Pyrite oxidization accelerates bacterial carbon sequestration in copper mine tailings

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Abstract. Polymetallic mine tailings have great potential as carbon sequestration tools to stabilize atmospheric CO_2 concentrations. However, previous studies focused on carbonate mineral precipitation, while the role of autotrophs in carbon sequestration by mine tailings has been neglected. In this study, carbon sequestration in two mine tailings treated with FeS_2 and ^{13}C-labeled CO_2 was analyzed using ^{13}C isotope labeling, pyrosequencing and DNA-based stable isotope probing (SIP) to identify carbon fixers. Mine tailings treated with FeS_2 exhibited a higher percentage of ^{13}C atoms (1.76±0.06 in Yangshanchong and 1.36±0.01 in Shuimuchong) than the control groups over a 14-day incubation, as well an increase in the total organic carbon (TOC) content (0.20±0.11 mg/g in Yangshanchong and 0.28±0.14 mg/g in Shuimuchong). These data demonstrated the role of autotrophs in carbon sequestration with pyrite addition. Pyrite treatment led to changes in the composition of bacterial communities, and the genera *Sulfobacillus* (8.04%) and *Novosphingobium* (8.60%) were found to be dominant in these communities. In addition, the DNA-SIP results indicated that the *cbbL* gene copy number was 8.20-16.50 times greater than the *cbbM* gene copy number in ^{13}C-labeled heavy fractions. Furthermore, a *Sulfobacillus*-like *cbbL* gene sequence (cbbL-OTU1) accounted for 30.11-34.74% of all *cbbL* gene sequences in the ^{13}C-labeled heavy fractions of mine tailings treated with FeS_2. These findings highlight the importance of the RuBisCO form I-encoding gene, *cbbL,* in bacterial carbon sequestration and demonstrate the ability of chemoautotrophs to sequester carbon during sulfide mineral oxidation in mine tailings. This study is the first to investigate carbon sequestration by autotrophic groups in mine tailings through the use of isotope tracers and DNA-SIP.

Keywords: mine tailings; pyrite oxidation; autotrophic bacteria

1. Introduction

Soil ecosystems have great potential as carbon sinks to stabilize CO_2 and regulate climate change (White et al., 2000), and atmospheric CO_2 can be fixed into plants by photosynthesis and assimilated into soils as soil organic carbon (SOC) by decomposition and microbial activity (Deng et al., 2016;Antonelli et al., 2018). Currently, chemolithoautotrophic organisms fix atmospheric CO_2 by six pathways, including the widely distributed Calvin-Benson-Bassham (CBB) cycle, the reductive tricarboxylic acid (rTCA) cycle, the reductive acetyl-CoA

pathway and the recently discovered 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycles (Berg, 2011;Alfreider et al., 2017).

The ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme is the most prominent enzyme on earth (Raven, 2013) and is important in the CBB cycle. The CBB cycle is the most prevalent means of CO_2 fixation by autotrophs (Tabita, 1999;Berg, 2011). In addition, the genes encoding the large subunit of RuBisCO serve as autotroph markers (Alfreider et al., 2017;Berg, 2011). The *cbbL* and *cbbM* genes encode RuBisCO form I and form II, respectively, with 25 to 30% amino acid sequence identity (Tabita et al., 2008).

Compared with soil ecosystems, polymetallic mine tailings exhibit specific features, including a lack of organic matter, nutrients and nutrient-holding capacity (Lottermoser, 2010;Young et al., 2015), that restrict plant growth, and plant productivity is generally difficult to restore in mining wastelands (Hu et al., 2018;Li et al., 2017). The limited amount of organic matter in mine tailings also inhibits the activities of heterotrophic microorganisms, and therefore the microorganisms in these environments are lithotroph-dominant (Li et al., 2015). Therefore, these autotrophic microbes may play a role that cannot be ignored for organic carbon sequestration in mine tailings. In addition, polymetallic mine tailings have also considerable potential to stabilize the levels of atmospheric CO_2 (Harrison et al., 2013) through the carbonation of noncarbonate minerals, including the dissolution of silicates, hydroxides and oxides and the precipitation of carbonate minerals (McCutcheon et al., 2016;Meyer et al., 2014;McCutcheon et al., 2014). However, previous studies focused on carbonate mineral precipitation, while the role of autotrophs in carbon sequestration by mine tailings has been neglected.

Polymetallic mine tailings contain sulfide minerals (e.g., pyrite), and the oxidation of these sulfide minerals leads to a decrease in pH, also known as mine tailing acidification. Previous studies have noted that polymetallic mine tailings have lithotroph-dominant microbial compositions (Li et al., 2015) due to the limited amount of organic matter. Consequently, acidophilic, chemoautotrophic bacteria, including *Acidithiobacillus*, *Leptospirillum* and *Sulfobacillus* (Chen et al., 2013;Liu et al., 2014), can promptly participate in ferrous and sulfur oxidation in mine tailings, and these autotrophic taxa play leading roles in carbon cycling and energy flow during the mine tailing acidification process. However, the relationship between the oxidation of sulfide minerals and carbon sequestration by these acidophilic, chemoautotrophic bacteria is still unknown. In the present study, we conducted a microcosm experiment using mine tailings collected from two mines to determine the effects of sulfide mineral (pyrite) oxidation on carbon sequestration in mine tailings through the addition of pyrite. In addition, the main carbon fixers were analyzed through DNA-based stable isotope probing (DNA-SIP) and *cbbL* and *cbbM* gene analysis. Our objectives were to investigate whether sulfide mineral oxidation can stimulate carbon sequestration in mine tailings and to identify the key carbon sequestration groups in mine tailings during the acidification process.

