

# Construction and Characterization of *tetH* Overexpression and Knockout Strains of *Acidithiobacillus ferrooxidans*

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*Acidithiobacillus ferrooxidans* is a major participant in consortia of microorganisms used for bioleaching. It can obtain energy from the oxidation of  $\text{Fe}^{2+}$ ,  $\text{H}_2$ ,  $\text{S}^0$ , and various reduced inorganic sulfur compounds (RISCs). Tetrathionate is a key intermediate during RISC oxidation, hydrolyzed by tetrathionate hydrolase (TetH), and used as sole energy source. In this study, a *tetH* knockout ( $\Delta tetH$ ) mutant and a *tetH* overexpression strain were constructed and characterized. The *tetH* overexpression strain grew better on sulfur and tetrathionate and possessed a higher rate of tetrathionate utilization and TetH activity than the wild type. However, its cell yields on tetrathionate were much lower than those on sulfur. The  $\Delta tetH$  mutant could not grow on tetrathionate but could proliferate on sulfur with a lower cell yield than the wild type's, which indicated that tetrathionate hydrolysis is mediated only by TetH, encoded by *tetH*. The  $\Delta tetH$  mutant could survive in ferrous medium with an  $\text{Fe}^{2+}$  oxidation rate similar to that of the wild type. For the *tetH* overexpression strain, the rate was relatively higher than that of the wild type. The reverse transcription-quantitative PCR (qRT-PCR) results showed that *tetH* and *doxD2* acted synergistically, and *doxD2* was considered important in thiosulfate metabolism. Of the two *sqr* genes, AFE\_0267 seemed to play as important a role in sulfide oxidation as AFE\_1792. This study not only provides a substantial basis for studying the function of the *tetH* gene but also may serve as a model to clarify other candidate genes involved in sulfur oxidation in this organism.

*Acidithiobacillus ferrooxidans* is an extremely acidophilic (pH 2), chemolithoautotrophic bioleaching bacterium of great importance in the biomining industry. It derives energy from the oxidation of hydrogen, ferrous iron, sulfur, or reduced inorganic sulfur compounds (RISCs) for fixation of the carbon dioxide and nitrogen used for synthesizing sugar, protein, nucleic acid, and other important compounds necessary for its growth. In addition, it can grow in the absence of oxygen with ferric iron as the electron acceptor and sulfur as the electron donor. The unusual metabolism and physiological characteristics of *A. ferrooxidans* make it not only widely applied in the biomining industry but also very attractive to study theoretically from a fundamental biological point of view. Until now, research on its ferrous iron oxidation mechanism have achieved profound results (1–3), and many metabolic models have been posted, among which Quatrini's model, reconstructed by microarray transcript profiling, reverse transcription-quantitative PCR (qRT-PCR) analysis, molecular biology, and biochemistry, was widely recognized (4, 5). However, the study of RISC metabolism in *A. ferrooxidans* is more complex and complicated due to the involvement of multiple oxidation states of sulfur (from  $-2$  to  $+6$ ) and some nonenzymatic reactions. The few predicted models were based mainly on transcriptomic data and bioinformatic analysis and lack sufficient support from experimental data (4–6).

Tetrathionate is a key intermediate during RISC oxidation, and it can also be used as the sole energy source for the growth of *A. ferrooxidans*. Tetrathionate can be hydrolyzed to thiosulfate, sulfate, and sulfur by tetrathionate hydrolase (TetH) (7–9). Compared with TetH from the sulfur-oxidizing strain *Acidithiobacillus caldus* (10, 11), TetH from *A. ferrooxidans* was speculated to be phylogenetically distinct (11, 12) and even diverse in its catalytic reaction on account of different cofactor requirements (10, 13). In addition, the subcellular location of TetH in *A. ferrooxidans* has not been certain until now because it has been reported as a periplasmic (8), outer membrane-associated (12, 14), and extra-

cellular (15) protein. In any case, much progress has been made with regard to the *tetH* gene and TetH of *A. ferrooxidans*, such as the identification of the *tetH* gene, successful refolding of recombinant TetH, and crystallization of TetH (12, 13, 16). However, the detailed function of *tetH* and its association with other related genes in sulfur metabolism are still not completely understood.

The gene knockout technique has long been a convincing tool for research on gene functions and other studies in a variety of organisms (17–19). On account of the difficulties of genetic manipulation in acidophilic chemolithoautotrophic microorganisms, there have been only three reports on the construction of gene knockout mutants in the strains of *Acidithiobacillus* (20–22). In 2000, Liu et al. constructed a *recA* mutant of *A. ferrooxidans* ATCC 33020 by marker exchange mutagenesis, which first provided a method of investigating the functions of target genes by null mutation in *A. ferrooxidans* (20). In 2008, van Zyl et al. constructed *A. caldus arsB* and *tetH* mutants by the same method. The inhibition of the growth of an *A. caldus tetH* mutant on tetrathionate was detected; however, no further analyses on the characteristics of the mutant were carried out (21). Moreover, the strategy in the construction of the two above-described knockout mutants was based on marker exchange in that the kanamycin resistance marker was eventually introduced and integrated into the host genomes. This marker exchange mutagenesis not only is a potential threat to biosafety but also cannot be applied to the

