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Scientific Research

Phylogeny of Bacteria from Steelmaking Wastes and Their Acidic Enrichment Cultures

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Abstract

Currently, millions of tons of steel are produced worldwide. This has become a serious economic and environmental challenge because the ores used for steel production are nonrenewable resources and the production generates huge amount of waste. In this study, we identified and investigated the ability of bacteria from steelmaking waste with low and high zinc concentration to promote leaching of zinc, when enriched by acidic (pH 2) culture conditions. The bioleaching assays indicated removal of Zn, as in chemical leaching. Bacterial communities from crude and enrichment culture wastes were characterized by the 16S rRNA gene. Phylogenetic analysis of the generated clone libraries revealed predominance of Proteobacteria and Firmicutes. The Actinobacteria, Bacteroidetes, Cyanobacteria, and Deinococcus-Thermus phyla were also encountered. The clones were most closely related to cultivable heterotrophic bacteria. Different genera were identified including iron redox cycling and leaching bacteria such as *Chromobacterium*, *Aeromonas*, *Escherichia*, *Bacillus*, and *Ochrobactrum*. These data add significant new information on bacteria which survive in extremely acidic conditions. They are distantly related to typical acidophiles responsible for the leaching process, which makes them good candidates for future studies on metal bioleaching.

Keywords

Bacteria, 16S rRNA, Diversity, Zinc, Steelmaking Wastes

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1. Introduction

Steelmaking activities have been particularly intensive during the 20th and 21st centuries, resulting in the generation of huge amounts of waste (approximately 700 kg of waste per ton of steel produced) including the presence of metals such Zn, Cu, and Cr [1]. Indeed, most of the waste is being left without proper management all over the world and without any management whatsoever in Brazil and perhaps in other countries as well. The release of these wastes to the environment leads to contamination and consequent human exposure to the metals present in these rejects. The World Steel Association reported an increase of 6.8% in steel production in 2011 resulting in 1.527 billion tons, of which Brazil contributed about 35.2 million tons (www.iabr.com.br). Thus the waste generated by this industry can no longer be ignored. First steps towards solutions for its management would be the search for suitable technologies to enable the removal of metals from these wastes.

Bioleaching of metals has gained increased attention since it is innovative, environmentally friendly, and economical [2]. Indeed, bioleaching has been considered as an alternative strategy for the extraction of metals from complex ores or wastes, which may reduce costs up to 80% when compared with traditional chemical techniques [3]. This biotechnological process is based on the ability of microorganisms to oxidize ferrous iron and/or reduce sulfur compounds [4]. The predominant metal-sulfide-dissolving bacteria that have been extensively used for the bioleaching of sulfide minerals with commercial interest are extremely acidophilic [5] [6] although heterotrophic bacteria can also contribute to metal leaching [7].

The process of production of steel involves many stages, from reduction of iron ore at temperatures reaching 2400˚C (in a blast furnace) to metal plating, which uses a large amount of Zn to protect the steel from corrosion. Overall, the wastes contain tramp metals such as Zn, Cu, and Cr preventing their recycling for steel production due to possible damage to industry furnaces by these metals. Nevertheless, Zn is economically important due to its anti-corrosive and pharmaceutical properties. In this study, we identified and investigated the ability of heterotrophic bacteria from steelmaking wastes to survive in acidic conditions and to promote leaching of zinc.

2. Materials and Methods

2.1. Sampling and Chemical Composition of the Wastes

Steelmaking waste samples were collected at Usiminas (Ipatinga, Minas Gerais state, Brazil) using sterilized bottles. The wastes studied were crude thin sludge waste (TS), constituted by fine solid particles resulting from the Linz-Donawitz converter (or BOF), and crude sludge from treatment of electroplating effluent waste (STEE), both of which were released into the environment three days before sampling. X-ray fluorescence spectrometer (PW 2510 Sample Changer, Philips) analysis of the wastes revealed 1.9% and 29.8% Zn, 57% and 5.5% Fe, and 11% and 40% Ca in the TS and STEE wastes, respectively. The efficiency in Zn extraction was compared using an unpaired t-test, performed by PAST data analysis package. The level of significance was considered at $p \leq 0.05$.

2.2. Acidic Enrichment Culture

Acidic enrichment cultures were established by blending 10 g of the separate TS and STEE wastes into 100 mL of Leathen medium [8], and were respectively named TSC and STEEC. Prior to the bioleaching experiments the pH values of the crude STEE and TS wastes were 7 and 5, respectively. To prepare primary enrichment of heterotrophic bacteria, crude TS and STEE wastes were added to flasks and then treated with H_2SO_4 to reduce the pH value to 2 and thereby provide adequately acidic conditions for bacterial growth. The flasks were then incubated at 30˚C with agitation (200 rpm) for 40 days. The pH was monitored daily and adjusted with H_2SO_4 as needed. At the end of this period, we obtained the primary bacterial enrichment samples from each type of waste, TSC and STEEC.

2.3. Bioleaching Assay

Bioleaching assays were performed in 500 mL Erlenmeyer flasks. Each flask contained 180 mL of Leathen medium, 10 g of autoclaved waste (5% w/v), and 20 mL of TSC or STEEC (10% v/v). The flasks were incubated at 30˚C with agitation (200 rpm) for 30 days. The pH value in the leaching solution was kept constant (pH 2) throughout the leaching process by adding H_2SO_4 as need. After this period, the wastes were filtered, dried at

110˚C for one hour, and the concentration of Zn was measured by X-ray fluorescence spectrometer. These samples were henceforth called TSB and STEEB depending on the origin of inoculum used to start the bioleaching assay.

