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A bacterial consortium isolated from an Icelandic fumarole displays exceptionally high levels of sulfate reduction and metals resistance

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ABSTRACT

The soils of three fumaroles and one mining site, all with high metal content, were surveyed for the presence of metal-resistant sulfate-reducing bacteria and their potential application in the bioremediation of acid mine drainages. By means of selective soil enrichments a bacterial consortium was isolated from an Icelandic fumarole that displayed very high sulfate reduction in the presence of a mixture of 0.75 g/L of Fe, 0.20 g/L of Zn and 0.080 g/L of Cu. Under these conditions the bacterial consortium reduced 91% of the added 3.9 g/L of sulfate after 28 days, precipitating 100% of the Fe, 96% of the Zn and 97% of the Cu during the same time. Both total bacterial numbers and numbers of culturable sulfate-reducing bacteria remained unchanged when grown in media containing metals, suggesting low or absent inhibitory effects of the metals on the bacterial consortium. PCR-DGGE profiles of the sulfate reducing bacterial communities obtained from the Icelandic fumarole sample showed that bacterial diversity decreased significantly after metal addition: from the original 12 ribotypes only two were detected in the metal-tolerant culture. Phylogenetic analysis of 16S ribosomal RNA gene sequences revealed that these two ribotypes were affiliated with the genera *Clostridium* and *Desulfovibrio*, with *C. subterminale, C. pascui, C. mesophilum and C. peptidovorans and D. desulfuricans* identified as their closest relatives.

1. Introduction

Acid mine drainages (AMD) are contaminated water bodies characterized by high concentrations of metals and sulfate and very low pH [1]. In Southern Portugal, AMD produced by abandoned mines located at the Iberian Pyrite Belt damage the adjacent riverine ecosystems through contamination of the aquatic biota and surrounding soils [2,3]. Although every mine is unique in terms of its AMD potential [1], the main AMD heavy metals in this region are usually Fe, Cu and Zn in concentrations of e.g. 500–1260 mg/L for Fe, 50–69 mg/L for Cu and 110–264 mg/L for Zn for AMD produced by the São Domingos and Aljustrel mines, respectively [4,5]. Sustainable and low cost bioremediation technology based on the activity of sulfate-reducing bacteria (SRB) has been considered one of the best alternatives to chemical treatment when mitigating environmental contamination caused by AMD [6]. Recent efforts have been made to find suitable SRB inocula for the remediation of AMD in Southern Portugal [4,7]. SRB are chemoorganotrophic, obligate anaerobic bacteria that use sulfate as a terminal electron acceptor for respiration processes during which the sulfate is reduced to hydrogen sulfide. When metal ions are present they react with biogenic sulfide to form sparingly soluble metal sulfides [8] and the use of anaerobic sulfate reduction by SRB has been reported in the treatment of a variety of sulfate-containing industrial and mining effluents [9,10]. Usually SRB display only a moderate tolerance to heavy metals (for *Desulfovibrio* species approximately between 10 μM and 10 mM [11]). The toxicity of different heavy metals to SRB has been summarized by Utgikar et al. [12] and Martins et al. [4]. The mechanisms of metal toxicity are varied, but ultimately result in the denaturation and inactivation of enzymes, and in the disruption of cell organelles and membrane integrity [8,13,14]. Metal tolerance displayed by SRB is mainly a secondary outcome of their metabolism due to metal sequestration as metal sulfides, but enzymatic reduction of heavy metals may also occur [11]. One way of increasing metal tolerance of SRB is to use mixed cultures since these tend to exhibit an increased capacity to perform sulfate reduction in the presence of higher metal concentrations when compared to pure cultures [11,15]. Alternatively, metal contaminated sites have often been surveyed as a potential source of SRB displaying naturally higher metal tolerance [16–19]. However, in these latter studies SRB were usually only a minor fraction of the isolated metal-tolerant bacteria in these soils [16–19]. In this con-

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text, we explored four metal-rich environments (three of which new for this purpose) for the presence of bacterial consortia displaying a naturally high resistance to the metals Fe, Cu and Zn, common in Portuguese AMD. The aim of this work was to obtain a more efficient inoculum for AMD bioremediation in Southern Portugal.

2. Materials and methods

2.1. Sample sources and sampling procedure

All investigated soils were collected as cores (four replicates per sampling site) from the upper 5.5 cm layer using a sampling ring (5.5 cm height × 5.0 cm diameter). Rings were sealed and transported at room temperature in the dark. Samples were collected from 4 different sites with high metal content:

(a) São Domingos, Portugal (37°37′13″N, 7°30′29″W): the soils from the margin of the São Domingos stream, which receives AMD discharges from an abandoned mine with the same name, previously reported to be metal contaminated (As, Cu, Fe and Zn) [3,20,21]. Samples were collected twice during early spring at a temperature of approximately 18 °C. The pH of the soil samples was 3.6.
(b) Hveragerõi, Iceland (64°0′33″N, 21°12′57″W): soil surrounding a fumarole in the hydrothermal area 10 km southeast of the Hengill volcano. Samples were taken in early spring at a temperature of approximately 0 °C. The pH of the soil samples was 5.0.
(c) Krýsuvík, Iceland (63°56′28″N, 22°06′27″W): soils surrounding a fumarole in a geothermal area at the Southern coastline of the Reykjanes peninsula. Samples were taken in early spring at a temperature of approximately 0 °C. The pH of the soil samples was 3.4.
(d) Furnas do Enxofre, Terceira Island, Azores, Portugal (38°44′36″N, 27°14′53″W): soils surrounding the fumaroles in the geothermal area Furnas do Enxofre, located at the volcanic centre of the island. Samples were taken twice in early fall at a temperature of 20 °C. The pH of the soil samples was 5.5.

