Metagenomic signatures of a tropical mining-impacted stream reveal complex microbial and metabolic networks

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HIGHLIGHTS

• The microbiome of a metal-rich tropical stream sediment was studied by metagenomics.
• The metagenome showed complexity of microbial interaction and metabolic processes.
• Functional analysis highlighted nitrogen and methane metabolic pathways.
• Metal stress seems to contribute to distinct life strategies in this environment.

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ABSTRACT

Bacteria from aquatic ecosystems significantly contribute to biogeochemical cycles, but details of their community structure in tropical mining-impacted environments remain unexplored. In this study, we analyzed a bacterial community from circumneutral-pH tropical stream sediment by 16S rRNA and shotgun deep sequencing. Carrapatos stream sediment, which has been exposed to metal stress due to gold and iron mining (21 [g Fe]/kg), revealed a diverse community, with predominance of Proteobacteria (39.4%), Bacteroidetes (12.2%), and Parcubacteria (11.4%). Among Proteobacteria, the most abundant reads were assigned to neutrophilic iron-oxidizing taxa, such as Gallionella, Sideroxydans, and Mariprofundus, which are involved in Fe cycling and harbor several metal resistance genes. Functional analysis revealed a large number of genes participating in nitrogen and methane metabolic pathways despite the low concentrations of inorganic nitrogen in the Carrapatos stream. Our findings provide important insights into bacterial community interactions in a mining-impacted environment.

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1. Introduction

Freshwater ecosystems are threatened by a myriad of pollutants, especially those generated by mining activities. Among these, iron ores mining is of great importance since iron is the most mined metal worldwide (Edwards et al., 2004). Brazil is one of the world’s largest producers of iron ores (Yellishetty et al., 2010), which considerably impact Brazilian ecosystems.

Iron exists in different oxidation states, but in biological systems, it can be found as Fe(II) or Fe(III). Redox cycling of iron in aquatic ecosystems can be closely tied to biogeochemical transformations of other elements, including carbon, nitrogen, and sulfur. In addition, iron cycling is important for pollutant transformation and mobility, meaning great environmental relevance of iron (Chiorse, 1984; Lovley, 2000; Emerson and Weiss, 2004; Picardal and Cooper, 2005; Roden and Emerson, 2007).

Iron redox reactions are often driven by microorganisms. Fe-reducing bacteria (FeRB), e.g., Geobacter sulfurreducens, couple reduction of Fe(III) with oxidation of organic matter or molecular hydrogen, while Fe-oxidizing bacteria (FeOB), which occur in many phyla (e.g., Nitrospirae, Firmicutes, Actinobacteria, Chlorobi, and
especially Proteobacteria), can use Fe(II) or molecular hydrogen as electron donors (Lovley et al., 1989; Jiao et al., 2005; Emerson et al., 2010; Byrne et al., 2015).

Recent advances in metagenomic technologies allow for more comprehensive assessment of the microbial biodiversity and functioning in aquatic environments. Sediments are important for metal cycling in these ecosystems because they can retain over 90% of metals and metalloid pollutants and have higher microbial diversity than bulk water (Lozupone and Knight, 2007; Mortatti et al., 2010).

To our knowledge, only a few other studies that involved pyrosequencing and denaturing gradient gel electrophoresis have shown the structure of microbial communities in iron-rich environments at circumneutral pH (Gorra et al., 2012; Wang et al., 2014; Emerson et al., 2015). Although FeOB are present under oxic and anoxic conditions, from extremely acidic to circumneutral pH, only a few genera of neutrophilic FeOB have been identified to date, e.g., Gallionella, Sideroxydans, Ferriphilus, Ferritrophicum, and Mariprofundus (Emerson et al., 2010; Kato et al., 2014). Therefore, iron-rich sediments of tropical freshwater streams are not sufficiently explored.

In this study, we performed a taxonomical and functional analysis of a microbial community from mining-impacted stream sediments of tropical freshwater streams are not sufficiently explored.

