was unique because there was only one culture of each consortium. Table 1 shows the combinations of pH, substrate and type of inoculum that yielded the seven consortia coupling sulfate-reducing activity with complete oxidation of the substrates. Figure 1 shows the time profiles of acetate production/consumption and sulfide production during the five successive transfers of the seven consortia. In most of the transfers, acetate accumulated between days 5 and 30; later, the communities consumed acetate and continued producing sulfide. Most probably, acetate accumulated due to the incomplete oxidation of lactate or glycerol; according to the stoichiometry 1 mmol L^{-1} of lactate can produce 0.5 mmol L^{-1} H$_2$S, 1 mmol L^{-1} acetate and 1 mmol L^{-1} CO$_2$, and 1 mmol L^{-1} of glycerol can produce 0.75 mmol L^{-1} H$_2$S, 1 mmol L^{-1} acetate and 1 mmol L^{-1} CO$_2$.[24]

The first two transfers of consortia 1 to 6 still had remains of the sediment as a consequence of the strategy of using 20% of slurry as inoculum. Nevertheless, from the third transfer onward, all of the cultures were planktonic and free of sediment, producing sulfide and consuming acetate in a more reproducible way. In the third transfer, sulfate-reduction and acetate consumption were slower compared with the previous transfers, possibly as a consequence of getting rid of the remaining sediment, all the seven consortia behave the same (Figs 1 and S2). The pH profiles showed that no matter which pH value each of the consortia started at, the pH increased to values between 6.1 and 7.3 (Fig. S3). Interestingly, the consortia started to consume acetate when the pH reached ≈5.5, this trend occurred in all the transfers (Figs 1 and S3). Attempts of developing consortia using acetate as the sole electron donor for sulfate-reduction, at initial pH 3 or 4, were unsuccessful due to the high concentration of undissociated acetic acid (9.8 and 8.4 mmol L^{-1} at pH 3 and 4, respectively). The sulfate-reducing rates of the successive transfers varied widely (Table S1) and did not show any clear tendency to increase; however, the sulfate-reducing rates decreased from transfer 1 to 3. Eventually, in the last two transfers (4 and 5), the sulfate-reducing activity increased in some cases.