The soils of the sampling sites in Iceland and on Terceira Island contain high concentrations of naturally occurring metals due to hydrothermal activity, whereas the high metal concentrations in the soils of São Domingos were of anthropogenic origin. For enrichment experiments, samples were processed immediately after arrival at the laboratory. Samples for DNA extraction were frozen at −80 °C prior to processing.

2.2. Multielemental analysis of the soils

Samples used for chemical analysis were air-dried, crushed to pass through a 2 mm sieve, and quartered to obtain representative sub-samples, which were ground to <0.1 mm in an automatic agate mill. Total digestion of the sub-samples was performed with a mixture of 14 M HNO_3, 28 M HF and 9 M $HClO_4$ (2:3:1) and heated to 90–100 °C for 24 h in Teflon digestion vessels. The concentration of the different metals was determined at the Central Research Services of the University of Huelva by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES Yobin-Ybon Ultima2). Multi-elemental standard solutions prepared from single certified standards supplied by SCP SCIENCE were used for calibration. Certified Reference Material (SRM-1640 NIST freshwater-type and inter-laboratory standard IRMM-N3 wastewater test material, European Commission Institute for Reference Materials and Measurements) were also analysed. Detection limits were calculated as the average and standard deviations from 10 blanks. Detection limits were: 200 μg/L for Fe; 50 μg/L for Zn; 5 μg/L for Cu; 2 μg/L for As and 1 μg/L for Co, Cr, Ni and Pb.

2.3. SRB enrichment

For the enrichment of SRB from the soil samples, Postgate B medium [22] was used supplemented with 0.03 g/L of the redox indicator resazurine and 7.8 g/L sodium lactate. The COD of the culture medium was 1.52 g/L O_2.

Since the SRB isolated should ultimately contribute to improve AMD remediation strategies in Southern Portugal, all enrichment and subsequent metal tolerance tests were carried out in the light and at 21 ± 3 °C because this temperature is an approximate average for the conditions prevailing in that region throughout the year. Replicate soil cores (about 50 g) from São Domingos, Hveragerõi, Krýsuvík and Furnas do Enxofre were placed in a 500 mL Schott flask with 400 mL of medium, closed with rubber rings to avoid oxygen diffusion, and incubated for 28 days. Growth of SRB was indicated by loss of the resazurine red colour (indicating the consumption of atmospheric O_2), the formation of an insoluble ferrous sulfide precipitate and the development of the typical H_2S odour. Soil-free SRB cultures were produced from these flasks as follows: the flasks were vigorously shaken for 2 min, opened and 10 mL of soil cultures were collected. The samples were centrifuged at 4000 rpm for 10 min, washed twice with Ringer's solution (6 g/L NaCl, 75 mg/L KCl, 0.1 g/L $CaCl_2$; 0.15 g/L $NaHCO_3$), the sediment was resuspended in 10 mL of fresh Postgate B medium and inoculated in duplicate into 120 mL serum bottles containing 90 mL of Postgate B medium. The bottles were sealed with butyl rubber stoppers and aluminium crimp seals, and oxygen was removed from the gaseous phase with nitrogen. The soil-free cultures were incubated for a further 28 days. All further cultures were produced by the same procedure, except that the 10 mL were collected with a sterile syringe through the butyl rubber stopper of the serum bottles.

2.4. Metal tolerance assessment

The soil-free bacterial cultures described in Section 2.3 were tested for their ability to grow and reduce sulfate when grown in Postgate B medium amended with Fe, Cu and Zn. All assays were performed in duplicate in 120 mL glass serum bottles in the light and at room temperature (21 ± 3 °C). All inoculations were performed as described in 2.3. $FeSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$ and $CuSO_4{\cdot}5H_2O$ were simultaneously added to the culture medium prior to sterilization to a final concentration of 0.5 g/L of Fe, 0.175 g/L of Zn and 0.08 g/L of Cu (referred to as L1 metal level, corresponding to the average concentrations of these metals in the AMD produced by the São Domingos mine). Total sulfate content of the mixture was 3.2 g/L. For the second experiment, the bacterial cultures displaying the highest sulfate reduction efficiency in the presence of the metals added were incubated in higher metal and sulfate concentrations, consisting of 0.75 g/L of Fe, 0.20 g/L of Zn, 0.08 g/L of Cu (referred to as L2 metal level) and 3.9 g/L of sulfate. For each assay a positive control and two negative controls were always carried out in duplicate. The positive control consisted of the tested culture inoculated in medium without metals; the negative controls consisted of the sterile Postgate B media, one with, and the other without, metal addition. Samples were taken at least weekly during 28 days with a sterile syringe and processed as described below.

2.5. Chemical analysis

Liquid samples were centrifuged at 4000 rpm for 10 min and the supernatant was transferred to clean tubes and used for mea-

surements. Redox potential (Eh) and pH of the culture media were measured immediately after sample collection and centrifugation using a pH/E Meter (GLP 21, Crison). The sulfate concentration was measured by UV-vis spectrophotometry (Hach-Lange DR2800) using the Sulfa4 method (Hach-Lange). For determination of the dissolved metals (Fe, Cu and Zn) the samples were acidified with nitric acid to the appropriate pH and analysed by atomic absorption spectroscopy using a Shimadzu AA-680 model spectrometer.