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
<i>A. ferrooxidans</i>		
ATCC 23270	Type strain	ATCC
ΔAF0029	ATCC 23270 Δ <i>tetH</i>	This study
<i>E. coli</i>		
JM109	<i>recA1 endA1 gryA96 thi-1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) <i>supE44 relA1λ<sup>-</sup> lac</i> (F <sup>+</sup> <i>proAB lacI<sup>f</sup></i> ZΔM15)	39
DH5αλ <i>pir</i>	F <sup>-</sup> <i>hsdR17 thi-1 gyrA Δ(lacZYA-argF) supE44 recA1φ80dΔ(lacZ)M15 relA λpir</i>	40
S17-1λ <i>pir</i>	Tp <sup>f</sup> Sm <sup>r</sup> <i>recA thi pro hsdR hsdM<sup>+</sup></i> RP4::2-Tc::Mu::Km Tn7λ <i>pir</i> lysogen	41
SM10	Km <sup>r</sup> <i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu</i>	42
Plasmids		
pUC19	Ap <sup>r</sup> , ColE1 replicon, cloning vector	TaKaRa
pKIT	Km <sup>r</sup> , R6K replicon, <i>oriT<sub>RP4</sub></i> , I-SceI site	22
pKIT-HA1	pKIT containing HA1	This study
pKIT-KtetH	pKIT containing homologous sequences flanking the <i>tetH</i> gene from <i>A. ferrooxidans</i> ATCC 23270	This study
pMSD1	Sm <sup>r</sup> , pBBR1 replicon, <i>mob</i> positive	43
pMSD1-I-SceI	pMSD1 containing the <i>I-SceI</i> gene	22
pUC19-probe	pUC19 containing the probe sequence	This study
pUC18	Ap <sup>r</sup> , ColE1 replicon, cloning vector	TaKaRa
pEXT20	Ap <sup>r</sup> , ColE1 replicon, <i>tac</i> promoter, <i>lacI<sup>f</sup></i>	44
pEXT20-tetH	pEXT20 containing the <i>tetH</i> gene from <i>A. ferrooxidans</i> ATCC 23270	This study
pUC18-tac-tetH	pUC18 containing the <i>tetH</i> gene under the control of the <i>tac</i> promoter	This study
pMSD1-tac-tetH	pMSD1 containing the <i>tetH</i> gene under the control of the <i>tac</i> promoter	This study

construction of multiple mutations in one strain, as the selectable markers available for *Acidithiobacillus* in an acidic environment are quite limited. In 2012, a markerless gene replacement system was constructed based on the homing endonuclease I-SceI in *A. ferrooxidans*, and the function of the *pfkB* gene was characterized by comparing the properties of the *pfkB* knockout mutant with those of the wild type (22). It provided us with a powerful tool to investigate the physiological functions of candidate genes involved in the complex sulfur metabolic pathways in *A. ferrooxidans*.

In this study, we describe a *tetH* knockout (Δ*tetH*) mutant constructed with the markerless gene replacement system and a *tetH* overexpression strain of *A. ferrooxidans*. The function of the *tetH* gene in *A. ferrooxidans* was characterized by comparing the properties of the Δ*tetH* mutant with those of the wild type and the *tetH* overexpression strain grown on different substrates; we examined the growth, enzymatic activities, and transcriptional analysis of related genes involved in sulfur oxidation by qRT-PCR. This study can not only provide a substantial base for investigating the function of the *tetH* gene in *A. ferrooxidans* but also serve as a model for future research on other related genes involved in the cascade of sulfur oxidation in this organism.