2. Materials and Methods

2.1 Sampling of mine tailings

Samples of mine tailings were collected from the Tongling Yangshanchong (30°54′N, 117°53′E) and Shuimuchong (30°55′N, 117°50′E) mine tailing ponds of copper mines in Anhui Province, East China. Samples of oxidized mine tailings on the surface (0-20 cm) were collected using a steel corer in October 2015. Mine tailing samples stored in sterilized plastic bags were transported to the laboratory in an ice cooler and stored at -20°C before analysis. The properties of the mine tailings were as follows: Yangshanchong acidic samples, pH 3.21, total nitrogen (TN) 0.11 $g \cdot kg^{-1}$, total organic carbon (TOC) 16 $g \cdot kg^{-1}$, SO_4^{2-} 13.32 $g \cdot kg^{-1}$, As_T 63.29 $mg \cdot kg^{-1}$, Fe_T 133.46 $g \cdot kg^{-1}$, Cu_T 1.95 $g \cdot kg^{-1}$, Pb_T 27.58 $mg \cdot kg^{-1}$, and Zn_T 205.44 $mg \cdot kg^{-1}$; Shuimuchong acidic samples, pH 2.92, TN 0.11 $g \cdot kg^{-1}$, TOC 18 $g \cdot kg^{-1}$, SO_4^{2-} 8.84 $g \cdot kg^{-1}$, As_T 51.77 $mg \cdot kg^{-1}$, Fe_T 117.59 $g \cdot kg^{-1}$, Cu_T 2.53 $g \cdot kg^{-1}$, Pb_T 30.43 $mg \cdot kg^{-1}$, and Zn_T 176.59 $mg \cdot kg^{-1}$.

2.2 DNA-SIP microcosms

There were a total of four treatments in the microcosms of the two mine tailings. For each mine tailing, fresh mine tailings (equivalent to 10.0 g d.w.s.) were mixed with a total of 2 g of sterile pulverized FeS_2 at approximately 60% maximum water-holding capacity as the FeS_2 treatment, followed by incubation at 25°C in the dark for 14 days. Yangshanchong mine tailing samples (YM) cultured with FeS_2 are abbreviated as YM_FeS_2, and Shuimuchong mine tailing samples (SM) cultured with FeS_2 are abbreviated as SM_FeS_2. In addition, fresh mine tailings at approximately 60% maximum water-holding capacity without any additive were used as the control groups and abbreviated as YM_ck and SM_ck. For each treatment, the microcosms were incubated with 10% ^{13}C-CO_2 or ^{12}C-CO_2, and both treatments were constructed in triplicate for DNA-SIP analysis.

2.3 Chemical properties analysis

Carbon isotope composition was analyzed by a Delta V Advantage Mass Spectrometer (Thermo Fisher Scientific, Inc., USA) coupled with an elemental analyzer (Flash2000; HT Instruments, Inc., USA) in continuous flow mode. The ^{13}C atom % was calculated as follows:

$$^{13}\text{C atom \%} = \frac{[^{13}C]}{[^{13}C]+[^{12}C]} \times 100$$

The TOC content was analyzed using an element analyzer (Vario MACRO cube, Elementar Inc., Germany). The carbon isotope composition and TOC content were analyzed after performing a soil acidification pretreatment method to remove inorganic carbon as described previously (Wang et al., 2015). The pH of the mine tailing samples was measured using a pH meter (tailings:water=1 g:5 mL) at the end of the microcosm experiment. The Fe^{2+} and Fe^{3+} in the soils were extracted by HCl. The Fe^{2+} in the extract was measured by a spectrophotometric method after mixing with phenanthroline and trisodium citrate, and the Fe^{3+} in the extract was reduced to Fe^{2+} by hydroxylammonium

chloride and measured by a spectrophotometric method (Heron et al., 1994). The total sulfate ion content was determined via ion chromatography after extraction by sodium hydroxide, as described previously (Yin and Catalan, 2003).

2.4 DNA extraction and SIP gradient fractionation

Total DNA was extracted from each sample with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Cleveland, OH, USA) according to the manufacturer's instructions. DNA-based stable isotope probing (DNA-SIP) fractionation was performed as previously described (Zheng et al., 2014), and a total of 14 gradient fractions were generated for each sample. The refractive index of each fractionated DNA was measured via an AR200 digital hand-held refractometer (Reichert, Inc., Buffalo, NY, USA).

2.5 Real-time quantitative PCR analysis of the *cbbL* and *cbbM* genes

Real-time quantitative PCR analysis was performed on a CFX96 optical real-time detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA) to determine the copy numbers of the *cbbL*, *cbbM* and 16S rRNA genes in DNA gradient fractions from the YM_FeS_2 and SM_FeS_2 DNA-SIP microcosms. The K2f/V2r primer pair (K2f: 5'-ACC AYC AAG CCS AAG CTS GG-3' and V2r: 5'-GCC TTC SAG CTT GCC SAC CRC-3') (Nanba et al., 2004), the cbbMF/cbbMR primer pair (cbbMF: 5'-TTC TGG CTG GGB GGH GAY TTY ATY AAR AAY GAC GA-3' and cbbM-R: 5'-CCG TGR CCR GCV CGR TGG TAR TG-3') (Campbell and Cary, 2004) and the 515F/907R primer pair (515F: 5'-GTG CCA GCM GCC GCG G-3' and 907R: 5'-CCG TCA ATT CMT TTR AGT TT-3') (Zhou et al., 2011) were used to amplify the *cbbL*, *cbbM* and 16S rRNA genes, respectively. The reactions were performed in a 20-μL mixture containing 10.0 μL of SYBR Premix Ex Taq (TaKaRa), each primer at 0.5 μM, and 1 μL of DNA template. qPCR analysis of the *cbbL*, *cbbM* and 16S rRNA genes was performed under the following conditions: 40 cycles of 30 s at 95°C, 30 s at 55°C (*cbbL* and 16S rRNA genes) or 57°C (*cbbM* gene), and 45 s at 72°C. Standard curves were obtained using 10-fold serial dilutions of linearized recombinant plasmids containing the *cbbL*, *cbbM* and 16S rRNA genes with known copy numbers. The amplification efficiencies were 90-100%, which were obtained with R^2 values greater than 0.99.