2.6. Bacterial enumerations

The concentration of SRB in the cultures and soil samples was determined by cultivation according to the three-tube most probable number (MPN) assay with serial dilutions in Postgate E medium [22]. Culture samples were serially diluted with Ringer's solution and used directly for MPN enumerations. For the soils, a core of five grams was resuspended in 45 mL of 0.1% sodium pyrophosphate containing 0.1% Tween 80 in Erlenmeyer flasks and shaken for 5 min at 200 rpm. The supernatant was appropriately diluted with Ringer's solution and used for determination of the MPN. The tubes were incubated at room temperature ($21 \pm 3\,^{\circ}C$) for 5 days and the formation of black colonies indicated the presence of SRB.

Direct bacterial counts were performed by epifluorescence microscopy according to the following procedure: the bacterial dilutions used for the determination of SRB concentrations were fixed with 3.7% formaldehyde/PBS solution, filtered onto a polycarbonate filter (0.2 μm pore size, OMR International) and stained with 2 drops of 0.01% acridine orange (Merck). Bacterial cells were visualized using a Leica DMLB epifluorescence microscope equipped with a 1000-fold objective and a I3 filter (Illumination path BP450–490, dichroic mirror 510). For statistical evaluation, at least 30 microscopic fields (40.85 by 40.85 μm) and a minimum of 1000 cells were chosen randomly and enumerated. The experiment was performed in triplicate.

2.7. Whole community DNA extraction

DNA was extracted from the original soils and from the bacterial cultures with the Ultraclean™ Soil DNA Isolation Kit (Mo BIO) following the manufacturer's instructions. The starting material used in the extraction procedure was 0.5 g of soil and microbial pellets obtained by centrifugation (3 min at 10,000 rpm) from 3 mL of the cultures. Extractions were performed in duplicate. For the soil samples, a core of about 5 g was mixed thoroughly and small portions were transferred randomly to the extraction tubes in a total of 0.5 g. At the end of the extraction, DNA from each source was pooled for further use.

2.8. Molecular fingerprinting analysis

Changes in the composition of the original bacterial communities after metal addition were monitored by PCR-DGGE.

2.8.1. PCR amplification

PCR amplification (T1 Thermocycler, Biometra) of the bacterial 16S rRNA gene from total community DNA was performed using the primer pair F984GC/R1378 according to the protocol of Heuer et al. [23] (dimethyl sulfoxide was replaced by 4% acetamide). The reaction mixture contained: 1 μL template DNA (ca. 20 ng), 5 μL of 5x GoTaq Flexi Buffer, 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 4% (w/v) acetamide, 100 nM of each primer and 2 U/25 μL GoTaq polymerase (Promega). A GC-rich sequence (GCclamp) was attached to primer F984GC to prevent complete melting during separation in the gel [24]. The amplification protocol was as follows: 5 min denaturation at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 53 °C, and 2 min at 72 °C and extension step at 72 °C for 10 min.

2.8.2. DGGE

DGGE analysis followed the methodology described by Heuer et al. [25] using a TGGE/DGGE System (TTGE-2401, CBS Scientifics). The gradient was composed of 32–60% denaturants (7 M urea and 40% formamide) and 20–30% acrylamide. PCR products were loaded side by side onto a gel, together with duplicates of a control 16S rRNA gene marker composed of GC-clamped fragments (positions 984–1378) of 11 known bacterial strains with different electrophoretic motilities to control the quality and accuracy of the run. The gel was run in duplicate in 1x Tris-acetate-EDTA buffer at a constant voltage of 150 V for 15 h at 60 °C and silver stained as described by Heuer et al. [25].

2.9. Molecular characterization of the metal-tolerant bacterial cultures

The bacterial culture which removed the highest percentage of sulfate in the presence of L2 concentrations of the tested metals (0.75 g/L Fe, 0.2 g/L Zn, 0.08 g/L Cu) was characterized by cloning and sequencing assays of the 16S rRNA gene.

2.9.1. PCR amplification of the 16S rRNA gene

All PCRs were performed in a T1 Thermocycler (Biometra). For the amplification of the 1.5 kb fragment of eubacterial 16S rRNA gene, universal primers BAC-8F and BAC-1492R [26] were used. The reaction mixture (25 μL) contained: 1 μL DNA, 5x Promega buffer, 1 mM $MgCl_2$, 200 μM dNTPs, 200 μM of each primer and 0.625 U of *GoTaq* DNA Polymerase (Promega). Amplification conditions were: 3 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C. Final extension at 72 °C was 5 min. After amplification 5 μL of PCR products were loaded onto a 1% (w/v) agarose gel and run at 120 V for 30 min in order to confirm amplification and fragment size. The DNA was visualized using ethidium bromide staining and transilluminatior.

2.9.2. Cloning assays and RFLP analysis

PCR products were purified (EZNA Cycle Pure Kit, Omega biotek) and ligated into the cloning vector pGEM®-T Easy (Promega) followed by transformation into competent host cells (*E. coli* XL1-blue), according to the manufacturer's instructions. Individual colonies were screened by direct PCR amplifications with the SP6 and T7 primers according to the manufacturer's instructions. RFLP analysis of the inserts was performed using the restriction enzymes HhaI and MspI (Promega) for 2.5 h at 37 °C using buffer C (Promega). Restriction fragments of the digested PCR products were separated (1.5 h, 80 V) in a 2% (w/v) TAE agarose gel. The DNA was visualized using ethidium bromide staining and transillumination.