## MATERIALS AND METHODS

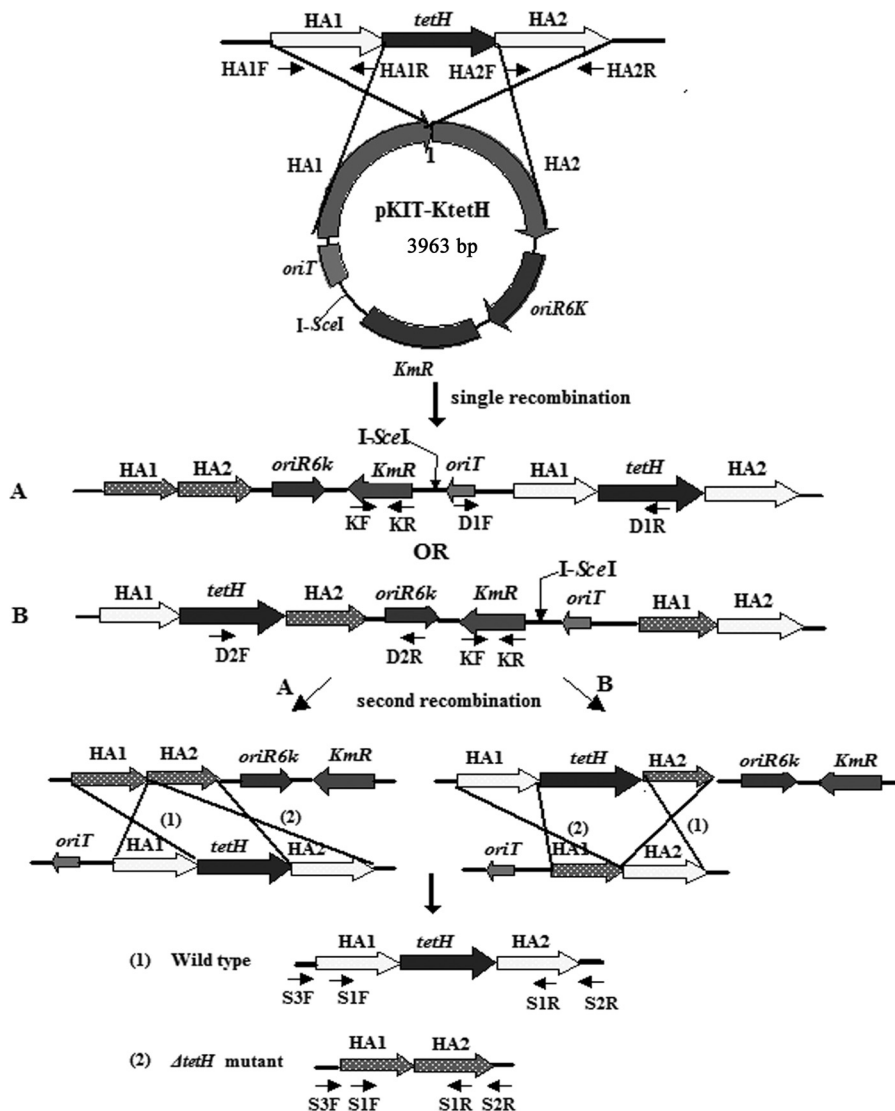
**Media, bacterial strains, and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured at 37°C in Luria-Bertani (LB) broth with shaking at 180 rpm or on LB agar plates with kanamycin (50 μg/ml) or streptomycin (50 μg/ml) added as required. The strains of *A. ferrooxidans* were cultured at 30°C with shaking at 180 rpm in 9K basal salts solution (23) adjusted to pH 2.0 with H<sub>2</sub>SO<sub>4</sub> supplemented with 3.3% (wt/vol) FeSO<sub>4</sub>·7H<sub>2</sub>O, 1% (wt/vol) S<sup>0</sup> or 0.5% (wt/vol) K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> as an energy source. The concentrated solution of FeSO<sub>4</sub>·7H<sub>2</sub>O and K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> was filtered and added aseptically at the time of inoculation, and S<sup>0</sup> was sterilized by intermittent boiling for at least 3 h and added after inoculation.

The compositions of mating medium and solid 2:2 medium for culturing *A. ferrooxidans* were described previously (24). Kanamycin (100 μg/ml) and streptomycin (100 μg/ml) were used for selection in *A. ferrooxidans* on solid 2:2 medium plates, and kanamycin (300 μg/ml) and streptomycin (300 μg/ml) were used in 9K liquid medium as required.

**Genetic manipulations.** General molecular biological techniques, including the restriction enzyme digestion, ligation, gel electrophoresis, and transformation of a plasmid, were performed as described elsewhere (25). The isolation of genomic DNA from cells of *A. ferrooxidans* ATCC 23270, the isolation of plasmids, and the recovery of DNA fragments from agarose gels were performed by using, respectively, a bacterial DNA kit, a plasmid minikit I, and a gel extraction kit of Omega Bio-Tek according to the manufacturer's instructions. Restriction enzymes and T4 DNA ligase were purchased from TaKaRa and used as recommended. Primer synthesis and DNA sequencing reactions were performed by the Shanghai Biosune Biotechnology Company. The Southern blot analysis was performed with Amersham Hybond-N<sup>+</sup> positively charged nylon transfer membranes (GE Healthcare) and a digoxigenin (DIG)-High Prime DNA labeling and detection starter kit I (Roche) and accomplished according to the manufacturer's instructions.

**Construction of the *tetH* gene knockout mutant of *A. ferrooxidans* ATCC 23270.** The plasmids involved in the construction of the *tetH* gene knockout mutant are listed in Table 1, and the designed primers are listed in Table S1 in the supplemental material. A schematic chart for the construction of the Δ*tetH* mutant of *A. ferrooxidans* ATCC 23270 is shown in Fig. 1.