2.6 Pyrosequencing of the 16S rRNA gene

The composition of the bacterial communities in different samples was assessed by pyrosequencing of the 16S rRNA genes. The 16S rRNA gene from the ^{13}C-labeled DNA fraction, which had CsCl buoyant densities of 1.738 $g \cdot mL^{-1}$ in the heavy fraction in YM_FeS_2 and 1.734 $g \cdot mL^{-1}$ in the heavy fraction in SM_FeS_2, was also amplified for pyrosequencing. The 515F/907R primer pair was used for amplification of the V4-V5 regions of the 16S rRNA gene.

Primers were tagged with unique barcodes for each sample. Each sample was amplified in triplicate, and the products were pooled. Negative controls using sterilized water instead of soil DNA extract were included to check for primer or sample DNA contamination. The qualities and concentrations of the purified barcoded PCR products were determined using a NanoDrop spectrophotometer. The bacterial community composition of each sample was assessed by Illumina MiSeq sequencing of the 16S rRNA gene using the MiSeq Reagent Kit v3.

Read merging and quality filtering of the raw sequences were performed using QIIME software with the UPARSE pipeline. The 'identify_chimeric_seqs.py' command was used to identify chimeric sequences according to the UCHIME algorithm, and chimeric sequences were removed with the 'filter_fasta.py' command. Operational taxonomic units (OTUs) were clustered with 97% similarity, and OTU picking and taxonomy assignments were performed with the 'pick_de_novo_otus.py' command for subsequent analysis. The OTUs containing less than 10 reads in the ^{13}C-labeled DNA fractions were removed. The raw amplicon sequence data of the 16S rRNA genes have been deposited in the GenBank sequence read archive under accession number SRP155504.

2.7 Clone library construction of the *cbbL* and *cbbM* genes

Clone libraries of the *cbbL* and *cbbM* genes were also constructed from the ^{13}C-labeled DNA fractions with CsCl buoyant densities of 1.738 g·mL^{-1} in the heavy fraction in YM_FeS_2 and 1.734 g·mL^{-1} in the heavy fraction in SM_FeS_2. The K2f/V2r and cbbMF/cbbMR primer pairs were used to amplify the *cbbL* and *cbbM* genes, respectively. Triplicate amplicons were pooled, ligated into the pGEM-T vector (Promega, Fitchburg, WI, USA), and transformed into competent DH5α cells. One hundred and eighty-eight *cbbL* gene sequences and one hundred and eighty-three *cbbM* gene sequences were obtained by Sanger sequencing of randomly selected positive clones. OTU clustering with 97% similarity was also performed with mothur. Representative OTU sequences of the *cbbL* and *cbbM* genes obtained from clone library sequencing have been deposited in GenBank under accession numbers MH699091 to MH699105.

2.8 Data analysis

Bray-Curtis distance matrices for the overall bacterial community composition among the given samples were calculated in R v.3.3.2 using the 'vegdist' function of the vegan package and visualized by nonmetric multidimensional scaling (NMDS) in Origin 8. A heatmap of dominant genera with relative abundances above 0.2% was applied for plotting in the R environment with the pheatmap package. The translated *cbbL* and *cbbM* sequences from the heavy fractions were used to construct a phylogenetic tree by the neighbor-joining method using the MEGA package, version 7.0.

3. Results

3.1 Pyrite oxidation and carbon sequestration

For the two mine tailing control groups, YM_ck and SM_ck, the pH values (3.25±0.09 in YM_ck and 2.98±0.04 in SM_ck), sulfate (SO_4^{2-}) contents (13.15±2.58 mg/g in YM_ck and 8.95±2.19 mg/g in SM_ck), and total organic carbon contents (16.75±0.09 mg/g in YM_ck and 18.55±0.12 mg/g in SM_ck) exhibited no significant changes after 14 days of incubation (Fig. 1). The addition of pyrite decreased pH values by 0.48±0.16 and 0.41±0.07 in YM and SM, respectively. Pyrite addition also increased the SO_4^{2-} content by 252.96% and 262.35%, Fe^{2+} content by 329.47% and 240.38%, and Fe^{3+} content by 137.47% and 140.37% in YM and SM, respectively. Together these data indicate the occurrence of pyrite oxidization and acidification in mine tailings with the addition of pyrite. Additionally, the TOC content increased by 0.20±0.11 mg/g in YM and 0.28±0.14 mg/g in SM. Furthermore, the ^{13}C atom % values in YM_FeS_2 and SM_FeS_2 were higher than those in the control groups YM_ck and SM_ck, which exhibited ^{13}C atom % values of 1.76±0.06 and 1.36±0.01. This result indicated that the fixation of ^{13}C-CO_2 occurred in these mine tailings with the addition of pyrite. The CO_2-fixing capacities of autotrophs under FeS_2 addition were 9.50±0.91 mg/kg·d in YM and 3.69±0.11 mg/kg·d in SM.