Non-inoculated controls consisted of autoclaved crude TS and STEE acidified and subjected to the same conditions as the TSC and STEEC tests. However, the pH of control flasks was monitored daily and continuously adjusted to pH 2 with H_2SO_4.

2.4. DNA Extraction and PCR Amplification of 16S rRNA Gene

Total DNA from waste (crude TS and STEE) and primary enrichment (STEEC and TSC) samples were isolated by using a Max™ Power DNA Isolation Kit for soil and water (MO Bio Laboratories) according to the manufacturer's instructions. The DNA samples were stored at −20˚C until further processing.

The bacterial 16S rRNA gene fragment was amplified using touchdown PCR according to Freitas *et al*. [1], using the primer set 8f (5'AGAGTTTGATCMTGGCTCAG 3') and 907r (5'ACGGHTACCTTGTTACGACTT 3') [9].

2.5. Cloning, Sequencing, and Clone Library Analysis

Bacterial 16S rRNA gene fragments were gel-purified using the Silica Bead DNA Gel Extraction Kit (Fermentas, Canada), cloned into the vector pJET1.2/blunt (Fermentas, Canada) according to the manufacturer's instructions, and transformed into electrocompetent *Escherichia coli* XL1Blue. Partial 16S rRNA gene sequences were obtained using the pJET1.2 forward and reverse primers and a Mega BACE 1000 capillary sequencer (GE Healthcare, United Kingdom) according to the manufacturer's instructions. Further, the sequences were checked for quality, aligned, and edited to produce a consensus using the programs Phred v. 0.020425 [10], Phrap v. 0.990319 [11], and Consed 12.0 [12]. Chimeras were checked and omitted using Bellerophon software (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl). Closely related sequences from Greengenes [13] were identified by blast tool using the Silva database. Phylogenetic relationships were inferred with ARB software [14]. The operational taxonomic units (OTUs) were set at 97% level identity using the DOTUR software [15]. Library coverage was calculated using the equation $C = 1 - (n/N)$, where n denotes the number of unique OTUs and N is the number of sequences analyzed in the library [16]. The diversity of the OTUs was further examined using DOTUR software, LIBSHUFF statistics [17], and rarefaction analysis. A comparative analysis was performed in order to detect OTU sequences shared among the four libraries. This analysis was performed using the DOTUR software [15] to detect sequence similarity at 97% level. The partial 16S rRNA gene sequences generated were deposited in the GenBank database under accession numbers KC164772-KC164863.

3. Results

3.1. Bioleaching of Zinc by Bacteria from Steelmaking Waste

In an attempt to extract Zn from the steelmaking wastes (STEE and TS) bioleaching assays were performed using bacteria from enrichment cultures of these wastes (STEEC and TSC). During bioleaching experiments the pH initially increased over the first seven days, being thus adjusted to pH 2 as indicated in the Materials and Methods section. After this time, acidity remained stable in the test flasks but not in the control flasks, which needed pH adjustments throughout the study period. Bioleaching efficiency was calculated by difference between the Zn contents in the crude TS and STEE samples and TSB and STEEB residues determined by X-ray fluorescence analysis. The data obtained revealed that STEEB (76%) and TSB (53%) presented efficiency in Zn extraction. Chemical leaching of Zn in the control flasks reached 82% and 47% for the STEE and TS wastes, respectively. Both leaching assays were equally efficient to remove Zn ($p = 0.02$).

3.2. Phylogenetic Affiliation

To reveal the phylogenetic identity of the bacteria, 16S rRNA gene clone libraries were constructed from crude TS and STEE wastes and from the TSC and STEEC enrichments. A total of 324 partial 16S rRNA gene sequences were obtained upon removal of chimeric sequences. These sequences were clustered into 94 OTUs spanning six bacterial phyla, mostly represented by cultivated heterotrophic bacteria. Clone libraries coverage

accounted for >67% of the bacterial diversity. Rarefaction curves generated from our data did not reach an asymptote, indicating an amount of undetected diversity, especially for the STEEC (**Figure 1**). The phylogenetic distributions of the OTUs and the resulting phylogenetic trees are shown in **Figure 2** and online **Resources 1-4**, respectively.

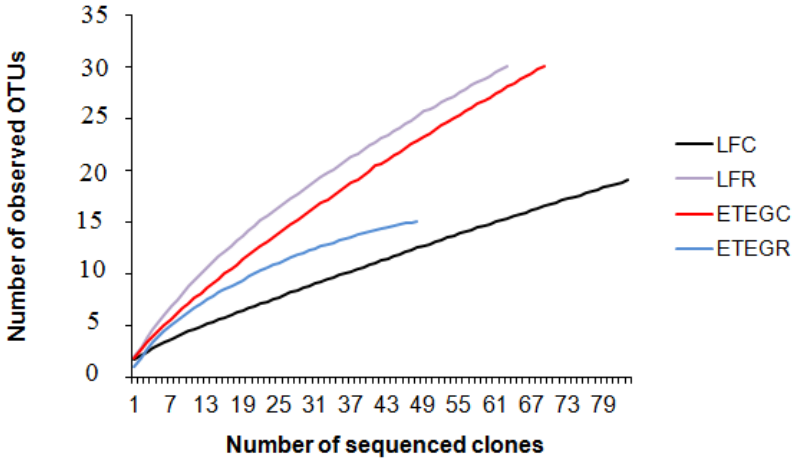


Figure 1. Rarefaction analysis of bacterial 16S rRNA gene sequences from crude sludge from treatment of electroplating effluent (STEE), enrichment culture from treatment of electroplating effluent (STEEC), crude thin sludge (TS), and enrichment culture from thin sludge (TSC). The total number of sequenced clones is plotted against the number of OTUs observed in the same library. The OTUs were defined at the ≥97% identity level (species level).