2.9.3. Sequence analysis of the cloned inserts

The clones exhibiting a unique restriction pattern were selected for 16S rRNA gene sequencing at CCMar (Centro de Ciências do Mar, Universidade do Algarve) using the Genetic Analyzer 3130xl (Applied Biosystems) according to standard procedures. The obtained sequences were edited with the Biological Sequence Alignment Editor (Bioedit). Closest matches to the sequence queries were searched using the Basic Local Assignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST).

Table 1
Most common heavy metals in the investigated soils. Values are means of duplicate samples.

Metal content (mg/Kg)								
Sampling site	As	Co	Cr	Cu	Fe	Ni	Pb	Zn
Hveragerõi	0	65	183	135	92,108	80	0	115
Krísuvyk	0	61	203	102	149,253	91	0	85
Furnas Enxofre 1	0	19	9	8	153,596	5	85	468
Furnas Enxofre 2	0	31	31	7	110,716	1	62	96
São Domingos 1	210	21	79	524	119,430	32	314	413
São Domingos 2	311	20	78	1,037	80,816	26	415	927

3. Results

3.1. Multielemental analysis of the soil samples

As shown in Table 1, all analysed samples had a high Fe content. The Furnas do Enxofre samples contained the lowest overall metal concentrations while São Domingos samples displayed the highest total metal concentration. As for the AMD metals tested, sample 2 collected near the mine of São Domingos had the highest concentrations of Cu and Zn. The soil samples from the Hveragerõi fumarole also contained non-anthropogenic high levels of Cu and Zn and were similar in metal composition to the Krysuvík soil samples.

3.2. Enrichment of SRB from the soil samples and selection of metal-tolerant cultures

3.2.1. Sulfate reduction performance of the enrichment cultures

When incubated in metal-free Postgate B medium the enrichment cultures derived from the soil samples of the fumaroles at Hveragerõi and Furnas do Enxofre, and from the São Domingos mining site, displayed sulfate reducing activity (Fig. 1) but differed in the amount of sulfate consumed. The Hveragerõi cultures reduced 99% of the initial 3.2 g/L sulfate after 28 days of incubation, while São Domingos and Furnas do Enxofre reduced only 46% and 41% of total sulfate, respectively, after the same period of time. The cultures derived from the soil samples of the Krysuvík fumarole did not display sulfate-reducing activity (1%) and no bacterial growth was observed: these samples were not tested any further. The Hveragerõi, São Domingos and Furnas do Enxofre cultures were exposed to Fe, Zn and Cu concentrations equivalent to those present in Portuguese AMD (L1 metal level). In the presence of metals the Furnas do Enxofre cultures lost their sulfate reduction activity (1.5% sulfate reduction after 28 days, Fig. 1) and metal concentrations in the medium remained unchanged. The São Domingos cultures displayed low sulfate-reducing activity in the presence of L1 metals (12.0% after 28 days). Unlike the other samples, the Hveragerõi cultures kept their full sulfate-reducing ability, reducing 99.6% of total sulfate after 28 days in Postgate B medium containing L1 metals.

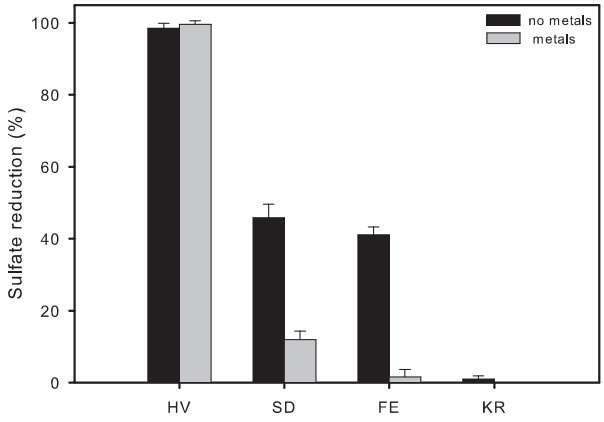

Fig. 1. Sulfate reduction in the enrichment cultures derived from different soils after 28 days of incubation at room temperature (21 ± 3 °C) with and without L1 metals. In the non-inoculated media sulfate concentrations remained unchanged (data not shown). Error bars represent the mean unsigned error. HV: Hveragerõi, SD: São Domingos; FE: Furnas do Enxofre; KR: Krisuvik.

3.2.2. Variation of cell numbers during the screening assay

No SRB were detected in the culture obtained from the Krysuvík soil (Table 2) which is in accordance with the lack of sulfate-reducing activity in the assays (Fig. 1). As for the enrichment cultures derived from the Hveragerõi, São Domingos and Furnas do Enxofre soil samples, the culture displaying the highest sulfate reduction performance (Hveragerõi, Fig. 1) also contained the highest SRB concentrations after 28 days (7.6×10^6 CFU/mL, Table 2), followed by São Domingos (3.3×10^6 CFU/mL) and Furnas do Enxofre (5.4×10^4 CFU/mL). Total bacterial concentrations enumerated in the same cultures were higher (2.1×10^7 cells/mL in Hveragerõi, 6.1×10^6 cells/mL in São Domingos and 4.8×10^5 cells/mL in Furnas do Enxofre). MPN enumerations of SRB in the soil samples showed the same tendency as in the enrichment cultures: Hveragerõi soil samples were richer in culturable SRB (1.3×10^3 CFU/g soil), than São Domingos with 9.1×10^2 CFU/g soil and Furnas do Enxofre with 1.1×10^2 CFU/g soil. As for the total bacteria enumerated in the soil samples, while the Hveragerõi

Table 2
Numbers of SRB and total bacterial cell counts in the Hveragerõi, São Domingos, Furnas do Enxofre and Krísuvyk samples at different stages of the experiment. Enumerations were carried out at room temperature (21 ± 3 °C).