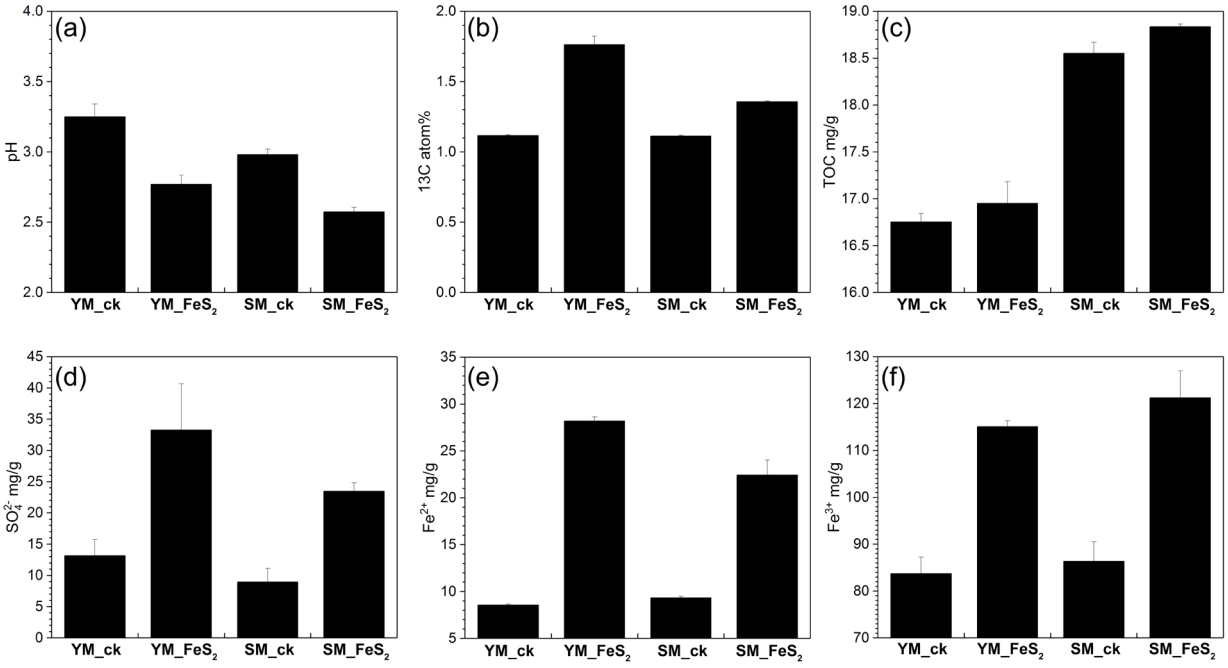

Fig. 1 pH values (a), ^{13}C atom % (b), TOC (c) contents, SO_4^{2-} (d), Fe^{2+} (e) and Fe^{3+} (f) in mine tailings. The error bars indicate the standard errors of three subsamples for each tailing sample. To determine ^{13}C atom % (b), all analyzed samples were treated with ^{13}C-CO_2 in microcosms. YM_ck, control group of Yangshanchong mine tailings; SM_ck, control group of Shuimuchong mine tailings; YM_FeS_2, Yangshanchong mine tailings treated with FeS_2; SM_FeS_2, Shuimuchong mine tailings treated with FeS_2.

3.2 Bacterial communities present during the process of pyrite oxidation

A total of 220877 usable sequences (mean 24541, minimum 9362, maximum 28400) were obtained from total genomic DNA. The ordering of samples by NMDS according to their OTU composition and Bray-Curtis similarity measures (Fig. 2) demonstrated separation of the bacterial community structure in both the YM and SM samples.

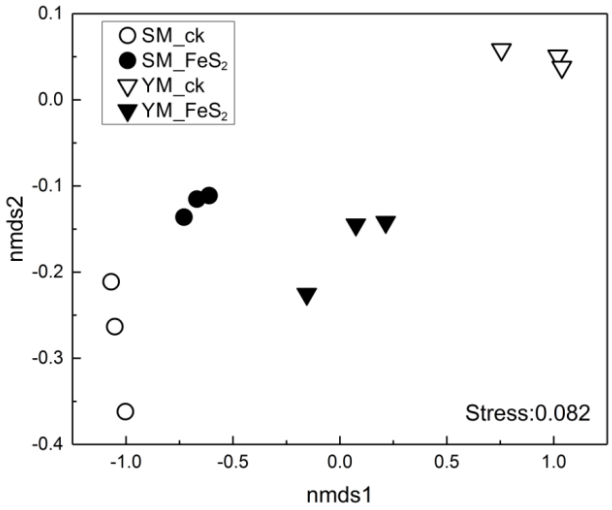

Fig. 2 Nonmetric multidimensional scaling (NMDS) of the overall bacterial community composition by Bray-Curtis distance matrices in mine tailings.

In this study, a total of 8 bacterial phyla and 4 proteobacterial classes were frequently identified in the two mine tailings, including Proteobacteria (mainly composed of classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Deltaproteobacteria), Firmicutes, Acidobacteria, Actinobacteria, WPS-2, Planctomycetes, AD3 and Nitrospirae (Fig. 3). In the Yangshanchong mine tailings, pyrite addition significantly increased the relative abundances of AD3, Nitrospirae and unclassified Proteobacteria by 0.75% (P=0.008), 0.59% (P=0.019) and 6.33% (P<0.001), respectively. In parallel, in Shuimuchong mine tailings, FeS_2 addition significantly increased the relative abundances of Firmicutes, Planctomycetes, unclassified Proteobacteria, Alphaproteobacteria, Betaproteobacteria, and Deltaproteobacteria by 15.69% (P<0.001), 0.97% (P<0.001), 5.88% (P=0.002), 4.35% (P=0.001), 8.61% (P<0.001) and 0.21% (P=0.003), respectively. However, in SM, the percentages of AD3, Acidobacteria, Actinobacteria and Gammaproteobacteria in SM by 0.97% (P=0.002), 7.43% (P=0.002), 1.35% (P=0.016) and 4.85% (P=0.002) were decreased under pyrite addition.