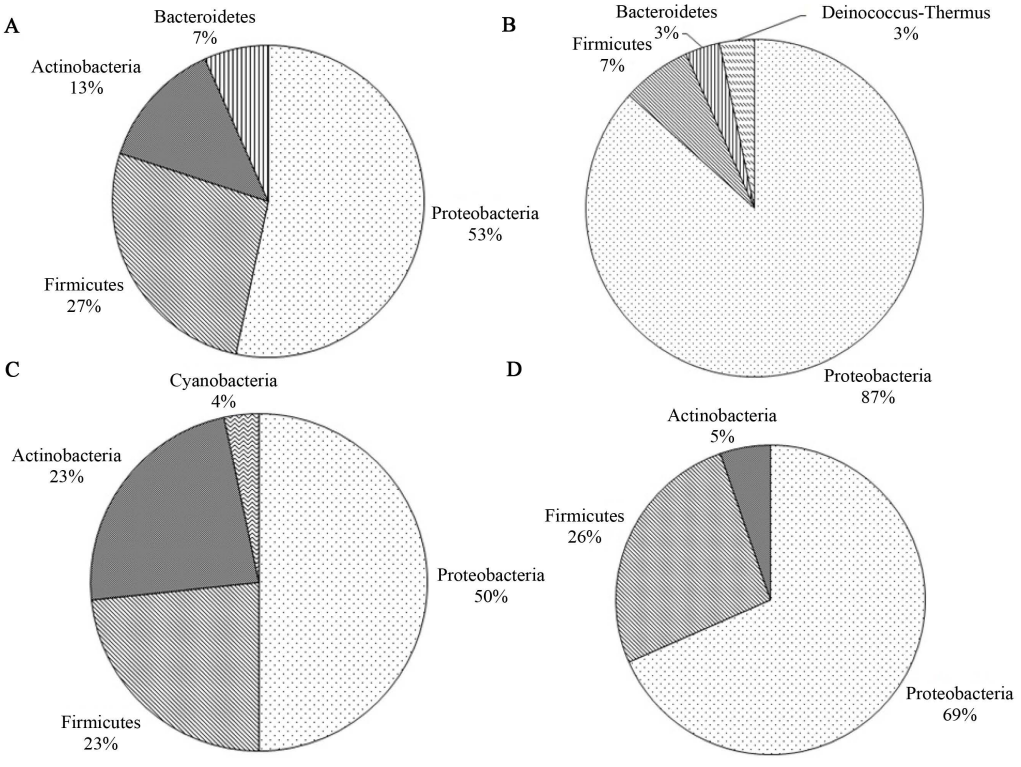
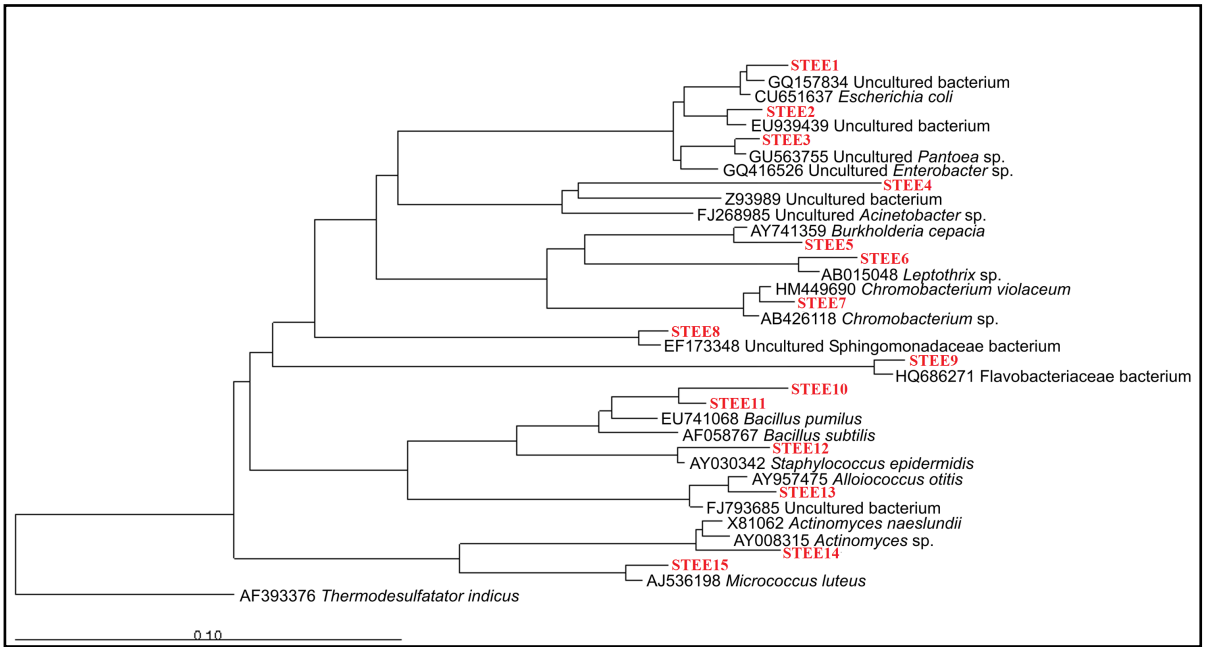
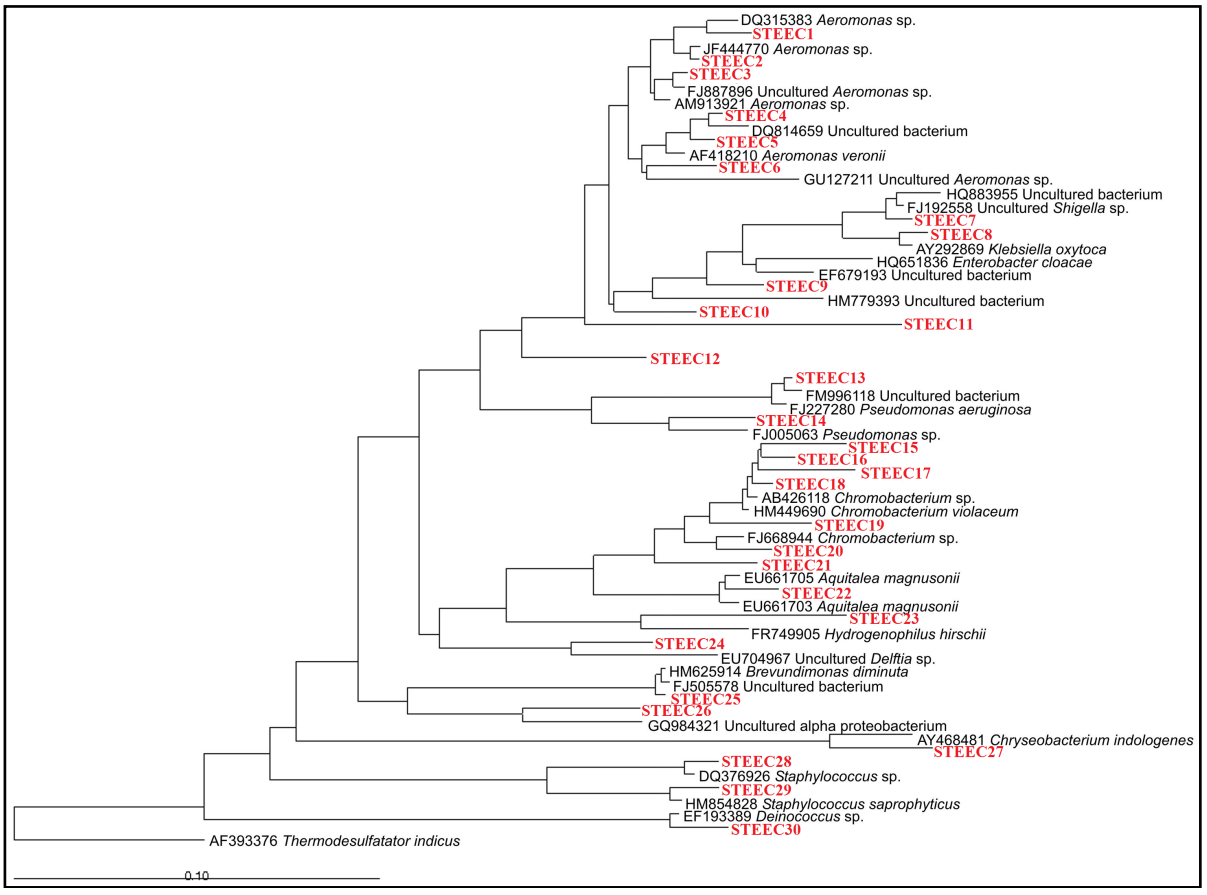


