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Sulfate Reduction, Molecular Diversity, and Copper Amendment Effects in Bacterial Communities Enriched From Sediments Exposed to Copper Mining Residues

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ABSTRACT–Sulfate–reducing bacterial communities from coastal sediments with a long–term exposure to copper–mining residues were studied in lactate enrichments. The toxicity of excess copper may affect sulfate-reducing bacterial communities. Sulfate reduction was monitored by sulfate and organic acid measurements. Molecular diversity was analyzed by 16S rRNA, dissimilatory sulfate reduction *dsrAB*, and copper translocating define here at first mention phospho type–adenosine triphosphatases (P–ATPases) *cop*–like gene sequence profiling. The influence of copper amendment was tested in these enrichments. Results showed fast sulfate reduction mostly coupled to incomplete organic carbon oxidation, and partial sulfate reduction inhibition due to copper amendment. The The 16S rRNA clonal libraries analysis indicated that δ – and γ –*Proteobacteria* and *Cytophaga*–*Flexibacter*–*Bacteroides* dominated the enrichments. The The *dsrAB* libraries revealed the presence of *Desulfobacteraceae* and *Desulfovibrionaceae* families related sequences. Copper produced significant shifts (for instance, a decrease in the relative abundance of sulfate–reducing microorganisms) in the enriched bacterial community structure as determined by terminal–restriction fragment length polymorphism (T–RFLP) profiling and multivariate analyses. Clonal libraries of *cop*–like sequences showed low richness in the enriched microbial communities, and a strong effect of copper on its relative abundance. Novel copper–P_{IB}–ATPase sequences encoding copper resistance were detected. The present study indicates that copper does not significantly affect sulfate reduction and genetic diversity of taxonomic and dissimilatory sulfate–reduction molecular markers. However, the diversity of copper resistance genetic determinants was strongly modified by this toxic metal.

Keywords–Sulfate–reduction; Coastal sediments; Sulfate–reducing bacteria; Copper; Molecular diversity Acronyms are not allowed in keywords. Spell out or replace. You can add gene after dsrAB and leave in keywords, but spell out P-ATPases.

INTRODUCTION

Bacterial sulfate reduction plays a key role in the biogeochemical cycles of carbon and sulfur. The dissimilatory sulfate–reducing bacteria (SRB) are responsible for the main part of carbon mineralization in sulfate rich natural environments, accounting for the oxidation of ≥50% of the total organic carbon in ecosystems such as marine and salt marsh sediments, and microbial mats [1,2]. Sediments constitute an important reservoir and sink for organic matter and environmental pollutants. In particular, sediments associated with mining activity residues (i.e., wastewaters, tailings) contain high sulfate and heavy metal concentrations that are transported and accumulated in water bodies. Some studies report that heavy metals inhibit dissimilatory sulfate reduction in SRB [3,4].

Sulfate reducing bacteria are ubiquitous in sediments. They can use H_2 and a variety of organic compounds (small fatty acids, alcohols, and carbohydrate monomers, among others), as electron donors [5,6]. The sulfide generation leads to the formation of insoluble metal sulfides, reducing the bioavailability of metal ions in natural ecosystems [7]. The essential sulfate respiration step, the six–electron (bi)sulfite reduction to sulfide, is catalyzed by the dissimilatory sulfite reductase enzyme encoded by the *dsrAB* genes [8]. Due to its crucial functional role, high degree of conservation and appropriate matching phylogenetic topology with 16S rRNA, *dsrAB* gene sequences have been used as suitable molecular markers for SRB [8].

The diversity and activity of SRB have been determined in several sulfidogenic systems, using cultivation and culture independent approaches. Based on 16S rRNA and *dsrAB* gene sequences, recent studies on SRB diversity have

been reported, particularly in estuarine and marine sediments, and contaminated sites polluted with organic compounds and heavy metals [9–13]. ~~That~~ These These kinds kinds of studies ~~has~~ have have provided insights on the abiotic factors that control the abundance and distribution of different groups of SRB. However, less is known about the diversity of sulfate–reducing communities in response to metal toxicity that may be important for SRB activity [4,14,15].