	Sampling sites				
		Hveragerõi	São Domingos	Furnas Enxofre	Krísuvyk
Soil samples	SRB (CFU/g)	$1.3 \pm 0.11 \times 10^3$	$9.1 \pm 0.12 \times 10^2$	$1.1 \pm 0.22 \times 10^2$	0
	Direct counts (cells/g)	$1.92 \pm 0.26 \times 10^8$	$2.7 \pm 0.22 \times 10^8$	$3.6 \pm 0.41 \times 10^6$	$5.4 \pm 0.3 \times 10^5$
Enrichment cultures	SRB (CFU/mL)	$7.6 \pm 0.3 \times 10^6$	$3.3 \pm 0.21 \times 10^6$	$5.4 \pm 0.29 \times 10^4$	0
	Direct counts (cells/mL)	$2.1 \pm 0.04 \times 10^7$	$6.1 \pm 0.07 \times 10^6$	$4.8 \pm 0.11 \times 10^5$	–
Liquid cultures with L1 metals	SRB (CFU/mL)	$5.3 \pm 0.42 \times 10^6$	0	0	–
	Direct counts (cells/mL)	$1.6 \pm 0.04 \times 10^7$	–	–	–
Liquid cultures with L2 metals	SRB (CFU/g)	$1.6 \pm 0.55 \times 10^7$	–	–	–
	Direct counts (cells/mL)	$1.1 \pm 0.03 \times 10^7$	–	–	–

–: Not determined. L1: 0.5 g/L Fe, 0.175 g/L Zn, 0.080 g/L Cu; L2: 0.75 g/L Fe, 0.200 g/L Zn, 0.080 g/L Cu.

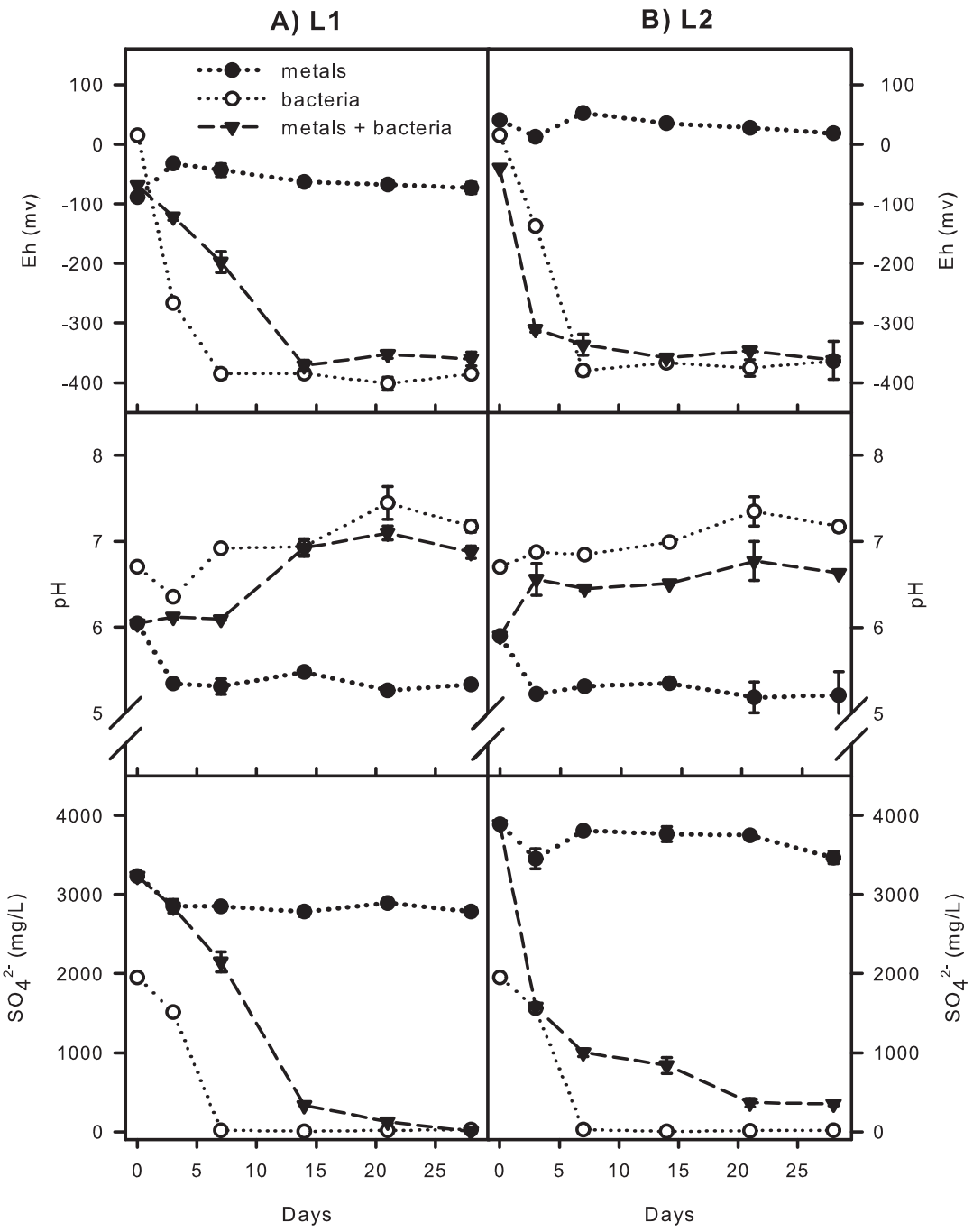

Fig. 2. Variation of the Eh, pH and SO_4^{2-} concentration in the Hveragerõi cultures incubated with L1 (A) and L2 (B) metals at room temperature (21 ± 3 °C). Error bars represent the mean unsigned error. There was no variation of any parameter in the negative control (non-inoculated Postgate B medium). L1: 0.5 g/L Fe, 0.175 g/L Zn, 0.080 g/L Cu; L2: 0.75 g/L Fe, 0.20 g/L Zn, 0.080 g/L Cu.

and São Domingos soil samples contained 10^8 cells/g soil the Furnas do Enxofre and Krísuvyk soil samples contained around 100- to 1000-fold fewer bacteria (10^6 cells/g soil and 10^5 cells/g soil, respectively).