2. Materials and methods

2.1. The study site

The Carrapatos stream (CS: 19°58’15.4”S and 43°27’50.7”W) is located in the Iron Quadrangle (Minas Gerais state, Brazil), a region rich in ores, especially iron ones, explored by mining since the 19th century. One study (Reis et al., 2014) showed that CS can be classified as a mesotrophic, circumneutral (pH 7.9), and mesothermal (17 °C) environment. Furthermore, its sediment contains high metal concentrations, including magnesium (1416 mg kg⁻¹), manganese (2319 mg kg⁻¹) and especially iron (21,850 mg kg⁻¹) (Reis et al., 2014).

2.2. DNA extraction

A composite sample from CS sediment (100 g) was collected, and DNA was extracted using the PowerSoil DNA Extraction Kit (MoBio Laboratories, USA). Quantification of total DNA was performed using a Qubit fluorometer (Invitrogen—Life Technologies, USA).

2.3. Sequencing and analysis of the V3 and V4 regions of the 16S rRNA gene

For taxonomic profiling, primers S-D-Bact-0341-b-S-17 (5’CTACGGGNGGCWGCAG3’) and S-D-Bact-0785-a-A-21 (5’GACTACHVGGGTATCTAATCC3’) (Klindworth et al., 2013), targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene were used, with Illumina adapters added. The amplification step was performed with the KAPA HiFi HotStart ReadyMix (KAPA, Woburn, MA, USA), followed by purification with AMPure XP beads (Agencourt Bioscience, Beverly, MA, USA). Library construction was performed according to the manufacturer’s instructions for paired-end sequencing on the MiSeq platform (Illumina, Inc., USA).

Bioinformatic analysis was carried out in the Mothur software, version 1.33.0 (http://www.mothur.org). Sequences of low quality (Q ≤ 20, with ambiguities and homopolymers above eight) or outside the range of 422–466 bp were discarded. Filtered reads were aligned and classified against the Silva v.223 16S rRNA database (Quast et al., 2013). Mitochondrial and chloroplast reads and reads that did not match any reference sequence were discarded. Chimeric reads were identified and removed by means of Uchime (http://drive5.com/uchime). The remaining reads were grouped into operational taxonomic units (OTUs), with 97% similarity, by the average neighbor method. Construction of the rarefaction curve, calculated for the evolutionary distance of 0.03, was conducted according to the Mothur pipeline (Schloss et al., 2009). The nucleotide sequences were deposited to GenBank [GenBank ID: SRR2087753].

2.4. Shotgun sequencing and analysis

To obtain a functional profile of the metagenome, whole-community shotgun sequencing from the CS sediment was performed on a SOLiD™ v.4 sequencer (Applied Biosystems), according to the manufacturer’s protocol. Briefly, 10 μg of total DNA was randomly fragmented using the Covaris™ S2 system (Life Technologies, USA). DNA fragment library (200—250 bp long) was then constructed. Emulsion PCR was carried out to clonally amplify fragments on sequencing beads, followed by enrichment, deposition in a plate, and sequencing. The DNA fragment size of the library was verified using the Agilent 2100 Bioanalyzer.

Bioinformatic analysis comprised an initial step of error screening in the SOLiD™ Accuracy Enhancer Tool (SAET) software (http://solidsoftwaretools.com/gf) and conversion of sequences from color space to letter space format by means of the encodeFasta.py software (http://gnome.googlecode.com/svn/trunk/pyGenotypeLearning/src/pytools/encodeFasta.py). Filtered reads were assembled in the Metavelvet software, as described by Namiki et al. (2012). Contigs were analyzed by means of the Metagenomics RAST Server (MG–RAST v3.3) (Meyer et al., 2008), which provides quality control (duplicate removal and size or base quality screening) prior to annotation. Functional analysis was conducted using SEED and KEEG subsystems (available on the MG-RAST Server), with an e-value of 10⁻³ and identity of 60% (Mitra et al., 2011; Costa et al., 2015). Sequencing data of the present study are available at MG-RAST (code 4520540.3).