Microbial communities that thrive in metal–stressed conditions should have metal tolerance systems. For example, SRB are able to reduce, accumulate and tolerate a variety of metals [9,15]. Membrane cation–transporting pumps are important components of cellular detoxification systems with selective structures for metal transport [16]. The P_{IB}–type ATPases are a subgroup of P–ATPases membrane proteins, present in most organisms that transport heavy metals across biomembranes [16]. Consequently, the determination of metal specific P–ATPases diversity is also required for the understanding of microbial adaptation to metal–stressed environments.

In northern Chile, at and around Chañaral Bay (26°15' S; 69°34' W) copper mining industries discharged untreated residues for more than 60 years [17]. Between the years 1938 and 1975, the Chañaral coastline received ~~about~~ approximately approximately 150 million tons of raw copper mining flotation tailings. In 1976, the tailing discharge point was moved 10 km north of Chañaral Bay to Caleta Palito (26°15.8' S; 70°40.6' W) releasing, until 1989, more than 130 million tons of wastes [17]. In 1983, the Chañaral situation was classified by the United Nations Environmental Program as one of the most serious cases of marine contamination in the Pacific Ocean. Since 1990, Canal Palito, the actual discharge

channel releases tailings–free is this a direct quote? If not, remove markes wastewater containing up to 31.5 µM of total copper, at a rate of 200 to to 250 L/s [17]. A previous study in the area demonstrated that bacterial communities from the sediment compartment were the most impacted by copper pollution imposing a high toxic environmental pressure [18]. Hence, these sediments constitute an adequate model to evaluate the effects of copper in these and other copper–exposed bacterial communities.

The aim of ~~this work~~ the present study the present study, using samples from the Chañaral site as a model, was to study the sulfate reduction, genetic diversity, and copper amendment effects in enriched sulfate–reducing bacterial communities from sediments chronically exposed to copper, applying culture independent methods based on the use of 16S rRNA, *dsrAB* and copper P–ATPases (*cop*–like) gene sequences, as molecular markers. We limit or delete the use of personal pronouns throughout The hypothesis that chronic exposure to excess copper leads sulfate–reducing bacterial communities to tolerate toxic copper concentrations and that the presence of this metal reduces molecular diversity, was tested.

MATERIALS AND METHODS

Sampling. Sediment and water samples were taken from the mouth of Canal Palito, (26°15.8' S; 70°40.6' W). For sediment sampling, 500 g (wet wt) duplicate samples were collected from the edge of the channel in the marine–mixing zone. Samples were taken from the first 10 cm depth and immediately put in sterile polyethylene bags. At the same point, duplicate 250 ml water samples were taken in sterile glass

bottles. All samples were stored in the dark at 4°C, and rapidly transferred to the laboratory for analytical procedures.

Culture medium and microbial inocula preparation. For culture purposes the marine Postgate C medium was used [6], with some modifications. Phosphate level and pH were lowered to minimize metal precipitation as phosphates and hydroxydes; iron concentration was increased to prevent its depletion by sulfide precipitation. The pH was adjusted to 7.20±0.05 with NaOH. Lactate (45 mM) was used as energy/carbon source. One liter of medium (prepared in deionized water) contained: 3.5 g Na_2SO_4, 0.1 g KH_2PO_4, 1.0 g NH_4Cl, 0.06 g $MgSO_4 \cdot 7H_2O$, 0.06 g $FeSO_4 \cdot 7H_2O$, 0.06 g $CaCl_2 \cdot 6H_2O$, 25 g NaCl, 1.0 g yeast extract and 0.3 g sodium citrate. For the preparation of microbial inocula, 5 g of sediment were aseptically suspended in two sterile 250 ml glass serum bottles containing 200 ml of autoclaved culture medium. Sodium dithionite (0.2 mM) and 0.001 mg resazurin were added to each bottle as reducing agent and redox indicator, respectively. Sterile butyl rubber stoppers were put and sealed with aluminum crimps. Following the Hungate techniques [19], oxygen was aseptically flushed away by gassing the bottles for 10 min with oxygen–free nitrogen. The bottles were left in the dark at 30°C for 5 d and used as inoculants for batch anaerobic incubations.