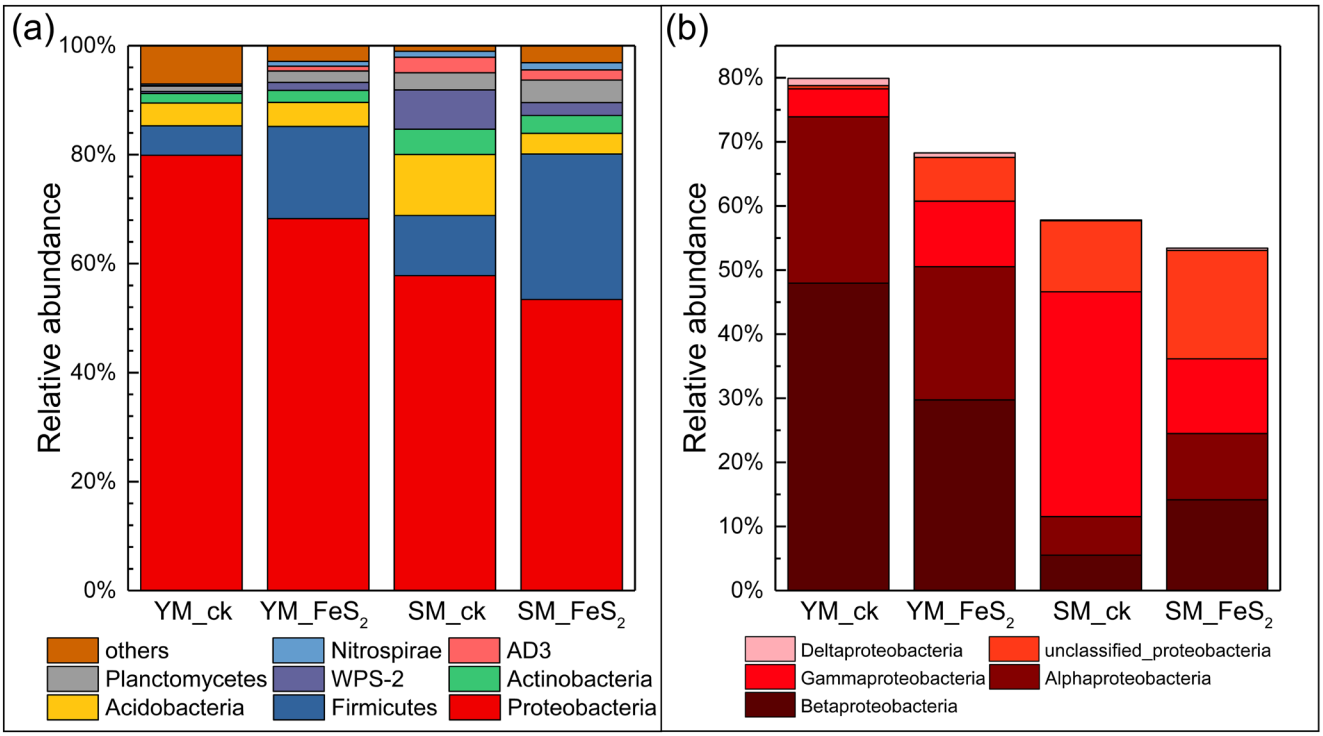

Fig. 3 Relative abundances (percentages) of the main identified bacterial taxonomic groups, i.e., the phyla Proteobacteria, Firmicutes, Acidobacteria, Actinobacteria, WPS-2, Planctomycetes, AD3 and Nitrospirae (a); and the classes Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria and Gammaproteobacteria (within the phylum Proteobacteria) (b). For each tailing sample, the relative abundances of the sequences assigned to a given taxonomic unit were calculated for each of three subsamples, and the average value was then used to represent the relative abundance of each tailing sample.

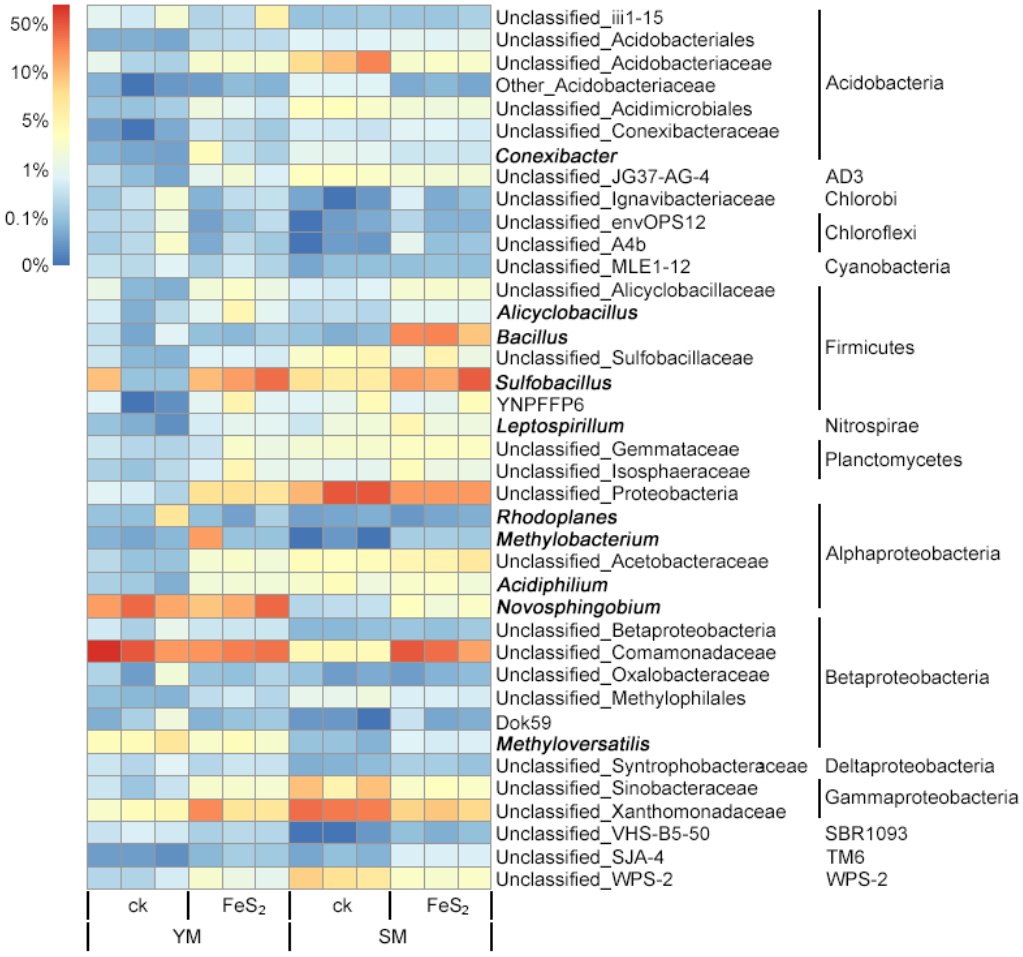

Fig. 4 Heatmap of the top genera with relative abundances above 0.2% in mine tailings.