Reproducibility of the acetate-dependent sulfate-reducing activity

In the fifth successive transfer, the cultures were devoid of sediment, and the sulfate-reducing activity remained. At this point, we considered that the consortia were cultivable and reproducible, as shown by the assays performed in triplicate (Fig. 2). From these results, it was possible to calculate the percentage of substrate used for sulfate reduction of each consortium based on the stoichiometry of sulfide production (Table 2). Consortia 2 and 7 used ≈75% of the electron donor (lactate or glycerol) to perform sulfate reduction, the rest of the consortia used close to 50% of the substrate for sulfate reduction that was the target activity of the culturing approach. These results indicated not only that the consortia were composed of sulfate-reducers, but also
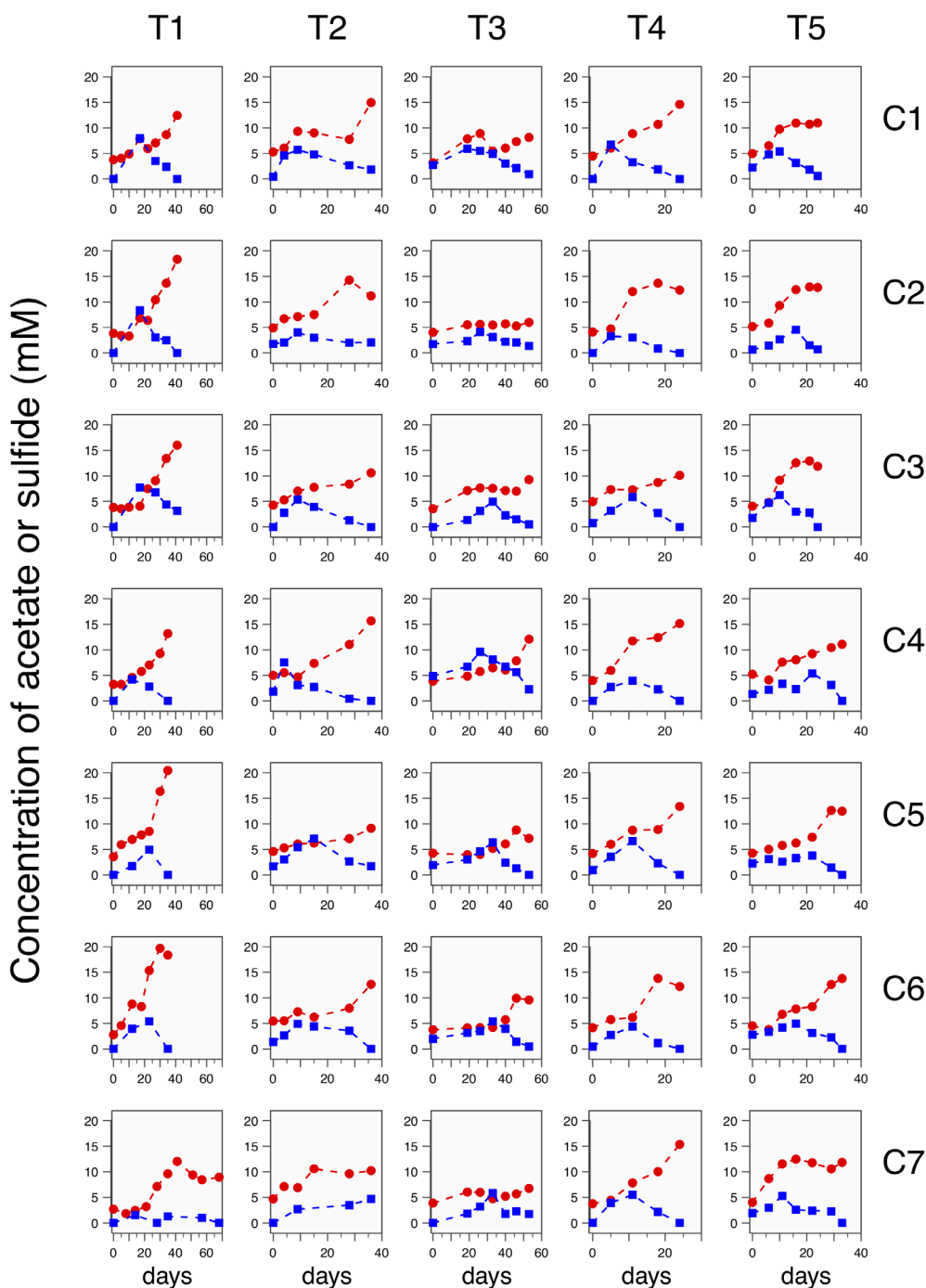
Figure 1. Kinetic profiles of sulfide (●) and acetate (■) of the seven consortia, Consortium 1 (C1) to Consortium 7 (C7), from successive transfer one (T1) to successive transfer five (T5). C1–C3 substrate lactate, initial pH 4; C4–C6 substrate lactate, initial pH 3; C7 substrate glycerol, initial pH 3.

that the successive transfer technique was accurate and appropriate for the cultivation of sulfate-reducers.

We also calculated the acetate consumption, once the acetate concentration reached a maximum and started to decrease, and the sulfide production rates (Table 2). The rates of acetate consumption varied between 0.20 and 0.44 mmol L^{-1}day^{-1}. Consortium 1, fed with lactate, showed the highest acetate consumption rate; the rest of the consortia also were able to use acetate as substrate at lower acetate consumption rates. Regarding sulfide production rates, these were between 0.22 and 0.28

(mmol L^{-1} day^{-1}), and Consortium 7 showed the highest sulfide production rate.

Range of favorable pH

We attempted to determine the most favorable pH at which the sulfate-reducing activity occurred comparing the rates of sulfide production at each pH (Fig. 3); the selection criterion was that the difference of the sulfide production rate obtained at the different initial pH values, was <0.2.

Table 1. Initial pH, electron donor and type of inoculum used to obtain the consortia

Consortium	Initial pH	Electron donor	Inoculum
1	4		
2	4		
3	4	Lactate	20% of slurry[a]
4	3		
5	3		
6	3		
7	3	Glycerol	10% of supernatant[a]

[a] Just in transfer 1; transfers 2 to 5 where inoculated with 20% of supernatant.