Batch anaerobic incubations. Batch cultures were carried out in 250 ml sterile serum bottles using the anaerobic preparation procedure described above. Two culture treatments were set: lactate, and a control without lactate. For every condition, four replicate bottles were inoculated with microbial suspensions obtained from the

inoculant bottles. Suspensions consisted of pellets obtained by centrifugation of 5 ml of culture (8,300 *g*, 5 min) and then carefully resuspended in 1 ml fresh medium. One batch culture set (S1-initial) was grown in the dark at 30° C for 5 d. At day 3, when pH was rising and hydrogen sulfide odor was detected, 5 ml of S1-initial cultures were transferred to corresponding fresh medium bottles, to obtain enriched cultures. The new culture series (S2-enriched) was grown in the dark at 30° C for additional 5 d, in the presence and absence of copper. For copper amendments, $CuCl_2$ (2 mM) was added into the medium at the beginning of S2-enriched treatments from a concentrated 0.02 µm–filtered solution (pH 2).

Analytical methods. The sediment sample was passed through a 1.25 mm sieve and after oven drying at 60°C, the <1.25 mm fraction was used to determine total copper concentration by inductively coupled plasma optical emission spectrometry (ICP–OES). Acid extraction was previously performed as described [20]. Sediment extractable copper was estimated by the diethylenetriamine pentaacetic acid (DTPA) extraction method [21]. The pH of water samples was determined with a pH–meter (HI 111; Hanna Instruments® , USA) and total copper was measured with ICP–OES. Sulfate concentrations were determined using a turbidimetric method based on the precipitation of barium sulfate [22].

All cultures were daily monitored for pH, sulfate, and organic acid determinations (duplicates) using the Hungate techniques [19]. After sampling, the cultures were gently gassed with oxygen–free nitrogen, for 10 min. The The pH was immediately measured. Organic acids supernatant concentrations were quantified by

high-performance liquid chromatography (HPLC). A gradient pump system (PU2089 Plus; Jasco, USA) coupled to an ultraviolet ultraviolet detector (FP2010 Plus; Jasco, USA) with a C_{18} column (5 µm, 4.6 by 150 mm; Kromasil® , Sweden) was used with a reversed–phase method [23].

Desoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR) amplification. DNA isolation from batch cultures was performed using a commercial kit (Ultra Clean Soil DNA™ ; Mobio Laboratories, USA). For cultures, cell pellets were obtained by centrifugation (8,300 *g*, 5 min). Extraction was done from 0.5 to 1 g samples, and the quality of the DNA was verified by 1% (w/v) agarose gel electrophoresis. DNA yields ranged from 2 to 3 µg/g of cell pellet. DNA preparations were stored at -20°C, prior to analyses and PCR amplification.

For 16S rRNA gene sequence amplification, the 8F (5'–AGA GTT TGA TCC TGG CTC AG–3') and 1392R (5'–ACG GGC GGT GTG TAC–3') universal oligonucleotide primer pair was used [24]. The The *dsrAB* gene sequences were amplified with the dsr1Fdeg and dsr4Rdeg primer pair which are specific for bacteria and archaea domains [25]. The The *cop*–like gene sequences amplification was carried out with the copAUF (5'–GGT GCT GAT CAT CGC CTG–3') and copAUR (5'–GGG CGT CGT TGA TAC CGT–3') primer pair (De la Iglesia, Ph.D thesis). Each 50 µl reaction mix contained: 1.25 U of *Taq* polymerase, 5 µl of 10×PCR buffer (200 mM Tris–HCl, pH 8, 500 mM KCl), 0.2 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 50 ng/µl bovine seroalbumin, 3 mM $MgCl_2$ (2 mM, for *dsrAB*, and *cop*–like gene sequences), and 10 to 50 ng of template DNA.

Polymerase chain reaction for 16S rRNA sequences amplification were carried out in a thermal cycler (2400; PerkinElmer® , USA), as follows: 5 min at 94°C for initial denaturation; 25 cycles of 45 s at 94°C, 1 min at 56°C for annealing, 2 min at 72°C; and a final extension for 7 min at 72°C. Polymerase chain reaction conditions for *dsrAB* gene sequences were: 20 s at 94°C for initial denaturation; 30 cycles of 15 s at 94°C, 20 s at 54°C for annealing, 2 min at 72°C; and a final extension for 1 min at 72°C. The The PCR conditions for *cop*–like gene sequences were the same as for 16S rRNA gene sequences, except for the annealing step which was made at 57°C. Expected amplified products (1.4, 1.9, and 0.75 kb, for 16 rRNA, *dsrAB*, and *cop*–like gene sequences, respectively) were visualized by electrophoresis in 1% (w/v) agarose gels prepared in 1×TAE buffer (40 mM Tris–acetate, 2 mM ethylenediaminetetraacetic acid [EDTA], pH 8), at 90 mV for 45 min. Agarose gels were previously treated with 1× SYBR® green DNA–binding dye (Invitrogen™ , USA). Spell out acronyms if possible.