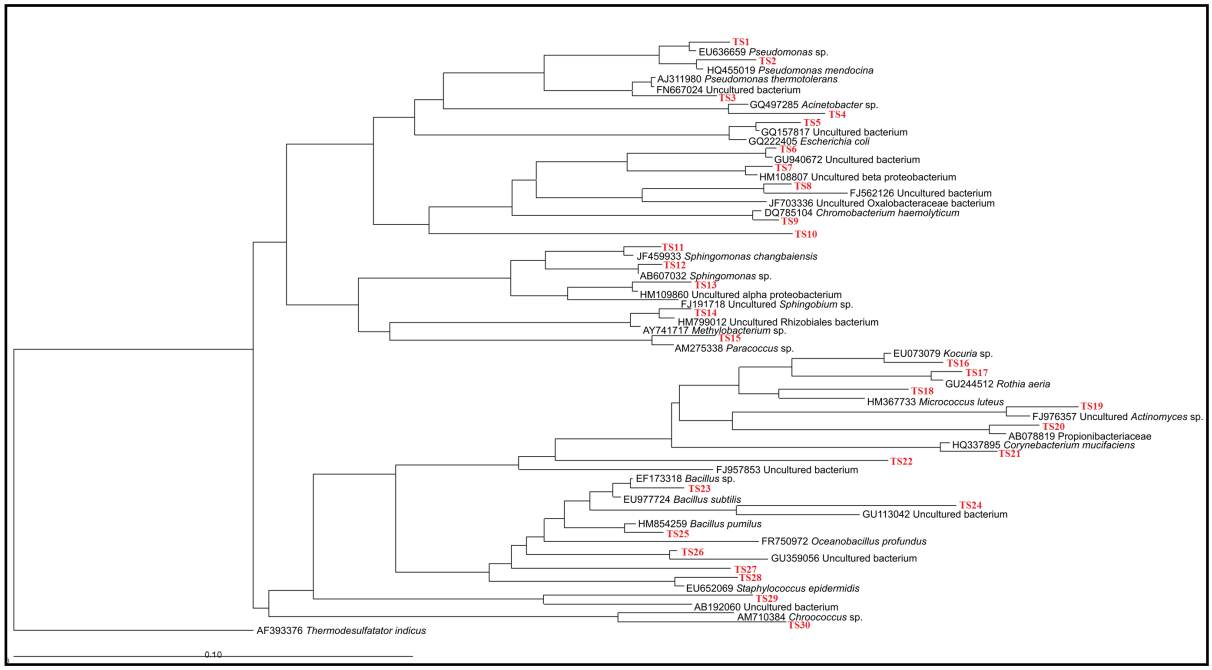
Figure 2. Phylogenetic ARB affiliation of bacterial 16S rRNA genes. The numbers indicate the percentage representative of each phylum in the library. (A) crude sludge from treatment of electroplating effluent (STEE); (B) enrichment culture from treatment of electroplating effluent (STEEC); (C) crude thin sludge (TS); and (D) enrichment culture from thin sludge (TSC).