The metagenome from the CS sediment was compared with two other metagenomes, one from an arsenic-rich sediment (Mina stream sediment [MSS]: MG–RAST ID 4519449.3) and another from a low-metal-concentration freshwater sediment (Férin [FER]: NCBI Bioproject ID 246439), described by Costa et al. (2015) and Gillan et al. (2014), respectively. These metagenomes were chosen for the comparative analysis on the basis of the study region similarity with CS (MSS) and availability of pristine-environment data (FER).

To identify possible significant differences with CS in the functional composition of each metagenome, we performed two-sided Fisher’s exact test (Fisher, 1958), with the p value > 0.05, using the Statistical Analysis of Metagenomic Profiles (STAMP; Parks and Beiko, 2010).

Metagenomic recruitment plots were used to identify the abundant species in the CS metagenome, by comparing the CS contigs with individual bacterial genomes available in the National Center for Biotechnology Information (NCBI) database, as reported by Costa et al. (2015).

2.5. Quantitative real-time PCR (qPCR)

qPCR was used to estimate the absolute number of copies of bacterial 16S rRNA in the sediment. The primers were 338F (5’TACGAGACGACACC3’) (Raskin et al., 1994) and 518R
with abundance below 2% were grouped into 3.5% of all the bacterial reads, this phylum harbored the fourth most ranging from 2% to 4%. Although Chloro, OP3, and Planctomycetes were present in lower proportions, Nitrospirae, Verrucomicrobia, Microgenomates, Candidate division fl

Other phyla such as Cyanobacteria, Chloro(11.4%) were the predominant phyla, accounting for 63% of all reads. Bacteroidetes (12.2%), and Parcubacteria (candidate division OD1:3.1. An overview of the taxonomic composition and abundance based on 16S rRNA and qPCR data

The high-quality reads were grouped into 34,827 OTUs. Table S1 summarizes the quality control dataset from the CS library. The number of reads and taxonomic assignments of these OTUs are presented in Table S2. The rarefaction curve (Fig. S2) and high Good’s coverage value (90%) indicated that the sequencing depth was sufficient to capture overall diversity. qPCR revealed the bacterial concentration of $1.04 \times 10^8$ cells/g in the CS sediment (Fig. S1).

The taxonomic data indicated high diversity in CS, spanning a wide spectrum of phyla (Fig. 1 and Table S2) in addition to unclassified bacteria (5.9% of all the reads). Proteobacteria (39.4%), Bacteroidetes (12.2%), and Parcubacteria (candidate division OD1:11.4%) were the predominant phyla, accounting for 63% of all reads. Other phyla such as Cyanobacteria, Chloroflexi, Acidobacteria, Nitrospirae, Verrucomicrobia, Microgenomates, Candidate division OP3, and Planctomycetes were present in lower proportions, ranging from 2% to 4%. Although Chloroflexi accounted for only 3.5% of all the bacterial reads, this phylum harbored the fourth most abundant OTU (Anaerolineaceae) of the CS metagenome. OTUs with abundance below 2% were grouped into “Other bacteria”, which included Chlamydiae, Actinobacteria, Elusimicrobia, Chlorobi, Spirochaetes, Gemmatimonadetes, Armatimonadetes, Saccharibacteria, and Gracilibacteria, representing 8.9% of all reads (Table S2).

Proteobacteria comprised broad diversity, with five identified classes: Beta- (44.61%), Delta- (19.8%), Alpha- (17.1%), Gamma- (13.1%), and Zetaproteobacteria (2.7%) (Fig. 1). The top 50 proteobacterial OTUs accounted for 48.4% of all the proteobacterial reads, comprising 46% of representatives of various orders or families, such as Gallionellaceae (15.3%), Comamonadaceae (7.5%), Methylcoccales (4.7%), Desulfuromonadales (3.7%), Maripridaee (2.5%), Rhodobacteraceae (2.5%), Hyphomonadaceae (2.4%), Rhizobiales (2.3%), Bdellovibrionaceae (1.1%), Sphingomonadales (1%), Rhodocyclaceae (0.9%), and Caulobacteraceae (0.5%). The remaining OTUs were classified only at phylum (0.05%) or class levels (Betaproteobacteria, 3.1%).