~~T–RFLP~~ Terminal–restriction fragment length polymorphism Terminal–restriction fragment length polymorphism reactions, data handling and statistical analyses. For terminal–restriction fragment length polymorphism (T–RFLP) analyses, primer 8F was labeled at the 5' end with the phenyl–1,4–dichloro–6–carboxyfluorescein fluorochrome define. The The 16S rRNA gene PCR labeled products were digested with 20 U of endonucleases *HhaI* or *MspI* in 20 µl reactions, at 37°C for 3 h. Digested PCR products were mixed with 0.1 volume volumes of 3 M sodium acetate (pH 5) and 2.5 volume volumes of 100% (v/v) ethanol, incubated at -80ºC for 1 h to

precipitate DNA, and centrifuged at 19,064 *g* for 15 min at 4°C. Pellets were washed with 70% (v/v) ethanol, vacuum dried, resuspended in 5 µl MilliQ® (USA) water and stored in the dark at -20°C, prior to analysis. DNA fragments were separated by capillary electrophoresis (3100 genetic analyzer; Applied Biosystems™ , USA) and fragment sizes were determined using the internal standard ROX 500, as reference.

HhaI and *MspI* T–RFLP profiles were obtained. Raw data sets consisted of peaks reflecting the size in base pairs (bp) of terminal restriction fragments (T–RFs) and the area of each peak, measured in fluorescence units. ~~T–RFs~~ (do not begin a sentence with an acronym) Terminal restriction fragments Terminal restriction fragments representing less than 0.5% of the total area and those with length sizes <50 and >500 bp were not included in the analyses. Data were standardized by calculating the area of each peak as a percentage of the total area. Profiles were plotted as peak area (relative abundance) against fragment size. Richness (S, number of phylotypes) was calculated by the number of T–RFs. The T–RFs areas were analyzed with the multivariate statistical software Primer 5 (Primer–E, UK). A similarity matrix was calculated using the Bray–Curtis coefficient [26]. Non–metric multidimensional scaling (NMDS) analyses were used to group data according to their similarity. One–way crossed analysis of similarity (ANOSIM), a multivariate randomization procedure was carried out to examine the statistical significance of grouping [27]. The output statistic, *R*, which indicates the magnitude of difference among group samples, takes a value of 0 if there is no separation of the bacterial community structure due to the factor analyzed, and 1 if total separation takes place

[27]. Values of *R*≥0.5 account for high differences, whereas between 0.25 and 0.5 differences are considered as moderate [18].

True T–RFs sizes of the 16S rRNA clone libraries (see below) were determined in silico with the software Vector NTI 9.0® (Invitrogen™ , USA), and used as a database for the assignation and abundance comparison of the corresponding T–RFLP profiles. Literature recommendations to minimize the drift between true and observed T–RFs were followed [28].

Clone analyses. 16S rRNA, *dsrAB,* and *cop*–like genes PCR products were ligated into the PCR–TOPO 2.1 TA cloning vector (Invitrogen™ , USA) prior to transformation into *Escherichia coli Escherichia coli* DH5–α cells, according to the electroporation protocol described by the manufacturer. Initial diversity analyses of cloned sequences were performed by restriction fragment length polymorphism (RFLP). Endonuclease restriction digestions were carried out as described above using *HhaI* or *MspI*. The restriction fragments were resolved by electrophoresis in 2% (w/v) agarose gels, in 1×TAE buffer at 65 mV for 2 h. Clones were grouped according to their restriction pattern. A representative number of clones per library was screened based on percent coverage value (*C*≥95%) calculated with the equation $C = [1 - (n_1/n)] \times 100$, where n_1 n_1 was the number of clones that occurred once (i.e., unique phylotype) and n if N represents number shouldn't it be formatted lowercase italic? was the total number of clones examined [29]. One to four representative clones were sequenced depending on the phylotype group size.