(i) **Construction of suicide plasmid pKIT-KtetH.** Plasmid pKIT was used as the backbone for constructing the suicide vector pKIT-KtetH. Two pairs of primers (HA1F/HA1R and HA2F/HA2R) were synthesized to amplify homologous arms 1 and 2 (HA1 and HA2) flanking the *tetH* gene from the *A. ferrooxidans* ATCC 23270 genome (GenBank accession number NC\_011761). The amplified HA1 sequence (positions 30709 to 31710) and HA2 sequence (positions 32974 to 33982) were first cloned into plasmid pUC19 for sequencing after digestion with EcoRI and HindIII, respectively. Then, HA1 and HA2 were obtained from their sequenced recombinant plasmids by digestion with EcoRI/XbaI and XbaI/SacI, respectively, and inserted into the corresponding sites in pKIT to



**FIG 1** Schematic chart for the construction of the *tetH* knockout mutant. The suicide plasmid pKIT-KtetH was introduced into *A. ferrooxidans* ATCC 23270 and integrated into the genome, producing either one of the two types of single recombinants (A and B). Then plasmid pMSD1-I-SceI, carrying the *I-SceI* gene, was introduced into the single recombinant, resulting in a double-stranded break at the *I-SceI* site and stimulating the occurrence of the second recombination. As a result, either a *tetH* gene knockout mutant or a restoration of the wild type was generated, and the kanamycin-resistant gene (*KmR*) was simultaneously eliminated from the chromosome. The positions of the primers used to identify various recombinants are indicated.

produce pKIT-KtetH, which was used to inactivate the *tetH* gene of *A. ferrooxidans* ATCC 23270.

**(ii) Selection of single crossovers.** The suicide plasmid pKIT-KtetH was transformed into *E. coli* S17-1 $\lambda$ pir under the selection of kanamycin resistance, and the resulting *E. coli* S17-1 $\lambda$ pir cells containing pKIT-KtetH were used as donors. The cells of *A. ferrooxidans* ATCC 23270 were applied as recipients. Conjugation conditions were modified with Peng et al.'s method (24). The donor cells were cultivated in liquid LB medium with kanamycin until exponential phase, and the recipient cells were grown in 9K-S<sup>0</sup> medium until stationary phase. Both the donor and recipient cells were collected by centrifugation (6,000  $\times$  g, 5 min), washed three times with 2:2 basal salt buffer, and then mixed at a ratio of approximately 1:1 to 1:~2. Then 100  $\mu$ l of the cell suspension (approximately 10<sup>8</sup> cells) was spotted onto the sterilized filter membrane (0.45- $\mu$ m pore size, 25 mm in diameter) placed on the mating medium. After incubation at 30°C for 5 to 7 days, the cells on the filter membrane were resuspended and diluted with 2:2 basal salt buffer and then plated on 2:2 solid plates

with or without kanamycin. After incubation at 30°C for 15 days, colonies grown on 2:2 plates were potential single-crossover mutants. As a control for spontaneous mutation, the recipient cells were plated on the same plates with kanamycin.

**(iii) Verification of single-recombination events.** Colonies grown on 2:2 plates with kanamycin were picked randomly, inoculated in 10 ml of 9K-Fe<sup>2+</sup> medium, and cultivated at 30°C for 5 days for recovery of growth. Then they were transferred to 9K-S<sup>0</sup> medium with kanamycin and cultivated at 30°C for 5 to 7 days. Cells at late exponential phase were collected for extraction of genomic DNA, which was applied as the template for PCR verification of single-recombination events. Primer pair KF/KR was used to amplify a partial sequence of the *aph* gene for kanamycin resistance, and primer pairs D1F/D1R and D2F/D2R were used to verify the two types (A and B in Fig. 1) of single crossovers.

**(iv) Screening for the *tetH* gene knockout mutant.** Plasmid pMSD1-I-SceI, which contained a streptomycin resistance gene and the *I-SceI* gene, was constructed previously (22). It was introduced into *E. coli* SM10

by transformation, and the transformed cells containing pMSD1-I-SceI were used as donors for conjugation. Single recombinant cells of *A. ferrooxidans* ATCC 23270 were used as recipients. The same conjugation procedure as mentioned above was performed, except that incubation at 30°C lasted for 48 to 72 h for the mobilization of pMSD1-I-SceI. To select transconjugants, the cells were plated onto 2:2 solid plates with or without streptomycin. After 15 days of incubation at 30°C, single colonies from 2:2 solid plates with streptomycin were picked and cultured in 10 ml of 9K-Fe<sup>2+</sup> medium at 30°C for 5 days for recovery of growth, followed by subculture in 9K-S<sup>0</sup> medium containing streptomycin for 5 to 7 days at 30°C. The cells were collected by centrifugation (6,000 × g, 5 min) for extraction of genomic DNA, which was applied as the template for PCR verification. Primer pairs S1, S2, and S3 correspond to primers S1F and S1R, S1F and S2R, and S3F and S2R, respectively, which were employed in the PCR verification to screen for the double-recombination mutants of *A. ferrooxidans* ATCC 23270.

(v) **Elimination of plasmid pMSD1-I-SceI from double-recombination mutants.** The target mutants were consecutively subcultured in 9K-S<sup>0</sup> medium without streptomycin at least 3 times and then plated on 2:2 medium without streptomycin. Single colonies were picked and grown again in 9K-S<sup>0</sup> medium, and then the cells were harvested for PCR verification to screen for the spontaneous loss of plasmid pMSD1-I-SceI. Primers SMF and SMR were used to amplify the streptomycin resistance gene fragment in pMSD1-I-SceI, so as to verify the elimination of plasmid pMSD1-I-SceI.