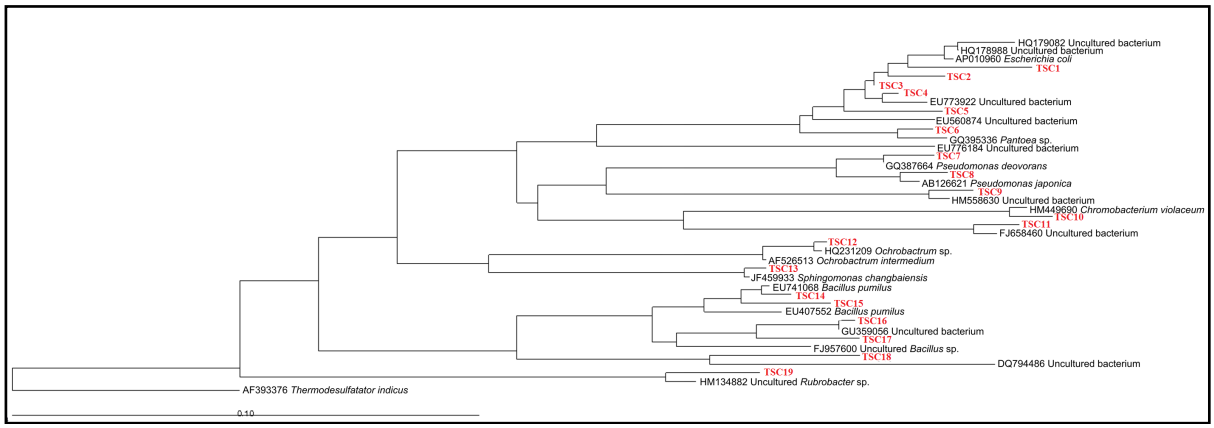
Resource 1. Phylogenetic neighbor-joining tree of bacterial OTUs from crude sludge from treatment of electroplating effluent (STEE) constructed using the ARB software.



Resource 2. Phylogenetic neighbor-joining tree of bacterial OTUs from enrichment culture from treatment of electroplating effluent (STEEC) constructed using the ARB software.



Resource 3. Phylogenetic neighbor-joining tree of bacterial OTUs from crude thin sludge (TS) constructed using the ARB software.



Resource 4. Phylogenetic neighbor-joining tree of bacterial OTUs from enrichment culture from thin sludge (TSC) constructed using the ARB software.

The Proteobacteria and Firmicutes phyla contained most of the OTUs identified in the crude STEE clone library (**Figure 2(A)**). Proteobacteria was represented by the Gammaproteobacteria (50%), Betaproteobacteria (37%), and Alphaproteobacteria (13%) classes.

Clone library analysis from the STEEC revealed that the acidic pH promoted the emergence of Deinococcus-Thermus and disappearance of Actinobacteria (**Figure 2(A)** and **Figure 2(B)**). Overall, the 27 OTUs harbored two phyla in common with the STEE clone library: Proteobacteria and Firmicutes, with a strong dominance of Proteobacteria, represented by Gammaproteobacteria (54%), Betaproteobateria (38%), and Alphaproteobacteria (8%) classes.

According to the phylogenetic analysis of the 16S rRNA gene sequences from the crude TS clone library, 30 OTUs were affiliated with the Proteobacteria, Firmicutes, Actinobacteria, and Cyanobacteria phyla (**Figure 2(C)**). Proteobacteria was the most abundant phylum, Firmicutes and Actinobacteria contributed evenly to bacterial community, whereas Cyanobacteria was present in lower ratios. Gammaproteobacteria (40%), Alphaproteobacteria (33%), and Betaproteobacteria (27%) classes were found.

The TSC clone library was composed of OTUs affiliated with Proteobacteria, Firmicutes, and Actinobacteria, with extensive variation in the proportional distributions of these phyla (**Figure 2(D)**). Gammaproteobacteria (70%), Alphaproteobacteria (15%), and Betaproteobacteria (15%) classes were also present. **Tables 1-4** show the classification of OTUs from all libraries down to genus and species level.

3.3. Comparisons of Bacterial Compositions Based on OTU Clustering

To determine the significance of differences between the clone libraries based on 16S rRNA gene sequences, we applied LIBSHUFF statistics, and the results revealed no significant differences in composition of bacterial communities.

To cluster sequences into OTUs and to distinguish between the shared and sample-specific OTUs, all sequence data were pooled together and analyzed using DOTUR (at >97% similarity). The OTUs were divided into three categories as plotted in a Venn diagram (**Figure 3**): core OTUs shared by all crude and enriched samples, OTUs shared by two or three samples, and sample-specific OTUs. Four bacterial communities shared 11 OTUs comprising the *Escherichia* and *Chromobacterium* genera as shown in the diagram. The genus *Ochrobactrum* was present only in the enriched samples of both waste types (STEEC and TSC). The diagram also reveals that all crude and enriched samples shared four bacterial communities (*Chromobacterium*, *Escherichia*, *Bacillus*, and *Ochrobactrum*).

The potential role of these bacterial communities in the Zn extraction processes from steelmaking wastes will be discussed in the following section.