No effect of metal addition on SRB cell counts was detected for the Hveragerõi cultures: after 28 days incubation with L1 metals, the Hveragerõi culture reached approximately the same concentration of culturable SRB (5.3×10^6 CFU/mL) as the metal-free culture after the same period. Contrary to the Hveragerõi cultures, cultivation of SRB after incubation in medium with L1 metals resulted in reduced cell counts for the São Domingos and the Furnas do Enxofre samples (Table 2), with only residual sulfate reducing activity being detected (Fig. 1). As a result of the screening assay, the Hveragerõi cultures were considered as having potential utility in bioremediation strategies and all further assays were conducted only with the L1 metal adapted Hveragerõi cultures.

3.3. Chemical parameters during metal adaptation

When the Hveragerõi culture was first inoculated in the L1 metal medium the redox potential (Eh) decreased to −372 mV after 14 days, remaining stationary until the end of the assay (Fig. 2A, Eh). This decrease was similar to the one observed in the metal-free cultures. However, when the L1 adapted Hveragerõi culture was subsequently inoculated in medium containing higher metal concentration (Fig. 2B, Eh) the Eh decreased faster, reaching −306 mV

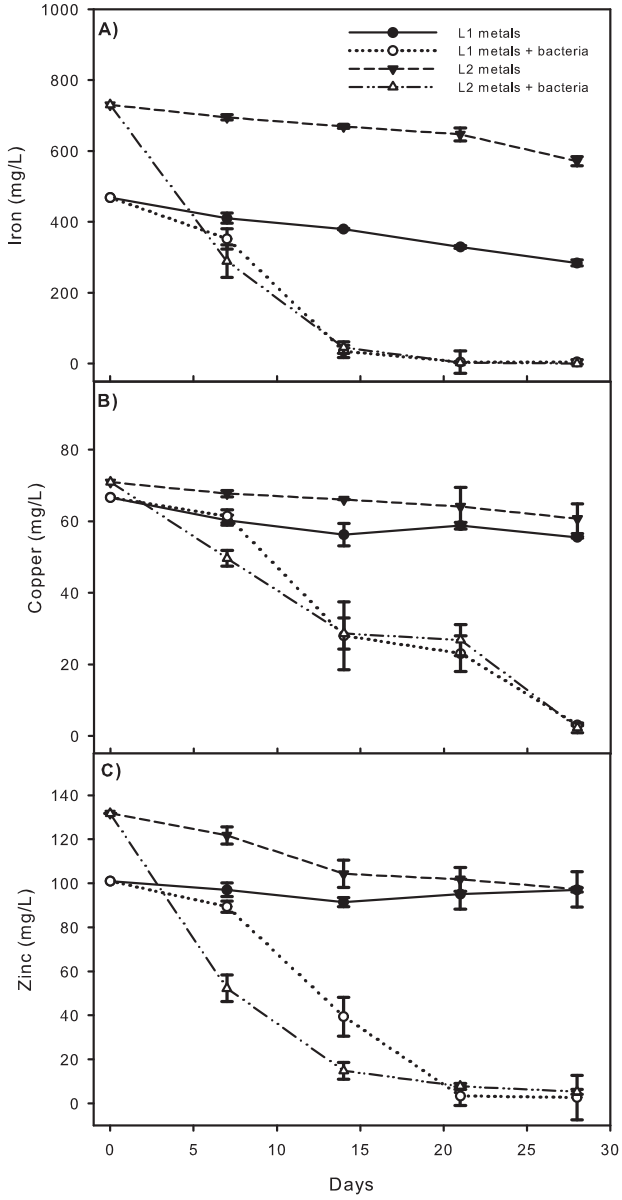

Fig. 3. Removal of Fe (A), Cu (B) and Zn (C) in the Hveragerõi cultures incubated in L1 and L2 metals at room temperature (21 ± 3 °C). L1: 0.5 g/L Fe, 0.175 g/L Zn, 0.080 g/L Cu; L2: 0.75 g/L Fe, 0.20 g/L Zn, 0.080 g/L Cu.

after only 3 days of incubation. As a result of metabolic activity of the SRB, the pH of the culture media increased with time in both cultures incubated with L1 and L2 metals (Fig. 2A and B, pH). The pH increase started after 7 days in the cultures incubated with L1 metals (from pH 6.0 to pH 7.0) and after 3 days in the subsequent cultures incubated with L2 metals (form pH 6.0 to pH 6.5). During incubation, sulfate reduction kinetics differed between treatments as shown in Fig. 2A and B: for the cultures incubated with L1 metals sulfate removal was highest between the 7th and the 14th days of incubation (average of 260 ± 32 mg/L of sulfate removed per day) whereas for the subsequent cultures incubated with L2 metals the sulfate removal was highest between day 0 and day 3 with an average of 770 ± 67 mg/L of sulfate removed per day. After 28 days of incubation only vestigial sulfate was detected in both L1 and L2 metal adapted cultures and Fe was removed by 99–100% (Fig. 3A), Cu by 95–97% (Fig. 3B) and Zn by 96% (Fig. 3C) in the L1 and L2 metal amended cultures, respectively.