(vi) **Southern blot analysis.** To confirm the genotype of the  $\Delta tetH$  mutant, Southern blot analysis was carried out with genomic DNA digested with EcoRV. The probe was designed by using a partial fragment of the downstream homologous arm, HA2, flanking the *tetH* gene from the *A. ferrooxidans* ATCC 23270 genome and was amplified with primers ProbeF and ProbeR. After digestion with EcoRI, the amplified 662-bp fragment was inserted into the corresponding site in pUC19, to give pUC19-probe for sequencing. The 650-bp EcoRI fragment digested from pUC19-probe was isolated and used as the probe for Southern blot analysis.

**Construction of the *tetH* gene overexpression strain of *A. ferrooxidans* ATCC 23270.** The strains and plasmids used to construct the *tetH* gene overexpression strain of *A. ferrooxidans* ATCC 23270 are listed in Table 1. The 1,717-bp DNA fragment containing the whole *tetH* gene (positions 31528 to 33232) with its signal peptide sequence was amplified with primers TF and TR by PCR using *A. ferrooxidans* ATCC 23270 genomic DNA as the template. After digestion with KpnI and BamHI, the amplified DNA fragment was inserted into the corresponding cloning sites (downstream of the *tac* promoter) in the vector pEXT20, producing pEXT20-*tetH*. The expression of the *tetH* gene under the control of the *tac* promoter was verified with SDS-PAGE. Then the 1,872-bp *tac-tetH* fragment was amplified with primers TTF and TR by PCR with pEXT20-*tetH* as the template. After digestion with PstI and BamHI, the amplified fragment was inserted into the corresponding cloning sites of vector pUC18 to produce pUC18-*tac-tetH* for DNA sequencing and then subcloned from pUC18-*tac-tetH* into the corresponding cloning sites of the broad-range-host vector pMSD1, to produce pMSD1-*tac-tetH*. The transfer of plasmid pMSD1-*tac-tetH* from *E. coli* SM10 into *A. ferrooxidans* ATCC 23270 was performed by using the same conjugation method as described above for plasmid pMSD1-I-SceI. The expression of the *tetH* gene in pMSD1-*tac-tetH* in *E. coli* was detected by SDS-PAGE.

**Characterization of the  $\Delta tetH$  mutant and *tetH* overexpression strain.** (i) **Growth properties.** The *A. ferrooxidans* ATCC 23270 wild type,  $\Delta tetH$  mutant, and *tetH* overexpression strain were cultivated in 9K basal salts solution with ferrous iron (Fe<sup>2+</sup>), sulfur (S<sup>0</sup>), and tetrathionate (S<sub>4</sub>O<sub>6</sub><sup>2-</sup>) as energy resources. Similar amounts of initial inoculum (approximately 10<sup>6</sup> cells) of each strain were employed for comparison. Growth in 9K-S<sup>0</sup> and 9K-S<sub>4</sub>O<sub>6</sub><sup>2-</sup> was monitored by measuring the optical density at 600 nm after the removal of elemental sulfur by low-speed centrifugation (400 × g, 5 min). Growth in 9K-Fe<sup>2+</sup> was monitored by

measuring the decrease in the ferrous iron concentration by the *o*-phenanthroline method (26). The decrease in the S<sub>4</sub>O<sub>6</sub><sup>2-</sup> concentration in 9K-S<sub>4</sub>O<sub>6</sub><sup>2-</sup> medium was measured by the method of cyanolysis (27, 28), which can also indicate the growth of the bacteria.

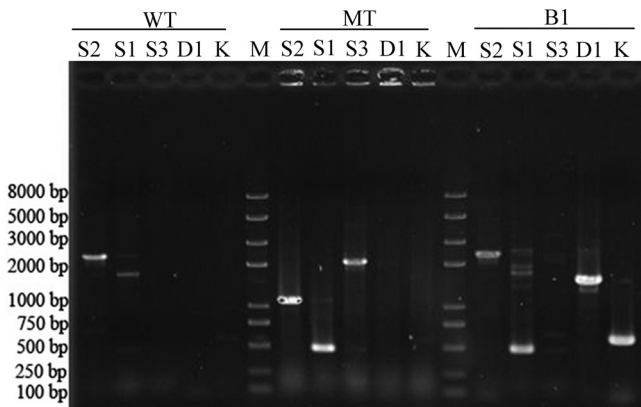
(ii) **TetH activity.** Cells at mid-log phase grown in 9K-S<sub>4</sub>O<sub>6</sub><sup>2-</sup> medium were collected by centrifugation at 13,800 × g for 10 min, washed twice with 0.1 M potassium acetate buffer (pH 3.0), and resuspended in the same buffer. The cells were sonicated intermittently on ice for 20 min. After centrifugation at 13,800 × g for 10 min, the cell debris was discarded and the supernatant was applied as TetH crude extract. Then, a 1-ml reaction mixture was prepared in 1.5-ml Eppendorf tubes, which contained 1 mM K<sub>2</sub>S<sub>4</sub>O<sub>6</sub>, 200 mM K<sub>2</sub>SO<sub>4</sub>, 0.1 M acetate buffer (pH 3.0), and 100 μl TetH crude extract. The reaction was initiated by adding the TetH crude extract at 30°C and lasted for 60 min. Then, the reaction tubes were immediately placed in cold ethanol (−20°C), with vibration for 2 min, followed by boiling for 90 s to stop the reaction. The samples were centrifuged at 13,800 × g for 1 min to precipitate the elemental sulfur produced in the reaction. The concentration of tetrathionate remaining in the supernatant was measured by the method of cyanolysis (27, 28). One unit of crude TetH activity (U) was defined as the amount of protein required for the hydrolysis of 1 μmol S<sub>4</sub>O<sub>6</sub><sup>2-</sup> per min.