4. Discussion

Environmental metal pollution is a serious problem and the treatment or recovery of desired metals from wastes is a major challenge for the sustainable use of non-renewable natural resources such as Zn. In this study, we performed assays under extremely acidic conditions, and showed that heterotrophic bacteria from steelmaking wastes were able to survive and had similar efficiency to extract Zn as in chemical leaching. However, the bioleaching assay could be considered more advantage since in this condition there was not need to addition acid

Table 1. Phylogenetic affiliation and distribution of bacterial 16S rRNA gene sequences analyzed from the STEE library.

Phylogenetic group	Closest sequences/microorganism	Acession no.	Identity (%)	Habitat of closest relative
Proteobacteria	*Escherichia* sp. (3)	HM028651	99.77%	Duck hatchery air
	Enterobacter hormaechei (2)	FJ976588	97.32%	Paddy field soil
	Pantoea sp. (3)	GU120653	99.26%	Mining waste land
	Acinetobacter sp. (1)	EU100397	98.47%	Effluent of pesticides factories
	Burkholderia sp. (1)	EF602552	93.19%	Sugarcane stem
	Leptothrix sp. (2)	AB015048	97.90%	Halophilic spa
	Chromobacterium sp. (13)	EF633687	98.94%	Spring water
	Alterierythrobacter epoxidivorans (2)	DQ304436	97.86%	Marine sediments of cold seep area
Bacteroidetes	*Chryseobacterium* sp. (2)	AM982789	97.69%	*Homo sapiens*
Firmicutes	*Bacillus* sp. (12)	FN687186	99.67%	Feather waste
	Bacillus sp. (2)	FJ615522	99.05%	Stratosphere
	Staphylococcus subsp. *aureus* (1)	CP002120	99.03%	Bloodstream of a patient
	Alloiococcus otitis (2)	AY957475	98.86%	1 year old child with otitis perforata
Actinobacteria	*Actinomyces* sp. (1)	AJ234049	98.39%	Canine and feline clinical specimens
	Micrococcus luteus (1)	AJ717367	99.21%	Alkaline groundwater

Table 2. Phylogenetic affiliation and distribution of bacterial 16S rRNA gene sequences analyzed from the STEEC library.

Phylogenetic group	Closest sequences/microorganism	Acession no.	Identity (%)	Habitat of closest relative
Proteobacteria	*Aeromonas* sp. (1)	DQ315383	97.93%	Silkworm
	Aeromonas sp. (7)	FJ847841	99.62%	Snails (*Helix aspersa*)
	Aeromonas sp. (1)	FN997620	96.61%	Estuarine sediment
	Aeromonas sp. (4)	U88662	98.87%	Environmental sources
	Aeromonas sp. (1)	U88662	95.89%	Environmental sources
	Aeromonas sp. (1)	AB472911	95.71%	Ascites
	Shigella sp. (1)	HM146924	99.62%	Rabbit intestine
	Enterobacter sp. (2)	EU272859	99.81%	Cotton rhizosphere
	Cedecea davisae (1)	AF493976	94.16%	Disinfecting footbaths
	Aeromonas sp. (1)	AM913921	94.92%	*Saccharina latissima*
	Aeromonas sp. (1)	EF111230	92.63%	Bogota river
	Aeromonas sp. (1)	AF063003	91.15%	Water
	Pseudomonas sp. (1)	FN663622	99.81%	Polluted pond water
	Pseudomonas sp. (1)	AF320993	93.41%	*Agaricus bisporus*
	Chromobacterium sp. (1)	EF633687	97.53%	Spring water
	Chromobacterium sp. (1)	EF633687	98.12%	Spring water
	Chromobacterium sp. (2)	EF633687	97.18%	Spring water
	Chromobacterium sp. (27)	EF633687	99.44%	Spring water
	Chromobacterium sp. (1)	EF633687	96.23%	Spring water
	Chromobacterium sp. (2)	DQ985277	98.31%	Blackbird wetland soil
	Chromobacterium sp. (1)	EF633687	96.24%	Spring water
	Aquitalea sp. (1)	AB277847	97.74%	Denitrification reactors
	Chromobacterium sp. (1)	EF633687	98.12%	Spring water
	Delftia sp. (1)	AJ237966	91.15%	Industrial waste water
	Brevundimonas diminuta (1)	EU977704	99.80%	Clean-room floor
	Ochrobactrum sp. (1)	AB508888	98.21%	Rice paddy soil
Deinococcus-Thermus	*Deinococcus* sp. (1)	EF193389	98.27%	Phyllosphere
Firmicutes	*Staphylococcus* sp. (3)	FJ773995	99.44%	Soil
	Bacillus sp. (1)	HM235923	99.45%	Tobacco cultivation soil
Bacteroidetes	*Chryseobacterium* sp. (1)	AY464462	98.50%	Agricultural setting

to maintain the pH 2. The bioleaching process is often based on acidophilic bacteria such as *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, and *Acidithiobacillus thiooxidans*, which have been implicated as being the most applicable bacteria involved in operation of biological metal-removal processes including Zn [18]-[20].

Table 3. Phylogenetic affiliation and distribution of bacterial 16S rRNA gene sequences analyzed from the TS library.