The results showed that there was not one favorable pH value but instead a range at which each consortium carried out sulfate reduction optimally (Fig. 3 and Table 2). On the one hand, the consortia developed at initial pH 4 and fed with lactate (consortia 1–3), performed better in the range of pH 4–6 than at pH 3.5 or pH 7. On the other, the consortia initially cultivated at pH 3.0 and fed with lactate performed better in a pH interval from 2.5 to 6.0 than at pH 7.0 (consortia 4–6). Consortium 7, fed with glycerol, showed a clear preference for acidic pH (3.0–5.5) to perform sulfate reduction. The initial optical density increased from a value around 0.019 ± 0.001 to values between 0.23 and 0.34 in all consortia, which is in agreement with the optical density values obtained in the sulfate-reducing activity assays (Fig. 2), confirming

that the microorganisms of the consortia are cultivable, showing growth and not just activity.

Microbial composition of the consortia

A total of 21 operational taxonomic units (OTUs) (genus level) were obtained per sample at 80–99% similarity (from 336 sequences) (Fig. 4). At the phylum level, all of the consortia were composed of members belonging to *Bacteroidetes* (20–70%), *Firmicutes* (6–58%) and *Proteobacteria* (2–17%). Other taxa were found exclusively in some consortia. For instance, only consortia 2 and 7 contained sequences resembling *Caldisericum* (2–7%); and sequences related to *Sphaerochaeta* (2–71%) were only present in consortia 1, 4 and 7. Interestingly, sequences related to the unclassified *Synergistetes JGI-0000079-D21* (2–11%) were present in all the consortia except in Consortium 7. Uncultured bacteria were retrieved from almost all of the consortia (2–9%) except from consortia 3, 4 and 6, whereas unclassified bacteria (nonrelative) amounted to 2–15%. According to the diversity indices (Table S2), consortia 3 and 7 showed the highest richness value ($S = 12$) in comparison with the rest of the consortia, but the Shannon–Wiener index indicated that Consortium 7 was the most diverse ($H = 2.106$) and the least diverse was Consortium 4 ($H = 1.145$). Consortium 6 (dominated by *Lentimicrobium*) and Consortium 4 (dominated by *Sphaerochaeta*) showed the lowest Simpson' index values, whereas the rest of the consortia were equally dominated. The rarefaction curves of all the consortia are shown in Fig. S4.

The consortia grouped in two different clusters (Fig. 4), consortia 1 and 3 (lactate, initial pH 4) showed the most similar microbial structure, as well as consortia 2 (lactate, initial pH 4) and 5 (lactate,
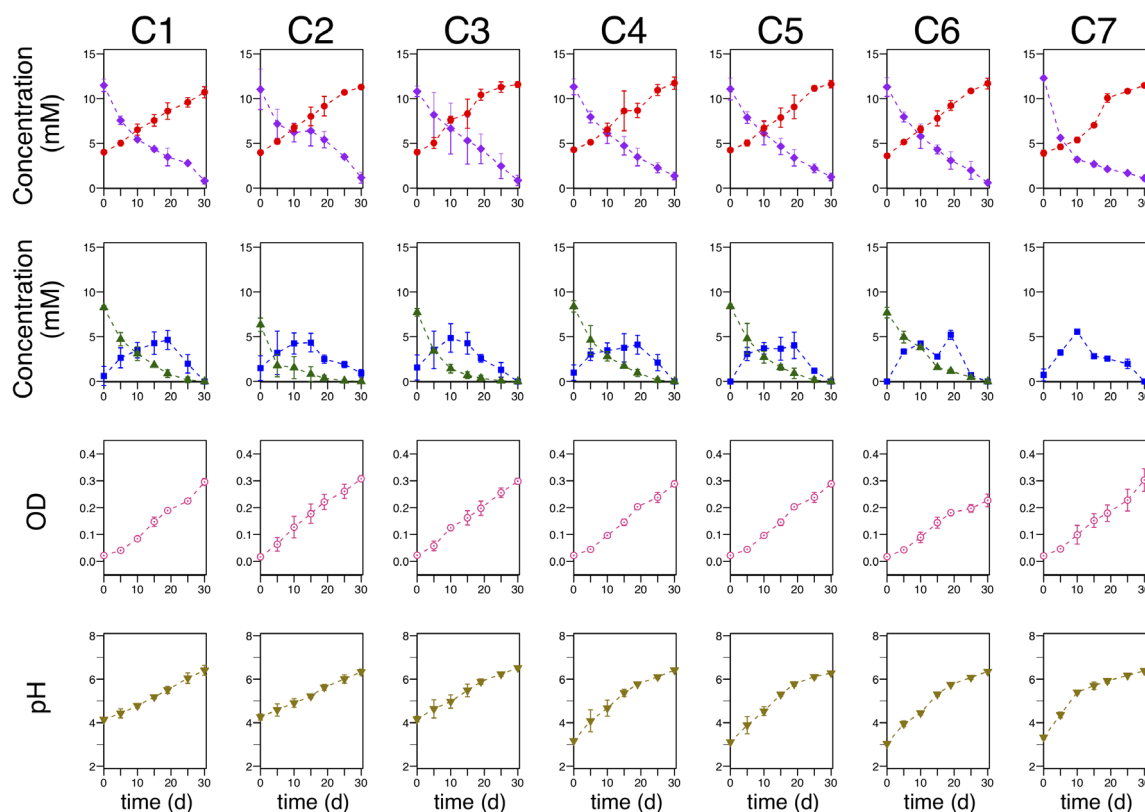