Sequencing and comparative analyses. Selected 16S rRNA, *dsrAB* and *cop*–like gene clones were sequenced (Macrogen™ , Korea). Sequence assemblage was done using the software Vector NTI 9.0® with the exception of *dsrAB* sequences due to fragment size sequencing limits. These latter clone libraries were divided into *dsrA* and *dsrB* partial fragments, according to the sequences amplified with the forward or reverse primer, respectively. Chimeras were screened using the Pintail software (http://www.bioinformatics-toolkit.org). The similarity between sequenced clones within each RFLP phylotype group was compared, and if identity percentages were ≥98% it was assumed that the sequences correspond to the same phylotype [30]. All sequences were subjected to a similarity search in the GenBank database (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) algorithm. Sequences with identity percentages <95% were assigned to the closest family level. For each molecular marker, nucleotide sequences were multiple aligned by the CLUSTAL W algorithm with related sequences selected from the GenBank database. The The *dsrAB* and copper P–ATPase nucleotide sequences were transformed to amino acid sequences. Only common gene regions were included in the analyses and gaps were treated as missing data. For the three cloning libraries, dendrograms were constructed with MEGA 4 software manufacturer? [31], utilizing the Neighbor–Joining method with 1000 bootstrap replications to support internal branches. To avoid tree bushes delete marks if this is not a direct quotation in the dendrograms, only one clone phylotype sequence per group was included.

Nucleotide sequences accession numbers. Sequence data from the clone libraries was deposited in GenBank under accession numbers: EU883388, FJ024708 to FJ024724, and FJ040818 to FJ040830.

RESULTS AND DISCUSSION

Caleta Palito sediment and water contain high copper concentrations. The sediments of Caleta Palito, with a long–term exposure to high levels of copper due to continuous release of copper–mining residues, were selected as study site [17]. This zone is essentially free of other pollutants [18]. Mean total copper concentration found in these sediments (1,054 mg/kg) is much higher than levels associated with non–contaminated sediments, being approximately approximately 30 times greater [32]. In addition, the level of available copper in Caleta Palito sediments (157 mg/kg), which includes dissolved and weakly sorbed copper [21], has been associated with very low biological diversity and abundance [18,32]. In the water samples from Caleta Palito, the average total copper was 236.1 μM and sulfate was 25.7 mM. The mean pH value for Caleta Palito water was 8.0.

Sulfate reduction is mostly coupled to incomplete oxidizing metabolism. The ability of Caleta Palito sediment enrichment sets (S1-initial and S2-enriched) to reduce sulfate in the presence of lactate as electron donor was tested. Significant levels (>95%) of sulfate were removed in anoxic batch cultures fed with lactate (Fig. 1). The faster sulfate depletion observed in S2-enriched compared with S1-initial treatment, suggested an increase in the activity of SRB during this enrichment (see below). Marginal sulfate removal was found in the absence of lactate (Fig. 1A, control). This

might be due to minor amounts of carbon sources present in the culture medium. No significant values were recorded in S2-enriched controls in the absence of lactate (data not shown).

Sulfate reducing bacteria oxidize a wide range of organic compounds either incompletely to acetate or completely to CO_2 [6]. Total consumption of lactate and lactate–derived acetate production was observed in S1-initial and S2-enriched treatments (Fig. 1B). Lactate is a preferred electron donor for SRB and the main reaction for sulfate reduction has a molar ratio of 2:1 [33]. Hence, the reduction observed of up to 38 mM sulfate would consume ~~about~~ approximately approximately 76 mM lactate. ~~About~~ Approximately Approximately 80% of the initial level of acetate (45 mM) was oxidized to acetate (Fig. 1B). This suggested a different stoichiometry in lactate oxidation and the dominance of incomplete oxidizing metabolism.

Sulfate–reducing enrichments are dominated by *Desulfovibrionales and* *Desulfobacterales*. Title is too long.