Phylogenetic group	Closest sequences/microorganism	Acession no.	Identity (%)	Habitat of closest relative
Proteobacteria	*Pseudomonas* sp. (1)	DQ192044	99.05%	Asphalt seeps
	Pseudomonas sp. (1)	DQ213044	98.50%	Yellow River estuary
	Pseudomonas sp. (1)	EU162043	96.84%	Compost
	Acinetobacter sp. (4)	HM489955	97.24%	Intestinal tract
	Escherichia coli (2)	FJ823386	98.92%	Soil
	Burkholderia sp. (8)	FJ603038	99.60%	Surface of weathered rock
	Burkholderia cenocepacia (2)	EF602551	99.63%	Sugarcane stem
	Massilia sp. (1)	AM237367	97.95%	Barnyard dust
	Chromobacterium haemolyticum (4)	DQ785104	99.24%	Sputum culture
	Stenotrophomonas maltophilia (1)	AJ293474	99.25%	Sewage
	Sphingomonas sp. (1)	FJ455064	96.11%	Aerial part
	Sphingomonas sp. (1)	AY749436	99.56%	Long term banking of genome resources
	Sphingomonas sp. (1)	EU931555	98.25%	Sugarcane roots
	Methylobacterium sp. (1)	AB252203	98.80%	Freshwater
	Paracoccus sp. (1)	AM275338	97.37%	Deep sea sediment
Actinobacteria	*Kocuria* sp. (1)	AY745813	98.09%	Eastern Chinese Sea
	Rothia aeria (1)	EU293888	100%	Human infection
	Micrococcus sp. (5)	AY745846	94.46%	Changjiang estuary
	Actinomyces sp. (1)	AJ234049	97.68%	Canine and feline clinical specimens
	Propiniobacterium acnes (2)	DQ672259	99.74%	Microdiscectomy
	Corynebacterium sp. (1)	AF537600	98.55%	Blood
	Corynebacterium sp. (1)	AF537593	87.99%	Blood culture
Firmicutes	*Bacillus* sp. (9)	FJ465012	98.81%	Soil at 30 - 50 m elevation
	Bacillus sp. (1)	GU171355	95.42%	Soil from lawn
	Bacillus sp. (6)	FN666893	99.51%	Landfill 3ft depth soil
	Bacillus sp. (2)	HM566654	97.64%	Soil
	Bacillus subtilis (1)	EU753871	93.09%	Lettuce
	Staphylococcus sp. (1)	GU797281	98.79%	Dental caries
	Turicibacter sp. (1)	NR_028816	85.76%	Blood culture of a patient with appendicitis
Cyanobacteria	*Chroococcus* sp. (1)	AM710384	91.85%	Freshwater reservoir

In contrast to these reports we reveal herein heterotrophic bacteria phylogenetically distinct from acidophilic bacteria.

It is well-established that metals can be removed from a variety of wastes by lowering the pH either by the

Table 4. Phylogenetic affiliation and distribution of bacterial 16S rRNA gene sequences analyzed from the TSC library.

Phylogenetic group	Closest sequences/microorganism	Acession no.	Identity (%)	Habitat of closest relative
Proteobacteria	*Escherichia* sp. (1)	GU594294	94.38%	Clinical samples
	Escherichia sp. (1)	HM576813	97.97%	Gastric ulcer swine
	Escherichia sp. (51)	FJ405310	99.66%	Swine
	Escherichia sp. (14)	AP010960	99.77%	Human infection
	Escherichia sp. (1)	DQ411026	96.19%	NOx removal system
	Enterobacter sp. (1)	GQ451698	99.32%	High biodiversity regions of India
	Pseudomonas sp. (2)	AF326375	97.75%	Isolated from pulpmill effluent
	Pseudomonas japônica (1)	AB126621	98.93%	Activated sludge sample
	Acinetobacter sp. (1)	GQ284532	98.22%	Mangrove sediment
	Chromobacterium sp. (1)	EF633687	98.94%	Spring water
	Alcaligenes faecalis (1)	AY959943	99.89%	Swine wastewater sludge
	Ochrobactrum anthropi (1)	DQ647056	99.64%	Nodules of *Cicer arietinum*
	Sphingomonas sp. (2)	EU682685	96.34%	Forest soil
Firmicutes	*Bacillus* sp. (1)	FJ615521	99.31%	Stratosphere
	Bacillus sp. (1)	HM629506	95.94%	Mangrove sediment
	Bacillus thuringiensis (1)	FJ462697	99.89%	Plant
	Bacillus sp. (1)	EF032682	96.02%	Goat skin
	Bacillus sp. (1)	FJ528074	91.69%	Farmland
Actinobacteria	*Rubrobacter* sp. (1)	FJ497714	98.30%	Vailulu'u Seamount