Figure 2. Profiles of sulfide (●); sulfate (◆); lactate (▲) and acetate (■); OD600 (○); and pH (▼) in the triplicate assays of the seven consortia (C1–C7) after successive transfer 5.

Table 2. Rates of sulfide production and acetate consumption, percentage of substrate used to perform sulfate-reducing activity, and the interval of favorable pH of the seven cultivable consortia

Consortium	Sulfide production rate (mmol L^{-1} day^{-1})	Acetate consumption rate (mmol L^{-1} day^{-1})	Percentage of substrate used to perform sulfate-reducing activity	Interval of favorable pH
1	0.22 ± 0.017	0.44 ± 0.076	53.9 ± 2.17	4.0–6.0
2	0.25 ± 0.008	0.20 ± 0.070	77.8 ± 8.75	5.0–6.0
3	0.25 ± 0.018	0.28 ± 0.053	60.6 ± 4.93	4.0–6.0
4	0.26 ± 0.019	0.39 ± 0.073	59.1 ± 4.54	3.0–6.0
5	0.26 ± 0.002	0.39 ± 0.123	58.3 ± 0.625	2.5–6.0
6	0.25 ± 0.011	0.34 ± 0.059	54.1 ± 2.09	3.0–5.5
7	0.28 ± 0.007	0.25 ± 0.018	[a]75.1 ± 3.74	3.0–5.5

[a] Theoretical value.

initial pH 3), because they grouped in the same branch. Consortia 4 and 6 (lactate, initial pH 3) clustered together in another branch having a different microbial structure from the rest of the consortia. Consortium 7 (glycerol, initial pH 3) showed a more similar structure to the cluster formed by consortia 2 and 5. The most dominant members of the communities at the genus level (21 OTUs) were mainly fermentative bacteria and SRM. The PCA showed no clear relationship between the initial pH value

(3 or 4) and the substrates (glycerol or lactate) with the composition of the microbial community in each of the seven consortia (Fig. S5).