We constructed a heatmap diagram (Fig. 4) that shows the top 39 dominant genera with relative abundances above 0.20% from the mine tailings. However, only a small number of genera were assigned to known taxa (accounting for 27.50% of total bacterial communities), including *Alicyclobacillus*, *Bacillus*, *Sulfobacillus*, YNPFFP6, *Leptospirillum*, *Rhodoplanes*, *Methylobacterium*, *Acidiphilium*, *Novosphingobium*, Dok59, and *Methyloversatilis* (Fig. 4). The *Sulfobacillus* (8.04%) and *Novosphingobium* (8.60%) genera accounted for 16.64% of the total bacterial communities and were the dominant taxa in the mine tailings. In addition, ferrous and sulfur-oxidizing bacteria, *Leptospirillum* and *Acidithiobacillus*, accounted for merely 0.80% and 0.02% of the total bacterial communities, respectively. In the Yangshanchong mine tailings, pyrite addition significantly increased the relative abundances of the genera *Alicyclobacillus*, *Leptospirillum*, *Sulfobacillus* and *Acidiphilium* by 0.67% (P=0.027), 0.74% (P=0.002), 8.86%

(P=0.043) and 1.57% (P<0.001), respectively. In the Shuimuchong mine tailings, FeS_2 addition significantly increased the relative abundances of *Alicyclobacillus*, *Bacillus*, *Sulfobacillus*, *Methylobacterium*, *Novosphingobium* and *Methyloversatilis* by 0.57% (P<0.001), 9.94% (P<0.001), 5.99% (P<0.001), 0.15% (P<0.001), 2.06% (P=0.004) and 0.56% (P<0.001), respectively.

3.3 DNA-SIP for autotroph identification in mine tailings

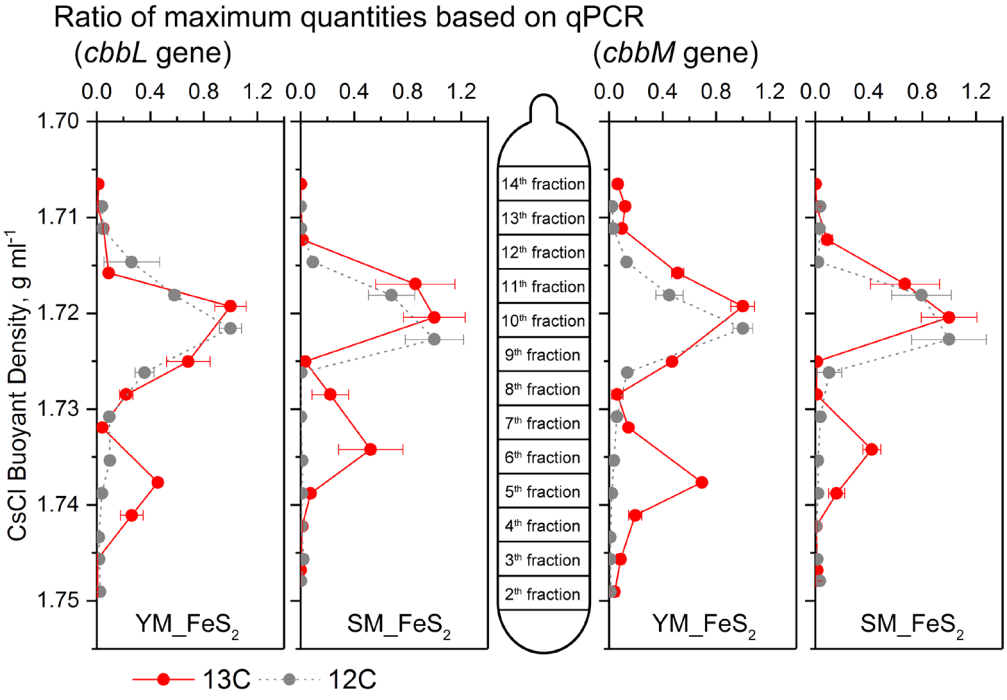

Fig. 5 Quantitative distribution of *cbbL* and *cbbM* gene fragments across the entire buoyant density gradients of the DNA fractions from microcosms treated with FeS_2 and incubated with ^{12}C-CO_2 or ^{13}C-CO_2. The normalized data consist of the ratio of the gene copy number for each DNA gradient to the maximum quantity for each treatment. The error bars represent the standard errors of triplicate microcosms, and each contains three technical replicates. *cbbL* and *cbbM* gene abundance qPCR data are shown in the supplementary materials.

For the quantitative analysis of *cbbL* and *cbbM* gene abundances, the buoyant densities of the DNA in isopycnic centrifugation gradients were used to assess the labeling efficiencies of *cbbL* or *cbbM* gene-carrying carbon fixers in the DNA-SIP microcosms (Fig. 5). *cbbL* and *cbbM* gene levels under ^{13}C-CO_2 treatment peaked at a density of 1.72 g·mL^{-1} in both the ^{12}C-CO_2 and ^{13}C-CO_2 treatments. In addition, a shift toward heavy fractions was observed for the *cbbL* and *cbbM* gene abundances in the ^{13}C-CO_2 treatment (Fig. 5), with buoyant densities of 1.738 g·mL^{-1} in YM_FeS_2 and 1.734 g·mL^{-1} in SM_FeS_2. By contrast, the highest copy numbers of the *cbbL* and *cbbM* genes under

^{12}C-CO_2 treatment appeared in the light fraction, with a buoyant density of 1.722-1.723 g·mL^{-1}.