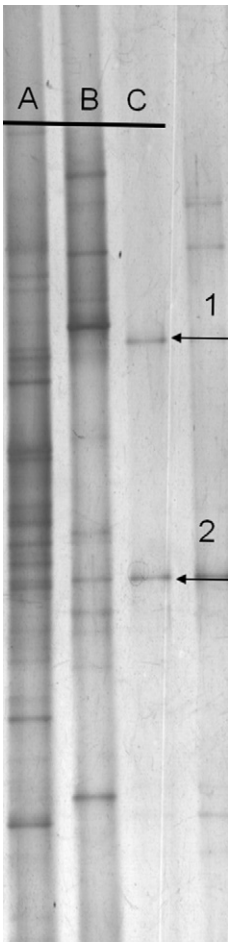

Fig. 4. 16S rRNA gene PCR-DGGE profiles of the bacterial communities from the Hveragerõi samples. Lane A: original bacterial community in the soil; lane B: bacterial community after metal-free culture enrichment, lane C: bacterial community after adaptation to L2 metals.

3.4. Changes in bacterial numbers and diversity in response to metal adaptation

During L1 and L2 metal adaptation total bacterial cells in the Hveragerõi cultures always reached a constant mean value of 10^7 cells/mL after 28 days, whereas the final concentration of culturable SRB increased from 7.6×10^6 CFU/mL (metal-free culture) to 1.6×10^7 CFU/mL (L2 adapted culture, Table 2). The fraction of culturable SRB was therefore 36% of total bacteria in the culture without metals, whereas in the L2 culture it was 145%. Moreover, and taking into account that inoculations were always performed with 10 mL of the previous culture in a total of 100 mL of medium, SRB concentrations at the beginning of incubation in L1 and L2 metal media averaged 10^5 CFU/mL, whereas they reached 10^6 CFU/mL and 10^7 CFU/mL, respectively, after incubation.

As for the changes in bacterial diversity, analysis of the DGGE profiles obtained by amplification of 16S rRNA gene fragments (Fig. 4) showed the presence of at least 23 bacterial ribotypes in the original soils (lane A). At least 12 ribotypes were still recovered after cultivation in Postgate B medium (lane B). Exposure to high doses of Fe, Zn and Cu led to a considerable decrease in bacterial diversity and only two bands in lane C (corresponding to the DNA extracted from the L2 metal cultures) could be detected. The bacterial group corresponding to band 1 in lane C was not present in detectable amounts in the original soils (lane A), indicating that it was selectively enriched by exposure to metals. Band 2 in lane C

Table 3
BLAST results obtained for the 16S rRNA gene sequence alignments of the metal-resistant Hveragerõi bacterial consortium.

No. of clones with identical RFLP patterns	Sequence length (bp)	Query coverage (%)	Closest match (BLAST search)	% Identity	EMBL accession no.
8	814	100	*C. subterminale*	99	FN997583
5	763	100	*C. peptidovorans*	99	FN997584
	763	100	*C. pascui*	98	FN997584
19	812	100	*D. desulfuricans*	99	FN997585
10	649	100	*D. desulfuricans*	100	FN997586
3	547	100	*Desulfovibrio* sp.	99	FN997587
8	841	100	*C. mesophilum*	100	FN997588
10	510	100	Uncultured *Clostridium* sp. strain	98	FN997589
7	511	100	*Clostridium* sp. culture clone	99	FN997590

was detectable in the original population (lane A) and is likely to correspond to a resilient bacterial population.

3.5. Characterization of the metal adapted sulfate-reducing community

The final L2 metal adapted culture was characterized by cloning and sequencing of the 16S rRNA gene. From 90 clones randomly selected and amplified with the primers BAC-8F and BAC-1492R, 70 contained the correct insert size. RFLP analysis of the 70 16S PCR products revealed 24 restriction patterns. Representatives of each RFLP pattern group (2–5 for each group, depending on the size of the group) were chosen randomly and sequenced. Alignment of the sequences obtained revealed high redundancy of several clones. Therefore the sequences were grouped into 9 identity clusters, the members of which displaying 100% identity (Table 3). The longest sequences of each identity cluster were deposited in the EMBL Nucleotide Sequence Database under the accession numbers shown in Table 3. All sequenced clones were identified as being very closely related (98–100% genetic similarity) to two genera only: *Clostridium* (Firmicutes) and *Desulfovibrio* (δ-proteobacteria). For those clones most closely related to *Clostridium* sp., five different types were detected: *Clostridium subterminale* (99% identity with 12 sequenced clones), *C. peptidovorans* (99% identity with 8 sequenced clones), *C. mesophilum* (100% identity with 6 sequenced clones) and other *Clostridium* sp. strains (98–99% of identity with nine sequenced clones). For the clones most closely related to *Desulfovibrio* sp., eight representatives of nineteen clones sharing the same RFLP pattern were 99–100% identical to *Desulfovibrio desulfuricans* 16S rRNA gene and six out of 13 were 98–99% identical to other *Desulfovibrio* sp. In order to establish a relationship between the sequencing data and PCR-DGGE results, we applied the PCR-DGGE protocol the *Desulfovibrio* sp. and *Clostridium* sp. cloned DNA sequences described in Table 3 and compared their electrophoretic mobilities with those of the L2 metal adapted culture (Fig. 3, lane C) on a second PCR-DGGE run. The *Clostridium* sp. clones displayed the same electrophoretic mobility as band 1 in Lane C, whereas the PCR-DGGE band observed for *Desulfovibrio* sp. clones displayed identical positioning on the gel as band 2 in lane C (Fig. 4).