Using glycerol or lactate as the electron donors, we retrieved sequences similar to *Desulfovibrio* (delta-Proteobacteria) representing 2–11% of the sequences in consortia 2, 4, 5, 6 and 7. Sequences similar (92–96%) to the genus *Desulfotomaculum* (Firmicutes) were obtained from consortia 1 and 3 representing
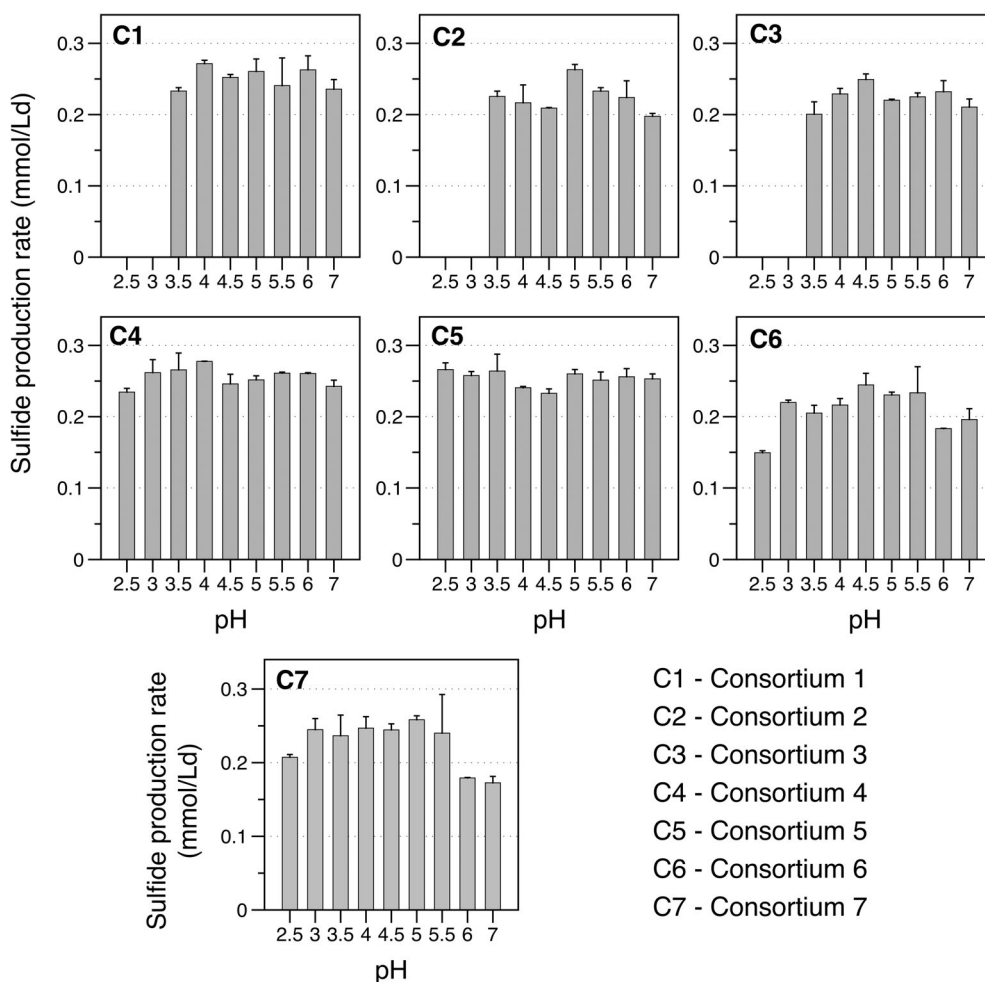

C1 - Consortium 1
C2 - Consortium 2
C3 - Consortium 3
C4 - Consortium 4
C5 - Consortium 5
C6 - Consortium 6
C7 - Consortium 7

Figure 3. Sulfide production rates obtained at different initial pH values for each cultivable consortium.
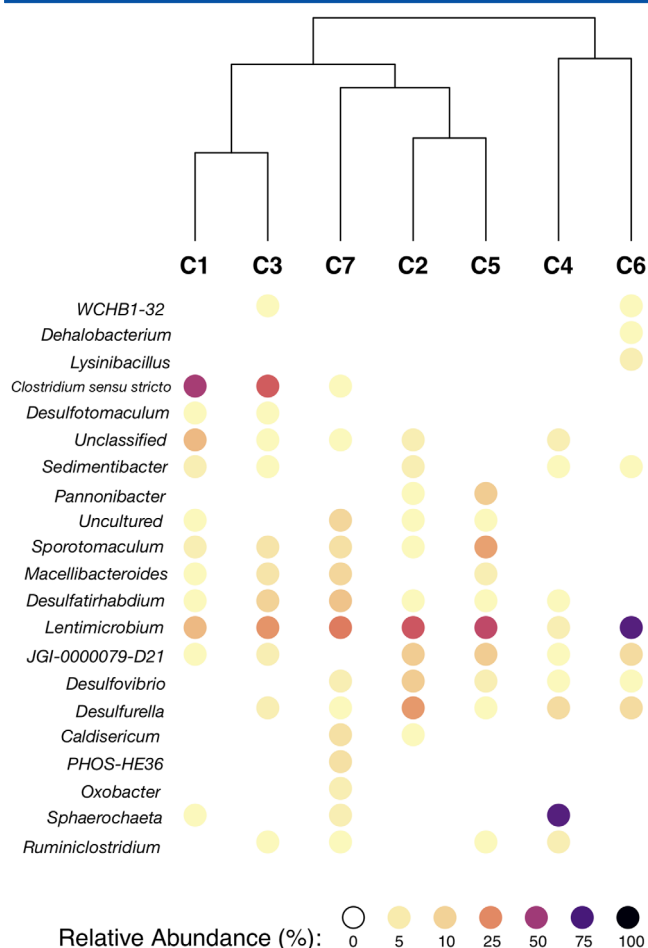
Figure 4. Dendrogram based on relative abundances of the 21 OTUs, at the genus level, obtained from the seven consortia (C1-C7).