Molecular diversity of bacterial communities was first studied by analysis of 16S rRNA clone sequences from lactate S2-enriched treatments (L entries). Although the number of clones used in this study for the 16S rRNA gene, and the other two (see next sections), gene markers is rather limited, it is still enough to support the conclusions drawn below. A dendrogram was constructed with them and using 16S rRNA sequences from selected bacteria isolated from ecosystems with analogous characteristics to the copper polluted sediments (e.g., anoxic, saline, marine, contaminated) (Fig. 2). The 16S rRNA sequences analysis showed significant similarities with several phyla and uncultured microorganisms related to obligate and

facultative anaerobic lineages. Similarity relationships were supported by high bootstrap values. Results showed clustering of the sequences within δ –*Proteobacteria*, γ –*Proteobacteria*, *Firmicutes*, *Spirochaetes*, and *Cytophaga*–*Flexibacter*–*Bacteroides* (CFB) groups. The main groups in L library (number of ribotypes *S*=10, number of clones *n*=48 format N for number in lowercase italic?, coverage *C*=96%) were δ - and γ - *Proteobacteria* and CFB bacteria. Clones closely related to SRB belonging to δ –*Proteobacteria* (relative abundance of 54.1%) dominated these enrichments. This class was represented by clone sequences belonging to the incomplete oxidizing *Desulfovibrio* genus, particularly to *D. capillatus* (L8) and *D. senezii* (L42) (Fig. 2). The clones clustered to the *Firmicutes* group were closely related to *Clostridium* (L20 and L55) and *Dethiosulfovibrio* (L23) genera (Fig. 2). Dissimilatory sulfite–reduction has been demonstrated in *Clostridium* species [34]. However, *Clostridium* species are not able to reduce sulfate to sulfite [34]. The sequences affiliated to the *Dethiosulfovibrio* genus, have a closer match with the halophilic species *Dethiosulfovibrio marinus*, a sulfur and thiosulfate reducing bacterium, unable to grow on either lactate or acetate [35]. Closely related sequences to the sulfur–accumulating *Spirochaeta* genus (L29) were also found in the library (Fig. 2). A symbiotic association between *Spirochaeta* and *Dethiosulfovibrio* species in sulfide–rich habitats has been reported [35] and may explain their presence.

Other abundant sequences were related to CFB marine species and to γ –*Proteobacteria* halophilic fermentors. The relative abundance of sequences related to uncultured bacteria (L21, L49, and L55) was 14.7% (Fig. 2). Interestingly, 16S rRNA cloning results agree with a bacterial diversity study carried out in >80 years

heavy metals polluted sediments from the continental shelf where the same major groups were found [36]. Although the S1-initial and S2-enriched bacterial inocula do not truly represent the original sample, they may still contain significant numbers of the representative, abundant bacterial groups thriving in the original sediment.

Since sulfate reducing microorganisms are a diverse phylogenetic group composed by bacterial and archaeal species [5], it was interesting to compare results from the 16S rRNA libraries with clone analysis using the functional *dsrAB* genes. In the *dsrAB* clone library (*S*=4, *n*=20, *C*=100%), all sequences clustered within the δ –*Proteobacteria* class, particularly *Desulfovibrionales* and *Desulfobacterales* (Fig. 3). ~~SRB~~ Sulfate–reducing bacteria Sulfate–reducing bacteria belonging to these Gram–negative bacteria orders have been described as dominant in marine and estuarine sediments. They seem to have a better–adapted metabolism to saline and high–sulfate environments than Gram–positive SRB, which have been described as dominants in freshwater environments [11,12]. *Desulfovibrio* related sequences accounted for 70% in L (L2 and L15) (Fig. 3). One clone type (L7), related to the genus *Desulfobacter*, which comprises slow growing acetate–oxidizers [37], was found (Fig. 3). Some acetate oxidation was verified after 5 d of incubation in lactate enrichments (Fig. 1B), which might be related to the relatively low abundance of these microorganisms during the relatively short incubation period of the enrichments. The presence of clones (L1) related to *Desulfocella halophila* was also determined (Fig. 3). This incomplete oxidizing species, unable to grow on lactate or acetate, probably utilizes pyruvate as energy/carbon substrate [38]. Pyruvate was detected (<8 mM), in S1-initial and S2-enriched lactate treatments (data not shown).