4. Discussion

The aim of this study was to obtain a bacterial culture with potential utility in the bioremediation of waters and effluents polluted with metals and sulfate. Metal-tolerant bacteria are likely to be isolated from soils with a high metal content [17–19]; consequently we sampled several metal-rich soils for SRB diversity. A 3-step assay procedure was conducted to determine the culture(s) with the highest potential for use in bioremediation: (1) samples of metal-rich soils were screened for SRB, (2) SRB cultures were enriched in metal-free culture media, and (3) the adaptation of the cultures to high metal and sulfate concentrations was characterized. The bacterial culture displaying the highest sulfate reduction efficiency in the presence of the tested metals was enriched from the Hveragerõi soils although total metal amounts were lower in Hveragerõi than in São Domingos. Hence, and unlike data reported in other studies [e.g. 17,18,19,27,28], no direct correspondence was observed in this study between the amounts or types of metals present in the inoculum source and the isolation of metal-tolerant SRB. Instead, there was a clear relationship between the numbers of culturable SRB originally present in the soils and the probability of isolating metal-tolerant SRB: the soil samples from Hveragerõi and all cultures derived from them contained more culturable SRB than the soils and cultures derived from São Domingos, Furnas do Enxofre and Krysuvík. This observation fits well with the main known mechanism of metal resistance for SBR—the production of sulfide. Sulfide production is a gratuitous metal resistance mechanism [29], meaning that the protection it confers to bacteria is a secondary effect and not the ultimate purpose of its production. Therefore, the amount of protection conferred will depend strongly on the amount of bacteria producing it [30]. However, it cannot be ruled out that other resistance mechanism of SRB, e.g. enzymatic metal reduction [11] may have also contributed to the high metal resistance of this consortium. In this case, a high initial population density is also beneficial for the acquirement of the resistance trait since the interactions between the initial population size and the cell death rate play a crucial role in the development of the resistant sub-population [30].

Recent studies have shown low sulfate reduction capacity of SRB in heavy metal containing media: Martins et al. [4] reported 110 days for the reduction of sulfate in the presence of 0.08 g/L of Cu and 0.15 g/L of Zn at room temperature, whereas Chockalingam and Subramian [31] used synthetic AMD containing lower concentrations of sulfate and metals (1615 mg/L of sulfate, 0.19 g/L Fe, 0.030 g/L Zn and 0.010 g/L Cu) than those used in this work and obtained sulfate reduction of 73%, together with the precipitation of 90% Fe, 89% Zn and 75% Cu after 55 days. Neculita and Zagury [32] used synthetic wastewater containing 5.5 g of sulfate and several heavy metals in batch assays containing different reactive mixtures as substrates. Sulfate removal occurred after a minimum of 90 days at room temperature. Hsu [33] reported high efficiency of sulfate removal (97–99% in 6 days) by a bacterial culture in the presence of 10 mg/L of copper and 300 mg/L of sulfate but the tested concentrations were far below those observed in our study. Especially copper and zinc are usually toxic to SRB at low concentrations (around 10 mg/L for copper [8,14,34,35] and 20 mg/L for zinc [8,34–36]). Azabou et al. [37] reported Zn precipitation as ZnS by a mixed SRB consortium at an initial concentration of 150 mg/L but inhibitory effects were detected at initial Zn concentrations of 25 mg/L. Additional cumulative toxic effects may occur if more than one metal is added to solution [38,39]. In comparison to the above mentioned works, the culture isolated from the Hveragerõi fumarole soil displayed an exceptionally high sulfate reduction performance (100% in L1 metal medium and over 91% in L2 metal medium within 28 days) in the presence of very high concentrations of three toxic AMD heavy metals. Hence this cul-

ture may help overcoming present constraints of bioremediation of AMD.

The metal adapted bacteria characterized in this study on the basis of the 16S rRNA gene sequences displayed very high similarity to members of the genera *Clostridium* and *Desulfovibrio*. Syntrophic relationships between *Desulfovibrio* sp. and *Clostridium* sp. have been described in environments without metals [40,41] but in media containing metals and sulfate only one genus was reported to be active [4,42,43]. In our study, *Clostridium* and *Desulfovibrium* species were cultivated and selected over several inoculation steps and are therefore likely to be simultaneously active which may explain the very high performance of this bacterial consortium. The special features of this mixed bacterial culture may help improve the technologies for simultaneous removal of sulfate and metals from contaminated waters.

5. Conclusions

Several metal-rich soils were surveyed for the presence of metal-tolerant SRB. From a single soil sample collected at a fumarole in the geothermal area of Hveragerõi, Iceland, a heavy metal-resistant, sulfate reducing bacterial culture was isolated that displayed high metabolic activity in the presence of high concentrations of Fe, Zn and Cu. The bacterial culture exhibited very high sulfate reduction associated with metal removal, suggesting that the consortium has potential utility in improving the efficiency of bioremediation technologies used in AMD decontamination. DNA sequencing revealed that the culture was composed of taxa from two genera: *Clostridium* and *Desulfovibrio*. A synergistic relationship between the two genera may explain the unusual biochemical features of this consortium.

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