The *cbbL* and *cbbM* gene sequences from the clone libraries in the ^{13}C-DNA heavy fraction treated with ^{13}C-CO_2 were used for phylogenetic analysis (Fig. 6). For the RuBisCO form I and II-coding genes, the *cbbL* gene copy numbers were 16.50-fold and 8.20-fold higher than the *cbbM* gene copy number in the heavy fractions of YM_FeS_2 and SM_FeS_2, respectively. In addition, a vast majority of the *cbb* genes may be associated with unknown groups, with the exception of cbbL-OTU1, cbbL-OTU6, cbbL-OTU9, cbbM-OTU5 and cbbM-OTU6. These OTUs were associated with *Sulfobacillus*, *Acidithiobacillus* and *Azospirillum* in the *cbbL* gene and *Acidithiobacillus* and *Thiobacillus* in the *cbbM* gene according to phylogenetic analysis based on amino acid sequences. The *Sulfobacillus*-like *cbbL* gene sequence, cbbL-OTU1, accounted for 30.11% and 34.74% of the total of *cbbL* gene sequences in the heavy fractions of YM_FeS_2 and SM_FeS_2, respectively. The *Acidithiobacillus*-like *cbbL* and *cbbM* genes accounted for merely 2.15% and 4.21% of the total *cbbL* gene sequences and 3.30% and 4.35% of the total *cbbM* gene sequences in the heavy fractions of YM_FeS_2 and SM_FeS_2, respectively. In addition, the *Sulfobacillus* genus had the highest relative abundance compared to any other genus based on 16S rRNA analysis (Table S2), accounting for 17.18% and 18.24% of the heavy fractions of YM_FeS_2 and SM_FeS_2, respectively. The *Leptospirillum* genus accounted for 1.32% and 1.58% of the heavy fractions of YM_FeS_2 and SM_FeS_2, respectively, and *Acidithiobacillus* accounted for 0.11% and 0.06% of the heavy fractions of YM_FeS_2 and SM_FeS_2, respectively.

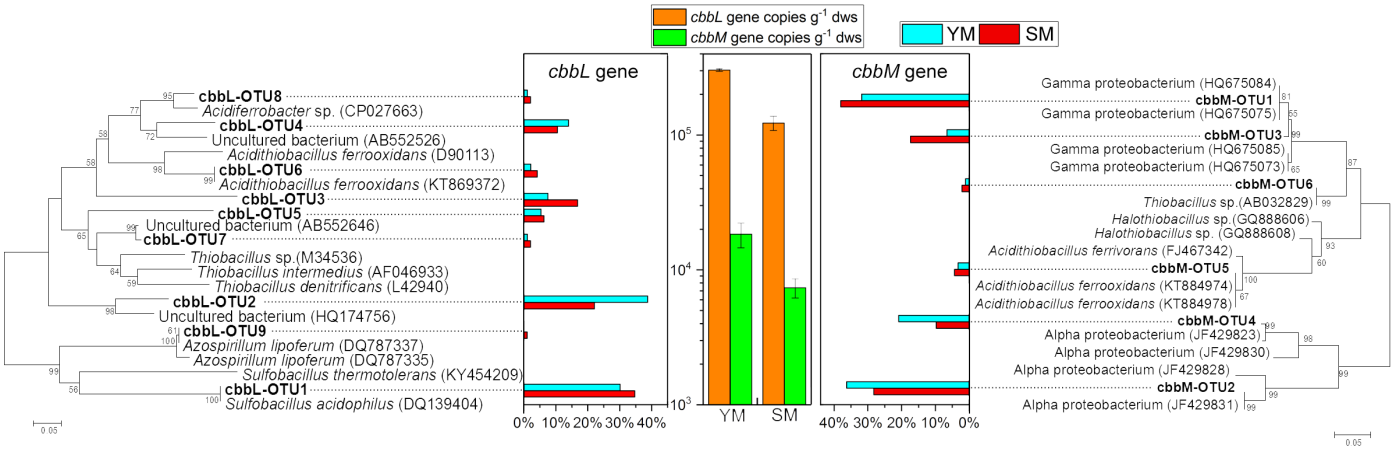

Fig. 6 Phylogenetic tree of the translated *cbbL* and *cbbM* sequences in the heavy fractions from YM and SM treated with FeS_2. Relative frequencies (%) are marked in the bar graph. Bootstrap values of >50% are indicated at branch points. The *cbbL* and *cbbM* gene copy numbers in the heavy fractions from FeS_2-treated YM and SM are shown in the middle of the figure. The cultured genera most related to OTUs from the *cbbL* and *cbbM* clone libraries are shown in Table S1.

4. Discussion

4.1 The effect of FeS_2 on the whole bacterial community in mine tailings

Acidic polymetallic mine tailings have strong potential for pyrite oxidation. In this study, a large amount of sulfuric acid was generated (increases of approximately 19.95 mg/g and 14.64 mg/g in YM and SM, respectively), and a persistent decline in pH was observed (pH decreased by 0.44 and 0.35 in YM and SM, respectively) in only 14 days. These changes clearly indicated oxidization of pyrite (i.e., acidification) in mine tailings. Previous studies have found that some bacterial phyla, such as Firmicutes and Nitrospirae, significantly increase (Chen et al., 2013;Liu et al., 2014) with the acidification process of mine tailings. In the present study, the bacterial composition in the different mine tailings varied greatly, and only the Firmicutes phylum increased in both tested mine tailings under pyrite addition. This group might participate in the oxidization of sulfide minerals (Chen et al., 2013), such as *Sulfobacillus*, which accounted for the majority of Firmicutes. Many other microorganisms might be inhibited under pyrite addition. Korehi et al. (2014) and Liu et al. (2014) also indicated that the ongoing oxidation process in mine tailings was accompanied by an increase in Firmicutes and a decrease in Actinobacteria and all classes of Proteobacteria except Gammaproteobacteria. In addition, Chen et al. (2013) and Liu et al. (2014) found that the relative abundances of Euryarchaeota belonging to archaea significantly increased with decreasing pH, which indicates that this taxon is an indicator of metal contamination (Hur et al., 2011). Euryarchaeota compete with β-Proteobacteria for ecological niches under such acidic conditions (Liu et al., 2014). However, in this study, only a few archaea were detected, which might be related to differences in primer affinities and samples.

The growth of microorganisms in bare mine tailings is usually limited by the availability of organic carbon (Schimel and Weintraub, 2003). Pyrite oxidization in mine tailings further enhanced the acidity of the mine tailings (pH decreased to 2.77 and 2.57 in YM_FeS_2 and SM_FeS_2, respectively). As a result, only microorganisms that were resistant to infertility and/or acidophilic conditions could maintain high activities. In the present study, *Conexibacter*, *Alicyclobacillus*, *Bacillus*, *Sulfobacillus*, *Leptospirillum*, *Rhodoplanes*, *Methylobacterium*, *Acidiphilium*, *Novosphingobium* and *Methyloversatilis* were the top genera with relative abundances above 0.2% in the mine tailings. Some specific taxa, including the genera *Alicyclobacillus*, *Sulfobacillus*, *Leptospirillum* and *Acidiphilium*, increased in both of the tested mine tailings under pyrite addition, indicating high consistency of dominant bacterial genera in different mine tailings. It is possible that in the case of pyrite oxidization and the availability of organic carbon, acidophilic and/or autotrophic bacteria could be stimulated (Deng et al., 2016;Antonelli et al., 2018), and the main carbon fixers found in these two mine tailings may be derived from the same groups.