Copper partially inhibits sulfate reduction and organic carbon oxidation in sulfate–reducing enrichments. Title is too long. The presence of copper clearly slowed down sulfate removal in the S2-enriched treatment (Fig. 1B). However, sulfate reduction levels after 4 d of incubation were the same as without copper (Fig. 1B). As lactate consumption was retarded by the presence of copper, lactate–derived acetate production was delayed and its levels remained almost unchanged (Fig. 1B). The effect of copper in sulfate reduction and organic acid consumption found in the enrichments was observed with high levels of this metal (2 mM) (does this represent to here? If so, use –), as these levels are much higher than values reported to be inhibitory and toxic (i.e., copper levels causing a cessation in sulfate reducing activity) in pure, mixed and enriched SRB cultures [4,15], and particularly, *Desulfovibrio* and *Desulfobacter* typical strains [39]. The observed effect of copper suggests that the delay of sulfidogenesis may be explained by a lower growth rate produced by denaturation of proteins, inactivation of enzymes and competition with essential cations [14]. As sulfate reduction is still ongoing in the copper supplemented enrichments, partial inactivation of enzymes might be occuring. On the other hand, as biogenic sulfide is generated during these enrichments, copper toxicity would decrease because copper sulfides are formed. However, these metal sulfides may cover the cell surface perturbing the access to sulfate and organic matter decreasing sulfate reduction [14]. In copper amended enrichment cultures obtained from metal contaminated sediments, the recovery of sulfate reduction has been linked with metal tolerance [15].

Copper modifies the genetic structure of sulfate–reducing bacterial communities.

Title too long. The effect of copper in sulfate reduction in S2-enriched treatments suggested the establishment of a different SRB community that may be better adapted to the presence of copper. Molecular diversity was also studied by clone analysis of copper amended S2-enriched treatments (LC entries). Clone richness (*S*=8, *n*=46, *C*=98%) was lower in the copper enrichments 16S rRNA library can LC be defined? but contained almost all the bacterial groups found in the L library (Fig. 2). The LC library showed also a decrease in γ – and δ –*Proteobacteria* relative abundance sequences. The latter class decreased from 54.1 to 34.7% and included clones (LC58 and LC61) related to the same *Desulfovibrio* species as in the L library (Fig. 2). On the other hand, clones related to CFB bacteria had a significant increase in their abundance. In the *dsrAB* copper enrichments library (*S*=4, *n*=20, *C*=100%) *Desulfovibrio* related sequences decreased from 70 to 55% (LC3 and LC5) and *Desulfobacter* related clones diminished too (Fig. 3). These results also indicated changes in the microbial community structure.

To evaluate the structure of the bacterial community enrichments, and to make a comparison between them, the T-RFLP technique using the 16S rRNA gene sequences was applied. This approach was used to have a broader view of the bacterial members of these communities. The number of relatively abundant bacterial species, estimated by the mean richness determined from 16S rRNA T–RFLP *HhaI* profiles (Fig. 4), indicated a decreasing trend from the lactate–only (*S*=17±1) to lactate–copper enrichments (*S*=14±1). Non–metric multidimensional scaling analysis and ANOSIM using T–RFLP data showed that the structure of the bacterial community of the lactate–only enrichments was clearly different from that of

the lactate–copper enrichment (*R*=0.68; $p<0.05$), revealing a significant perturbing effect of the copper addition. Results from T–RFLP profiles obtained with *MspI* showed the same tendencies (data not shown).

The trends observed by clonal analysis were also found for the putative phylogenetic assignation, based on 16S rRNA T–RFLP profiling. *Desulfovibrio* and CFB sequences found in the clonal analysis have adjusted *HhaI* T–RFs lengths within the 90 to to 95 bp range [22]. The most abundant signals (90 and 93 bp) were observed in the L and LC enrichment profiles being affected by the presence of copper (Fig. 4). Since most of the marker genes found in copper supplemented enrichments were also present in enrichments without copper, it seems possible that copper mostly affects the activity of the microbes than causes cell death.

Copper changed the diversity of copper P-ATPases. As copper tolerance plays an important role in the enriched bacterial communities, the presence of copper should also have some effect on the genetic diversity of copper resistance determinants. Therefore, an analysis using the copper P-ATPases, *cop*–like gene sequences, was carried out in S2-enriched treatments. Although novel P–ATPases sequences were detected (Fig. 5), low richness of copper P-ATPases sequences was found. In copper impacted areas this has been explained as an effect of high copper pollution [26]. All cloned sequences clustered in the P_{IB}–type ATPases division and included the two conserved motifs in copper P–ATPases [16,26]. The *cop*–like LC clone library (*S*=3, *n*=23, *C*=96%) contained all the sequences detected in the L clone library (*S*=2, *n*=26, *C*=100%) (Fig. 5). A *cop*–like sequence found only in the LC clone library (LC33) was very abundant (43.5%), indicating a strong effect of copper.