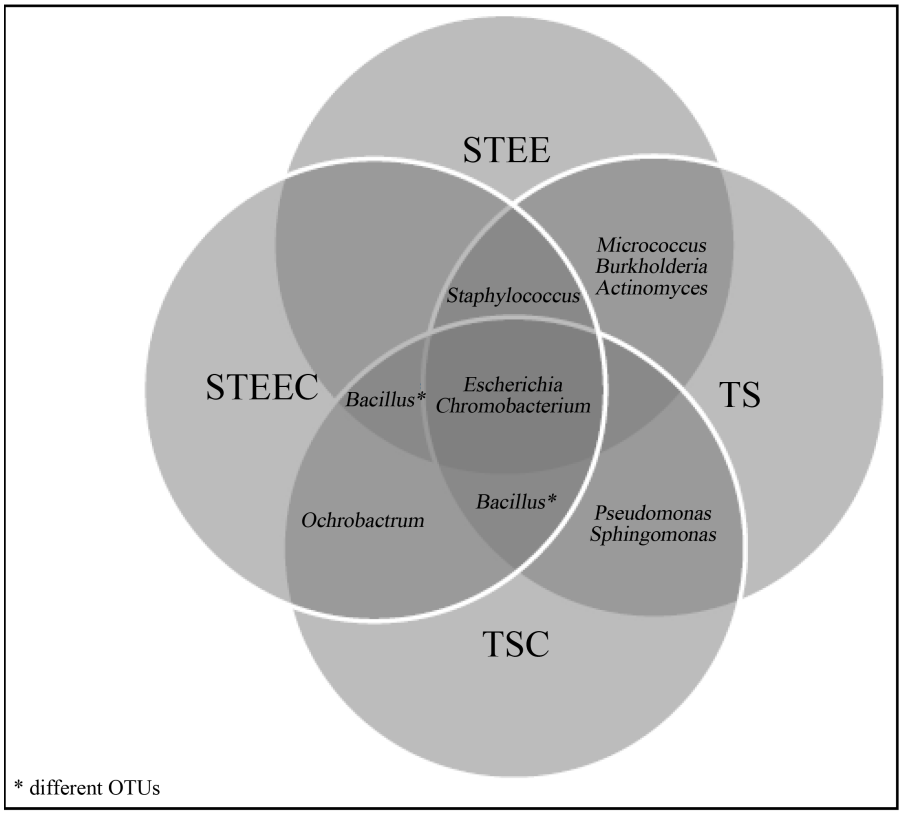


Figure 3. Comparative analysis of the OTU sequences shared among the four libraries.

addition of acids or by the production of acids by bacterial activity. As expected, we found that a chemical Zn leaching process also occurred in our un-inoculated steelmaking waste samples.

The pH increase observed at the first seven days in both wastes, could be explained by consumption of H^+ protons, which could be illustrated by the equation $Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow Fe^{3+} + H_2O$ [21]. Even during this period, bacterial growth could occur through the energy obtained in the Fe^{2+} oxidation. The Fe^{3+} formed would be further used as in the equation $4Fe^{3+} + ZnS + 2H_2O + O_2 \rightarrow Zn^{2+} + 4Fe^{2+} + SO_4^{-2} + 4H^+$, resulting in the release of protons and completing the iron redox cycling. Therefore, the combination of chemical reactions in acidic conditions, along with the emergence of bacterial metabolic activity favored the maintenance of a pH value of 2, at one week of leaching of the steelmaking samples analyzed. Thus, pH decrease in the bioleaching assays may be an indication of the bacterial activity enhancing the iron redox cycling and leading to an effective solubilization of Zn as suggested by Marhual *et al*. [22].

Using culture-independent molecular and enrichment culture approaches, this study provided insight into the bacteria from STEE, TS, STEEC, and TSC by revealing their phylogenetic identity. Overall, the steelmaking wastes harbored few lineages, suggesting an unfavorable environment for the bacterial communities present in them. Proteobacteria was systematically the phylum predominant (from 50% to 87%) in all the clone libraries. According to Chen *et al*. [23], in a metal-rich environment the proteobacterial account for up to 70% of 16S rRNA gene clone library sequences. Although the number of genera found was only eight, this ubiquitous phylum harbors several members associated with iron redox cycling and ability to leach metal in steelmaking wastes. Other phyla also disclosed herein have already been shown to harbor members with similar abilities [24]-[26].

Diverse bacterial taxa can be present in a metal-rich environment. However, metal-tolerant bacteria appear to be present as primary heterotrophic colonizers of exposed minerals [27]. Moreover, Johnson and Roberto [28] reported that heterotrophic bacteria can obtain carbon sources through the wastes products produced by the autotrophs and play a role in the mineral degradation processes. Given these data, it is likely that Zn leaching bacteria found in our steelmaking waste sample are also heterotrophic.

The *Chromobacterium*, *Escherichia*, *Bacillus*, and *Ochrobactrum* genera were common in the primary enrichment of both clone libraries, suggesting that they could play a role in Zn bioleaching of steelmaking wastes. Previous studies reported bioleaching ability of *Chromobacterium violaceum* to mobilize diverse metals, including Zn, from solid and electronic materials [29] [30]. An unexpected result was the occurrence of a strong dominance of *Escherichia* in the TSC waste. Indeed, it is not clear why and how this dominance occurs in such a poor carbon source environment and therefore further studies will be needed to clarify this matter. One possibility is the versatile behavior of *E. coli* which is able to tolerate and rapidly adapt to diverse stress environmental conditions such as low pH and strongly carbon-limited, among others (reviewed by [31]). Although Lin *et al*. [32] reported the ability of *E. coli* to survive at low pH, it is the first time that this genus has been connected with Zn extraction and the first time it has been reported in steelmaking wastes.

Representatives of *Bacillus* have been reported to efficiently solubilize metals and thus contribute to metal leaching without any benefit to themselves [7] [33]. Only one other study found *Bacillus* from steelmaking waste sources. That work, which was from our laboratory, reported an interesting dominance of this genus in Blast Furnace Sludge wastes [34]. We also found members of *Ochrobactrum* in our steelmaking wastes. Ozdemir *et al*. [35] reported that members of this genus are involved in the solubilization of other metals such as chromium, cadmium, and copper. However, we did not find such metals in our samples, indicating the ability of *Ochrobactrum* to solubilize metals other than those.

Aeromonas was only found in the STEEC clone library, where it shared dominance with *Chromobacterium*. Indeed, a previous study showed the presence of *Aeromonas* in uranium deposits [36] while another study revealed that members of this genus were able to reduce Fe^{3+}, nitrate, and sulfate [37].

This study is the first report of survival of heterotrophic bacteria from steelmaking wastes under extremely acidic conditions, such as *Chromobacterium*, *Aeromonas*, *Escherichia*, *Bacillus*, and *Ochrobactrum*. The data presented herein may be relevant for the management of this waste, particularly in the case of Zn extraction, making these bacteria candidates for future studies about metal bioleaching.

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