**RNA extraction and qRT-PCR.** Cells at mid-log phase grown in 9K basal salts media with different energy sources were collected by centrifugation, and each independent culture was performed with at least three replicates. Total RNA was extracted by using the RNeasy minikit from Qiagen according to the manufacturer's instructions. DNA was eliminated from the samples by incubating them with RNase-free DNase I (Fermentas) for an appropriate time, and the absence of contaminating DNA was determined by PCR. After we guaranteed the RNA quality and concentration by agarose gel electrophoresis and with a NanoDrop-1000 spectrophotometer (NanoDrop Technologies), cDNA was generated by using a RevertAid first-strand cDNA synthesis kit (Fermentas) for qRT-PCR analysis, which was performed with a LightCycler 480 (Roche) by using SYBR *Premix Ex Taq* from TaKaRa in triplicate. The thermal cycling conditions were 95°C for 2 min and 40 cycles of 95°C for 20 s, 54°C for 20 s, and 72°C for 15 s, followed by a final extension at 72°C for 2 min and subsequent melting curve analysis. Primers designed for qRT-PCR are listed in Table S1 in the supplemental material (from 29F to 2558R). Two genes, *alaS* and *map*, were applied for normalization (29). Relative gene expression values were calculated by using the method of Pfaffl (30).

## RESULTS

**Construction of the *tetH* knockout mutant of *A. ferrooxidans* ATCC 23270.** A markerless gene knockout system was successfully developed in *A. ferrooxidans* as described by Wang et al. (22). In order to verify the biological functions of the *tetH* gene in *A. ferrooxidans*, the construction of a  $\Delta tetH$  mutant was conceived based on this system. As shown in Fig. 1, the suicide plasmid pKIT-KtetH was first generated by inserting two homologous fragments (HA1 and HA2) flanking the *tetH* gene into the multiple cloning sites of plasmid pKIT and then transferred into *A. ferrooxidans* ATCC 23270 by conjugation. Plasmid pKIT-KtetH cannot replicate in *A. ferrooxidans* ATCC 23270 cells but can be integrated into the genome by homologous recombination via either of plasmid-containing HA1 or HA2 to generate the single-recombination event (two types), which can be selected under the pressure of kanamycin.

Ten colonies that grew on selective kanamycin plates were randomly picked and cultivated for the identification of a single recombination. A 571-bp fragment could be amplified from the kanamycin resistance *aph* gene by PCR using primer pair KF/KR in all tested colonies, which proved the occurrence of a single-recombination event in these colonies. Among the 10 colonies,



**FIG 2** PCR analyses of the wild type, single recombinant B1, and  $\Delta tetH$  mutant of *A. ferrooxidans* ATCC 23270. WT, MT, and B1 represent the wild type,  $\Delta tetH$  mutant, and single recombinant B1 of *A. ferrooxidans* ATCC 23270. S1, S2, S3, D1, and K represent the primer pairs S1F/S1R, S1F/S2R, S3F/S2R, D1F/D1R, and KF/KR, respectively. The numbers on the left indicate the sizes of the fragments based on the molecular size marker (lane M).

one named *A. ferrooxidans* B1 was chosen for further identification by PCR using primer pairs D1F/D1R and D2F/D2R. As shown in Fig. 2, a 1,533-bp fragment could be amplified by using primer pair D1F/D1R, which indicated that *A. ferrooxidans* B1 was the type A single recombinant (Fig. 1).