Among the 23 most frequent OTUs (>1000 reads), 10 OTUs were affiliated at the genus level, namely Gallionella, Maripridaee, Sideroxydans, and Woodsholea (Proteobacteria); Haliscomenobacter, Sediminibacterium, and Leadbetterella (Bacteroidetes); and Nitrospira (Nitrospira).

3.2. Functional analysis of the metagenome

The whole-community shotgun sequencing generated approximately 14.5 Gbp, corresponding to 289,015,844 reads 50 bp long. After MG-RAST quality filtering, 278,993,472 reads remained for further analysis (~13.9 Gbp of reads). Assembly of individual reads by means of Metavelvet resulted in 439,778 contigs, with the length of 157 ± 98 bp (mean ± SD).

Functional annotation of the CS metagenome revealed that 48% of the contigs (183,032/380,882) can be classified against protein databases available at MG-RAST. Among these, approximately 73% (133,356 contigs) were assigned to functional categories.

The comparison between the CS metagenome and reference genomes showed that the majority of contigs were assigned to Sideroxydans litoralis (24,064 contigs) and Gallionella capsiferriformans (19,255 contigs), which are neutrophilic FeOB (Fig. 2). Other bacterial species were reasonably well recruited, such as Leptothrix cholodni, which is also a FeOB, Nitrosospira multiformis ATCC 25196 and Nitrosomonas europaea ATCC 19718, both ammonia-oxidizing bacteria (Fig. 3).

A broad range of functional categories was detected by SEED subsystems (Fig. S3). The highest proportions of functional genes were categorized into the clustering-based subsystem (unknown function) and protein metabolism subsystem (10% each), followed by the miscellaneous subsystem (9%); RNA metabolism subsystem (7%); and virulence, disease, and defense subsystem (4%). The latter harbored heavy-metal (and metalloid) resistance genes, mainly cobalt–zinc–cadmium resistance (20%), copper homeostasis (12%), and arsenic resistance (9%).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper tool, available at MG-RAST, produced an integrated view of the CS microbiome metabolism. KEGG pathway analysis revealed that the CS metagenome contigs were distributed among the majority of the metabolic pathways, including 33 of 68 genes involved in nitrogen metabolism and 13 of 34 genes related to methane metabolism (Figs. S4 and S5). The SEED subsystem classification ranked the most abundant functions in the nitrogen metabolism subsystem, such as nitrate and nitrite ammonification, ammonia assimilation, and nitrogen fixation.

Functional comparative analysis between CS metagenome and MSS metagenome (arsenic-rich stream: Fig. 4) and between CS metagenome and FER metagenome (low-metal freshwater sediment; Fig. 5) revealed different profiles.
4. Discussion

The main purpose of this study was to enlighten the connections between microbial functions and community structure of a tropical stream sediment exposed to long-term mining activities.

The metagenomic data revealed a diverse bacterial community characterized by a complex metabolic network. Our results are consistent with other studies that showed the dominance of Proteobacteria in communities from metal-rich environments (Horner-Devine et al., 2004; Chen et al., 2008; Reis et al., 2013). Members of FeOB are distributed among a variety of phyla, such as Nitrosirae and Firmicutes, but Proteobacteria, especially all neutrophic iron-oxidizing Beta- and Zetaproteobacteria, are the most numerous (Hedrich et al., 2011). Noticeably, the assignment of Mariprofundus (iron-oxidizing Zetaproteobacteria), which was detected in our sample, contradicts the findings of Hedrich et al. (2011). Our results do not support the separation of neutrophic Fe oxidizing Beta- and Zetaproteobacteria by their habitat: freshwater (Betaproteobacteria) versus marine water (Zetaproteobacteria). This marine genus is a representative of a group that has the ability to produce an Fe-oxyhydroxide-encrusted helical stalk: apparently a requirement for the use of ferrous iron, Fe(II), as an energy source (Singer et al., 2011).