2% of the sequences, whereas *Desulfatirhabdium* (delta-Proteobacteria) was present in six consortia (91–93% similarity), with relative abundances between 2 and 13%. Sequences 94–96% similar to *Desulfurella* (delta-Proteobacteria) were found in all of the consortia except in Consortium 1, the relative abundance of sequences was between 2 and 22%.

DISCUSSION

Here, we report the enrichment and cultivation of seven sulfate-reducing microbial consortia able to consume acetate coupled to sulfate reduction at acidic pH. The microbial communities thriving in these enrichments carried out sulfate reduction, for over a year, in successive transfers using lactate or glycerol as the substrates. We pursued sulfate-reducing consortia free of sediment to avoid the 'endogenous noise' that the sediment may cause in their characterization and further studies with them.

The percentage of substrate used to perform sulfate reduction confirmed the main function of the consortia (Table 2). Although the consortia came from the same source of inoculum (sediment) and despite using the same substrate in six of them (lactate, consortia 1–6), each consortium showed different consumption rates, denoting the presence of distinct active members in each community, in agreement with their composition (Fig. 4), diversity indexes (Table S2) and PCA (Fig. S5). This result may be due to the unpredictable processes shaping the communities, such as random dispersal and stochastic

drift, as these forces have been identified to cause some systems to exhibit divergent communities when culturing microorganisms from a heterogeneous source, such as sediments or soils.[25, 26]

During the course of each transfer, all the consortia presented the same tendency to increase the pH gradually, from the corresponding initial pH 3 or 4 to values close to neutrality (Fig. S3). This fact is related to the conversion of a strong acid such as sulfuric acid to a weak acid like hydrogen sulfide and the CO_2 produced from microbial metabolism that in turn contribute to the alkalinity of the system and increment of pH.[27, 28] Therefore, if sulfate reduction occurs, the drift of the pH is unavoidable in batch assays and the initial conditions (pH and substrate) have a strong influence on the functional traits (consumption/production rates) of the communities developed under such conditions.[18]

We also observed that acetate accumulated and then consumed when the pH reached a value close to 5.0; at this pH, only 35% of acetic acid will remain undissociated, contributing to decreasing the potential toxicity of this organic acid (Figs 1, 2 and S3). Possibly, when reaching pH 5, acetotrophic SRM could have coupled the oxidation of acetate with sulfate reduction (Fig. 2). In this study, the consortia were cultivated at initial pH 4 or 3 (Table 1), which in principle constrained the cultures fed with lactate; it is well-known that organic acids (lactic and acetic, among others) are inhibitory at low pH because the undissociated form predominates and can cross the cell membrane lowering the intracellular pH.[29] The amount of the undissociated species depends on the dissociation constants; the pKa of lactic acid is 3.08 and for acetic acid is 4.76.[30] Therefore, in the experiments initiated at pH 4 or 3, the undissociated species of lactic acid amounted to 42% or 87%, respectively. In the case of undissociated acetic acid, the percentages were higher (84% at pH 4, 98% at pH 3). Most probably, these high percentages of acetic acid prevented the cultures from succeeding when we used acetate as the sole substrate. Sánchez-Andrea *et al.*[10] reported the inhibition of the acidophilic sulfate reducer *Desulfosporosinus acididurans* strain D with 5 mmol L^{-1} lactic acid at pH 5, whereas nonionic substrates (glycerol, H_2 and methanol) allowed sulfate-reduction at pH 4.0. Given that glycerol is nontoxic at acidic pH, because it does not ionize, this substrate has been used successfully to obtain sulfate-reducing consortia from natural environments;[10, 31, 32] nevertheless, the cultures obtained do not consume acetate.