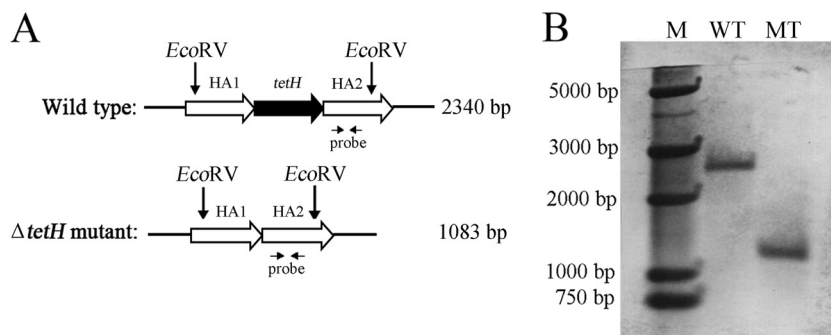
According to the strategy of the markerless gene knockout technique, a second recombination event could be stimulated by I-SceI. So, the broad-host-range plasmid pMSD1-I-SceI containing the *I-SceI* gene, which was constructed previously (22), was transferred from *E. coli* SM10 to *A. ferrooxidans* B1 by conjugation. Once transferred to cells of *A. ferrooxidans* B1, plasmid pMSD1-I-SceI could replicate and stably exist in the transconjugant under the selection pressure of streptomycin. During the growth of *A. ferrooxidans* B1 harboring pMSD1-I-SceI, the I-SceI expressed by the *I-SceI* gene from pMSD1-I-SceI could stimulate the event of double recombination in host cells. Since the second recombination event could occur at any time during cell growth and no selectable marker could be employed to concentrate the double-crossover strains, the screening of the  $\Delta tetH$  mutant had to be performed from a large number of colonies that grew on selective streptomycin plates by PCR. By using the specific primer pairs, different fragments would be amplified with the genomic

DNA from the  $\Delta tetH$  mutant, the wild type, or the single recombinant B1 (see Table S2 in the supplemental material).

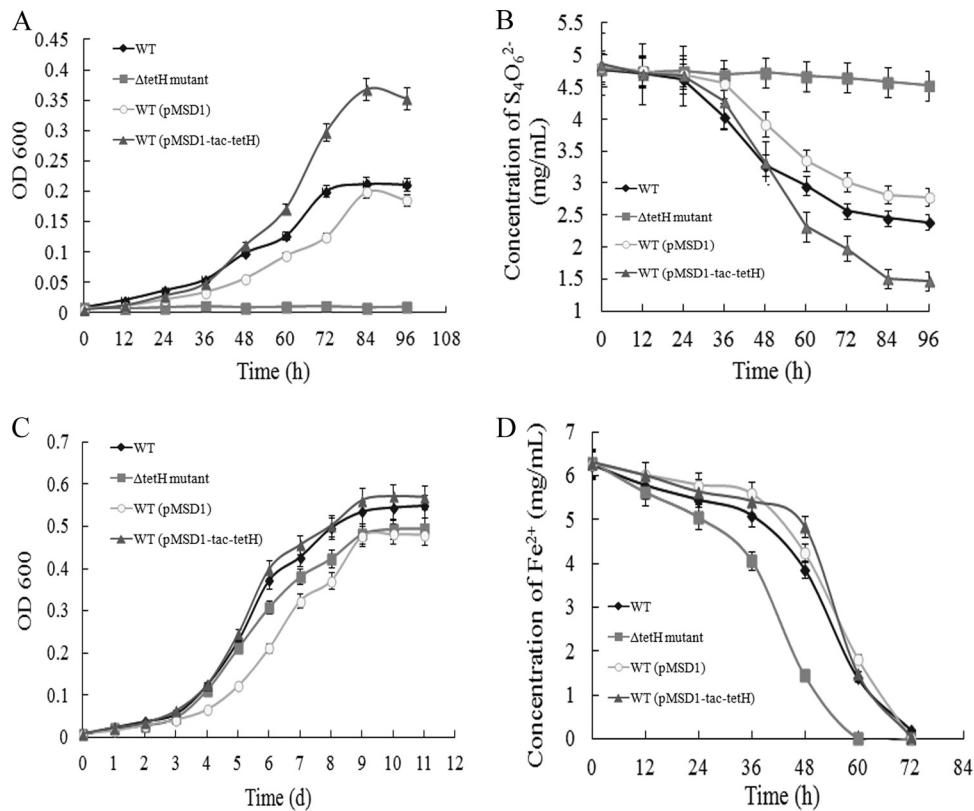
One potential mutant was finally purified by multiple subculturings alternately in liquid and solid media without streptomycin. During this period, plasmid pMSD1-I-SceI could be lost spontaneously, and the potential double-crossover  $\Delta tetH$  mutant lacking pMSD1-I-SceI was obtained as a result.

**Identification and genetic analysis of the  $\Delta tetH$  mutant of *A. ferrooxidans* ATCC 23270.** The identification of the potential  $\Delta tetH$  mutant of *A. ferrooxidans* ATCC 23270 was first carried out by PCR. By using primer pairs described in Table S2 and the genomic DNA of the  $\Delta tetH$  mutant as the template, the fragments amplified by PCR were analyzed and compared with the results obtained by using the genomic DNA of the wild type and of the single recombinant B1 as the template (Fig. 2). As shown in Fig. 2, an 1,122-bp fragment was amplified from the  $\Delta tetH$  mutant and a larger fragment (2,379 bp) was amplified from the wild type or the single recombinant B1 by using primer pair S2. While using primer pair S1, a 466-bp fragment from the  $\Delta tetH$  mutant and a 1,723-bp fragment from the wild type were obtained, and the expected 466- and 1,723-bp fragments were detected from the single recombinant B1. When primer pair S3 was employed, a 2,106-bp fragment could be amplified only by using the genomic DNA from the  $\Delta tetH$  mutant as the template (Fig. 2). The theoretically predicted long fragments (>3,000 bp) in Table S2 for each primer pair in the wild type and single recombinant B1 were too big to be amplified under the conditions used and could not be detected in Fig. 2. All the amplified fragments were in accordance with the expected sizes described in Table S2. The corresponding amplified fragments were also verified by sequencing.