The enrichment of the CS community in various neutrophilic FeOB groups points to their importance for iron cycling in this ecosystem. Other studies on the phylogenetic diversity and ecological properties of Gallionella-related FeOB revealed that this group is highly adapted to metal-rich environments because its genome harbors several resistance genes to different metals and metalloid, such as arsenic, mercury, cadmium, cobalt, silver, zinc, and copper (Emerson et al., 2013; Reis et al., 2014). Furthermore, Emerson et al. (2013) reported that both Gallionella and Sideroxydans have a rich genetic repertory for adaptation to stressful conditions, such as environmental sensing, motility, and chemotaxis; this repertory may allow them to survive in this mining-impacted ecosystem. Crenothrix-related OTUs (3566 reads) represented an abundant genus in the CS community. These filamentous FeOB are closely related to methanotrophs known to promote steel corrosion (Coetser and Cloete, 2005; Stoecker et al., 2006). Twenty-three OTUs (417 reads) were found to be affiliated with the Ferrophasetus genus, novel stalk-forming neutrophilic FeOB, recently described by Kato et al. (2014).
The most abundant genera affiliated with Bacteroidetes were *Haliscomenobacter*, *Sediminibacterium*, and *Leadbetterella*, which are not well studied at present. Members of this phylum are widely distributed in nature, especially in aquatic environments (Weon et al., 2005). The *Haliscomenobacter* genus comprises filamentous species that inhabit the pelagic zone of natural freshwater lakes and ponds (Hahn and Schauer, 2007; Santos et al., 2015). *Sediminibacterium* is rarely found in mining-impacted environments although it was previously recovered from metal-rich sediment (Reis et al., 2013). *Leadbetterella* was originally isolated from cotton-
The third most abundant phylum in the CS community is Parcubacteria. Members of this phylum have been identified in lakes, mainly under suboxic conditions, but they were barely studied. High abundance of this phylum was reported only in a few studies (Briée et al., 2007; Peura et al., 2012), including the present one. According to Peura et al. (2012), this finding could be explained by insufficient coverage of Parcubacteria members in previous bacterial PCR surveys, due to the use of general bacterial primers, which do not target this group. The primers used by Peura et al. (2012) and in our study span variable regions V3 and V4 of the 16S rRNA gene, thereby promoting much greater coverage of known sequences of Parcubacteria. Recently, Parcubacteria (OD1), Microgenomates (OP11), Saccharibacteria (GN02), and Gracilibacteria (TM7) phyla, represented by 15% of all the reads here, were proposed to form a new superphylum called Patescibacteria (Rinke et al., 2013; Sekiguchi et al., 2015; Shipunov, 2015). Genomic and metagenomic studies showed that members of this superphylum have reduced metabolic capabilities, which likely limit their cultivation (Kantor et al., 2013; Rinke et al., 2013; Brown et al., 2015). They are also involved in hydrogen production, sulfur cycling (Wrighton et al., 2012, 2014), and anaerobic methane oxidation (Peura et al., 2012). Because of the high proportion of members of this superphylum and genes related to the methane oxidation pathway, it is possible that these bacteria are responsible for methane oxidation in the CS sediment.

Anaerolineaceae-related reads represented a majority in the Chloroflexi phylum (77.6% of all the Chloroflexi reads). This phylum was previously identified as green nonsulfur bacteria and includes relatively unstudied organisms with diversified metabolism (Hug et al., 2013). The Anaerolineaceae family was formally proposed in 2006 and comprises heterotrophic and anaerobic bacteria inhabiting many environments, including sulfur springs and sulfide-rich anaerobic reactors (Yamada et al., 2006; Youssef et al., 2012; Dias et al., 2016). It is still unclear whether these bacteria can use sulfur compounds in their metabolic reactions (Youssef et al., 2012), but the presence of this taxon, in addition to other OTUs related to bacteria involved in sulfur cycling, e.g., Thiotrichales and Desulfuramonadales, may be an indication of sulfur compound transformations coupled to iron redox reactions in the CS sediment.