4.2 The effect of FeS_2 on bacterial carbon sequestration in mine tailings

Previous studies have shown that mine tailings provide an excellent substrate for carbon sequestration through the formation of carbonate due to the large surface area of the material grains (McCutcheon et al., 2016). Compared to soils and natural bedrock, mine tailings may possess higher carbonate precipitation rates (Wilson et al., 2009).

However, in this study, the ^{13}C content and TOC content in mine tailings increased slightly. DNA-SIP analysis demonstrated that a considerable amount of ^{13}C-CO_2 was assimilated by carbon fixers in the ^{13}C-CO_2-labeled mine tailing samples, leading to a significant shift of *cbbL* or *cbbM* gene-carrying genomic DNA into the heavy fraction. Both of these results indicated the contribution of microbial activities to carbon sequestration in mine tailings. To the best of our present knowledge, this report is the first to elucidate carbon sequestration by autotrophic groups in mine tailings based on isotope tracers and DNA-SIP. Previous studies have found that microbial photosynthesis accelerates carbonate mineral precipitation and further induces mineralization (McCutcheon et al., 2014;McCutcheon et al., 2016). However, in the present study, the microcosms were not cultured in the presence of illumination, and as a result, chemoautotrophic microorganisms, particularly iron and/or sulfide oxidizers (such as *Sulfobacillus*), were the dominant carbon fixers. In addition, only a few archaea were detected based on 16S rRNA gene sequencing, and the clone libraries of the *cbbL* and *cbbM* genes in the ^{13}C-labeled heavy fraction did not show archaeal sequences for Calvin cycle genes. These results indicated that bacterial carbon sequestration is mainly attributable to chemoautotrophic bacteria in pyrite oxidization of mine tailings. However, archaea may have higher activities in RuBisCO-mediated carbon metabolic pathways (Kono et al., 2017), which will require further study.

During pyrite oxidization in mine tailings, some acidophilic autotrophic microorganisms have very high activity levels. For example, the *Sulfobacillus* and *Leptospirillum* genera, both of which are vital ferrous and sulfur oxidizers, increased significantly during pyrite oxidization. Zhang et al. (2016) found genes for the CBB pathway and rTCA, but no other CO_2 fixation pathways, in a copper bioleaching microbial community. For the CBB pathway in this study, the *Sulfobacillus*-like *cbbL* gene was the primary carbon fixing-associated gene. Ňancucheo and Johnson (2012) found that among acidophilic prokaryotes isolated from mine-impacted environments, the ability to metabolize glycolic acid appeared to be restricted to Firmicutes (e.g., *Sulfobacillus*), and the glycolic acid in all of these acidophiles might be due to the activity of RuBisCO (Nancucheo and Johnson, 2012). Previous studies have confirmed the presence of *Sulfobacillus* in mine tailings (Coral et al., 2018;Yu et al., 2018); *Sulfobacillus* has the ability to oxidize or reduce Fe(III) and oxidize sulfur (Dold et al., 2005). This ability is important, as this genus likely leads to a high mineral dissolution rate by adhering to mineral surfaces and further enhancing sulfide mineral oxidation (Li et al., 2016;Becker et al., 2011). None of the *cbbL* or *cbbM* genes identified were highly homologous to genes in *Leptospirillum*, but this may be due to primer specificity. Nevertheless, Marín et al. (2017) found that the rTCA carbon fixation pathway genes were mainly found in by *Leptospirillum* spp. RuBisCO is the most prominent enzyme, and the gene coding for the large subunit of RuBisCO serves as a marker for the analysis of autotrophic organisms, including bacteria, using the CBB cycle (Berg, 2011). In addition, the *Sulfobacillus*-like *cbbL* gene dominated the ^{13}C-labeled DNA of the carbon-fixing taxa. Furthermore, the higher relative abundance of *Sulfobacillus* than *Leptospirillum,* according to 16S rRNA analysis, demonstrates the contribution of the *Sulfobacillus*-like *cbbL* gene to carbon sequestration. Even so,

while the number (or relative abundance) of autotrophs demonstrated their ability to sequester carbon, it did not reflect their ability to perform or their importance in ferrous and sulfur oxidation. For example, the reduced percentage of the genus *Acidithiobacillus* in the two mine tailings did not reflect the contribution of this genus to the oxidation of iron and sulfur. Falagán et al. (2017) highlighted the importance of thermotolerant acidophiles, such as *Acidithiobacillus* and the genus *Sulfobacillus,* in extracting and recovering metals from mine tailings. Furthermore, it has been known for many years that *Acidithiobacillus* can obtain energy by catalyzing the oxidation reaction of Fe^{2+} to Fe^{3+} from sulfites (Dold, 2014), and this may significantly speed up the rate of ferrous oxidization. The decreased level of total carbon, including low-molecular-weight carboxylic acids LMWCA, may also limit the activity of this bacterium (Dold et al., 2005).

In conclusion, this study was the first to elucidate carbon sequestration by autotrophic groups in the acidification process of mine tailings based on isotope tracers and DNA-SIP. Our results demonstrated higher ^{13}C atom % values with the addition of pyrite than in control groups after a 14-day incubation, as well as a significant increase in the total organic carbon content. The *Sulfobacillus* genus was dominant in the pyrite-treated bacterial communities and was also the primary carbon fixer with the RuBisCO form I-encoding gene *cbbL*. Finally, the *cbbL* gene may play a vital role in carbon sequestration in the sulfide mineral oxidation of mine tailings.

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