This sequence clusters with biochemically demonstrated copper–exporting P–ATPases (Fig. 5). The most abundant sequences in the enrichments (L1 and LC14) cluster with the *silP* sequence of *S. Salmonella typhimurium* spell out (Fig. 5), a gene involved in silver and copper resistance [40].

Following the hypothesis proposed in the present study, high levels of copper addition did not inhibit sulfate–reduction in bacterial enrichments. Interestingly, genetic diversity of taxonomic and dissimilatory sulfate–reduction molecular markers was scarcely affected by copper addition unlike copper resistance determinants. Changes produced by copper in these communities were mainly due to changes in the bacterial groups relative abundances.

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FIGURE LEGENDS

Figure 1: (A) Sulfate reduction (closed circles) in lactate (solid line) and control (dashed line) S1-initial treatments. (B) Sulfate (closed circles), lactate (closed squares), and acetate (closed triangles) concentrations in lactate (solid line) and copper amended lactate (pointed line) S2-enriched treatments. The mean concentrations were calculated from four biological replicates.

Figure 2: 16S rRNA dendrogram showing sequences from S2-enriched treatments. Relationships among representative sequences from clone libraries comparing lactate and lactate–copper enrichments (L and LC), and previously reported bacterial groups are shown in outer brackets. Clones and their best Basic Local Alignment Search Tool (BLAST) matched sequences are indicated (bold). Percentage values at the right of inner brackets show the nucleotide sequence identity between clones and the best matching reported sequences. Relative abundances of clones in the respective library are given (parentheses). The dendrogram was constructed from a Clustal W nucleotide sequences alignment using the neighbor–joining distance matrix method based on *Escherichia coli* (Z83204) positions 1 to 1392. *Burkholderia sedimenticola* (EU035613) was used as outgroup because it belongs to β – *Proteobacteria*. GenBank accession numbers are indicated.

Figure 3: Dissimilatory sulfite reductase *dsrAB* dendrogram showing sequences from S2-enriched treatments. Relationships among representative sequences from clone libraries comparing lactate and lactate–copper enrichments (L and LC), and different

sulfate–reducing microbial groups are represented. Clones and their best Basic Local Assignment Search Tool (BLAST) matched sequences are indicated (bold). Relative abundances of clones in the respective library are given (parentheses). The dendrogram was constructed from a Clustal W amino acid sequences alignment using the neighbor–joining distance matrix method based on approximately 260 amino acid representative sequences. The dendrograms with both *dsrAB* fragments were consistent, so only data from the *dsrB* gene sequences is shown. *dsrB* gene from *Archaeoglobus fulgidus* (NP_0629260) was used as outgroup because it belongs to *Archaea* sulfate–reducing bacteria which were not detected in the clonal libraries. GenBank accession numbers are indicated.

Figure 4: Representative terminal restriction fragment length polymorphisms (T–RFLP) profiles using 16S rRNA Polymerase chain reaction products from bacterial community DNA digested with the endonuclease *HhaI*. (**A**) S2-enriched lactate treatment. (**B**) S2-enriched copper amended treatment. bp =base pairs.

Figure 5: Copper translocating phospho type–adenosine triphosphatases define *cop*–like dendrogram showing sequences from the S2-enriched treatment. Relationships among representative sequences from clone libraries comparing lactate and lactate–copper enrichments (L and LC), and copper P–type ATPases from different bacterial groups are represented. Clones and their best Basic Local Alignment Search Tool (BLAST) matched sequences are indicated (bold). Relative abundances of clones in the respective library are given (parentheses). Sequences with biochemical demonstrated copper translocation function have an asterisk.

Sequences with demonstrated resistance function have a filled node. The dendrogram was constructed from a Clustal W amino acid sequences alignment using the neighbor–joining distance matrix method based on approximately 255 amino acid sequences. *cadA* P–ATPase from *Staphylococcus aureus* (P20021) was used as outgroup because it belongs to the IB2 group of heavy metal P–ATPases. GenBank accession numbers are indicated.