The functional analysis of the CS metagenome revealed dominance of clustering-based and protein metabolism subsystems, whose genes are thought to be related and linked functionally (Hu et al., 2010). Hence it is quite difficult to comment on the genes assigned to these subsystems or to speculate on their roles. We detected high abundance of contigs related to protein metabolism, including biosynthesis, degradation, folding, processing, and modification of proteins. This result reflects the high diversity, abundance, and metabolic activity of microorganisms in the CS sediment.

Although the Carrapatos freshwater sample showed low concentrations of inorganic forms of N, in contrast to the arsenic-rich freshwater (MSS) analyzed by Costa et al. (2015), the CS metagenome over-represents the nitrogen metabolic pathway. Reads related to N2 fixation were obtained here, possibly pointing to an activity of Sideroxydans, neutrophilic FeOB that possess three clusters of nif genes. This feature may allow these bacteria to occupy niches depleted of nitrogen and may confer a competitive advantage over other organisms. Furthermore, although ammonia-oxidizing bacteria were well represented in the shotgun results, only a few reads were detected in the 16S rRNA dataset (222/229,995 reads). This discrepant result may be due to the fact that shotgun sequencing does not depend on PCR amplification; thus, it is not affected by primer bias. Additionally, in a previous study on ammonia oxidizing bacteria in tropical stream sediments by Reis et al. (2015), the number of copies of the amoA gene on the same CS sediment sample was determined.

Environments that have high Fe concentrations may also have relatively high concentrations of other metals and metalloids,
including toxic ones (Gibbs, 1977). The resistance to antibiotics and toxic compounds was highlighted in the category of virulence, disease, and defense. Indeed, this resistance ensures survival of the microbial community in the contaminated environment of the CS sediments. Fluoroquinolones and methicillin are among the antibiotics whose resistance genes were detected in the CS sediment. These genes are also present in environments that receive human and animal waste as well as heavy metals and metalloid (e.g., cobalt, zinc, cadmium, copper, and arsenic) (Borrás et al., 2012; Li et al., 2012). Probably, antibiotic resistance and heavy-metal resistance genes were coselected, thus contributing to survival in this sediment. Emerson et al. (2013) reported genes responsible for arsenic resistance and genes that encode a heavy-metal efflux pump of the cation diffusion facilitator (CDF) family. This efflux pump is responsible for removal of structurally unrelated toxic metals like cadmium, cobalt, silver, zinc, and copper from the cytoplasm. These genes were found in the genomes of Sideroxydans lithotrophicus and Gallionella capsiferriformis.

Functional comparisons between the CS and MSS metagenomes and between the FER and CS metagenomes revealed that the functional categories recovered from the CS metagenome were more similar to those from the FER metagenome than to functional categories from the MSS metagenome. This finding suggests that the CS sediment harbors a metabolic network close to that of the reference sediment. Moreover, these comparisons unveiled overrepresentation of the virulence, disease, and defense subsystem (MSS). Our results also showed overrepresentation of stress response subsystems and subsystems of phages, prophages, transposable elements, plasmids (CS), all which contribute to survival of the microbial community in mining-impacted environments.

The diverse, abundant, and functionally complex bacterial community in the CS sediment thus represents a well-adapted community. This finding contradicts the ecological models that predict that community diversity decreases in response to stressors associated with toxic compounds (Odum, 1985). This apparent discrepancy may be due to the historical contamination of the Iron Quadrangle region. Indeed, other studies suggest that the composition and stability of bacterial communities in historically metal-contaminated sediments become diverse after approximately one century of exposition (Bouskill et al., 2010; Reis et al., 2013).

5. Conclusion

Both shotgun data and 16S analysis point to a diverse community in CS sediment, where many metabolic processes take place, as confirmed by detection of genes related to iron, methane, nitrogen, and sulfur pathways. Moreover, our data highlight the occurrence of rare taxa, like Leadbetterella, and to our knowledge, unprecedented findings about Woodsholea and Mariprofundus, previously recovered from marine environments. These results provide insights into the interactions and metabolic abilities of bacteria in mining-impacted sediments and may contribute to elucidate the role of bacteria in environments exposed to long-term metal stress.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.06.097.

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