We identified a range of pHs at which each consortium performed sulfate reduction (Table 2 and Fig. 3). All of the consortia showed the highest rates of sulfide production in a range of pH predominantly acidic (i.e. between 3 and 6), indicating that the enrichment technique was appropriate to obtain cultures with reproducible activity in a wide range of pH values. According to the previous classification of acidophilic microorganisms,[32] all of the consortia obtained in the present work could be considered as moderately acidophilic because the communities exhibited sulfate-reducing activity at pH <4. Overall, the performance of the seven consortia was very reproducible at acidic pH, which shows the robustness of the microbial communities; the consortia also consumed acetate, making them an asset for further application in the treatment of acidic effluents that contain metals. As expected, the structure of the consortia was not only composed of SRM and also included fermenters and chemoheterotrophs, in agreement with previous reports when enriching SRM from marine sediments or wastewater treatment reactors.[33, 34]

The majority of the consortia contained ≈2–9% of the sequences related to thus-far noncultivable microorganisms. The sequences related to known species were 80–99% similar to

their closest relative, denoting the relevance and potential novelty of some of the microorganisms in the consortia. Most of the fermenters had the lowest percentage of similarity 80%, highlighting their novelty.

In all of the consortia, at least one SRM was present in the community, and their global relative abundance was low (<17%), concurrently with previous observations in sulfate-reducing communities enriched from peatlands where SRM were present in low abundances.[35] Regarding the SRM found in the consortia, members of *Desulfovibrio* can incompletely oxidize a wide variety of substrates including lactate, ethanol and, in a few, glycerol.[24] They also can use hydrogen as electron donor, which was possibly produced by the fermenters present in the consortia. Microorganisms resembling *Desulfovibrio* could be responsible for the initial consumption of lactate or glycerol in the consortia leaving the residual acetate for other microorganisms able to consume it, such as *Desulfotomaculum* or *Desulfatirhabdium*. Some members of the genus *Desulfotomaculum* (Firmicutes) can degrade a great variety of simple organic compounds, including acetate, formate, ethanol, lactate and glycerol.[36] The genus *Desulfotomaculum* includes spore-forming microorganisms that enable them to survive and grow in habitats that exhibit desiccation periods and low pH.[37] This characteristic may explain their presence in the consortia because the primary inoculum (sediment) was retrieved from a semi-arid zone. Microorganisms resembling *Desulfatirhabdium* could be the main contributors to the sulfate-reducing activity in most of the consortia because they are classified as complete oxidizers that can use a wide variety of long- and short-chain fatty acids, including acetate.[38] The draft genome of *Desulfatirhabdium*, reconstructed from a metagenome, includes heavy metal and acid resistance traits that could be relevant for AMD remediation.[39]

Fermentative bacteria are ubiquitous in sulfate-reducing communities, and bacteria of the genera *Lentimicrobium*, *Clostridium*, *Sphaerochaeta*, *Sedimentibacter*, *Ruminiclostridium*, *Sporotomaculum* and *Macellibacteroides*, may compose anaerobic microbial communities. All of them gain energy from the fermentation of complex organic matter and most probably played a key role in providing hydrogen and acetate to sulfate reducers.[40–42] For instance, *Clostridium* and *Desulfovibrio* coexisted in mixed sulfidogenic cultures and cooperated in the resistance of heavy metals like Cu, Zn and Fe.[43]

Overall, the performance of the seven consortia showed that the successive transfer approach was appropriate to develop stable cultures of sulfate reducers from environmental samples (i.e. sediments) with lactate or glycerol as substrates at low pH (3 or 4). Despite the fact that obtaining the consortia was time-consuming (245 days), after five successive transfers, the cultures were devoid of the original sediment and this allowed us to corroborate the cultivability of the consortia and confirm that the sulfate-reducing activity remained. Our results showed that although the enrichments were cultivated at the same initial conditions, each one of the consortia turned out to be unique, as confirmed by the molecular analysis. These consortia, retrieved from the same source, represent an opportunity to use them as model communities that could help to understand the complexity of the natural community. Also, the value of the consortia is in their potential biotechnological application, given the reproducibility of the sulfate-reducing activity at acidic pH.

CONFLICT OF INTEREST

The authors declare that they not have any conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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