Next, the identification of the potential  $\Delta tetH$  mutant of *A. ferrooxidans* ATCC 23270 was performed by Southern blot hybridization (Fig. 3). As shown in Fig. 3A, the partial fragment of HA2 was designed as probe. After being labeled with digoxigenin, the 650-bp EcoRI fragment digested from pUC19-probe was used to probe the EcoRV-digested chromosomal DNA from the wild type and  $\Delta tetH$  mutant. An expected 2,340-bp band was obtained with the wild type, and a 1,083-bp band was obtained with the mutant (Fig. 3B), which suggested that the *tetH* gene was successfully knocked out of the genome in the  $\Delta tetH$  mutant of *A. ferrooxidans* ATCC 23270.



**FIG 3** Identification of the  $\Delta tetH$  mutant of *A. ferrooxidans* ATCC 23270 by Southern blotting. (A) Schematic chart of the genomic region bearing the *tetH* gene and showing the position of the probe and the sites of EcoRV. The sizes between the two EcoRV sites are indicated on the right. (B) Southern blot analyses of EcoRV-digested genomic DNA from the wild type (WT) and the  $\Delta tetH$  mutant (MT) of *A. ferrooxidans* ATCC 23270. Lane M represents the DNA molecular markers, and the sizes of the fragments are indicated on the left.

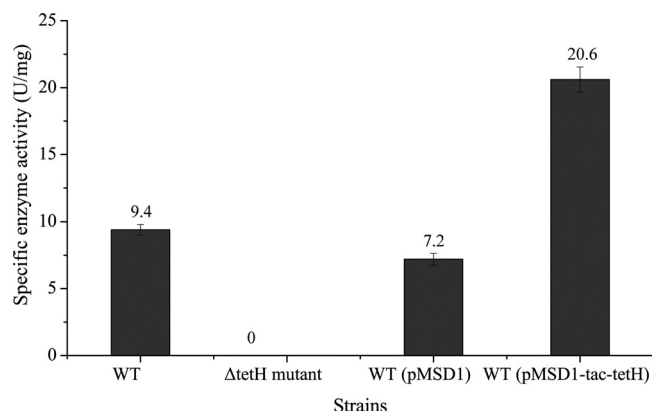


**FIG 4** Characteristics of the wild type,  $\Delta tetH$  mutant, and *tetH* overexpression strain of *A. ferrooxidans* ATCC 23270 grown in 9K basal salts medium to which were added different energy sources. (A) Growth in 9K-S<sub>4</sub>O<sub>6</sub><sup>2-</sup> medium; (B) concentration of S<sub>4</sub>O<sub>6</sub><sup>2-</sup> in 9K-S<sub>4</sub>O<sub>6</sub><sup>2-</sup> medium; (C) growth in 9K-S<sup>0</sup> medium; (D) ferrous oxidation in 9K-Fe<sup>2+</sup> medium. WT,  $\Delta tetH$  mutant, WT (pMSD1), and WT (pMSD1-tac-tetH) represent the wild type,  $\Delta tetH$  mutant, wild type carrying plasmid pMSD1, and *tetH* overexpression strain of *A. ferrooxidans* ATCC 23270, respectively. OD 600 indicates the optical density at 600 nm. All the measurements were performed in triplicate. Each data point is given as an arithmetic mean. d, days.

**Construction of the *tetH* gene overexpression strain of *A. ferrooxidans* ATCC 23270.** Since the *tetH* promoter in *A. ferrooxidans* has not been characterized, the expression of the *tetH* gene from *A. ferrooxidans* ATCC 23270 in *E. coli* cells was first tried by amplifying a fragment containing a longer upstream sequence (~300 bp) of the *tetH* gene. By inserting the amplified *tetH* fragment from the genome of *A. ferrooxidans* ATCC 23270 into pUC19, the recombinant plasmid pUC19-tetH was constructed. However, no obvious target band in the cell extract of *E. coli* JM109 containing pUC19-tetH could be detected with SDS-PAGE analysis. The result indicated that the promoter might be absent in the amplified fragment or that some regulators might be absent or not active in *E. coli*. Thus, the powerful *tac* promoter was substituted for its own unconfirmed promoter and the construction of plasmid pMSD1-tac-tetH was carried out as described in Materials and Methods. Expression of the *tetH* gene under the control of the *tac* promoter could easily be detected by SDS-PAGE in the cell extract of *E. coli* JM109 containing pUC18-tac-tetH (data not shown). Then, plasmid pMSD1-tac-tetH was transferred from *E. coli* SM10 into *A. ferrooxidans* ATCC 23270 by conjugation, and the *tetH* gene overexpression strain, *A. ferrooxidans* ATCC 23270 containing pMSD1-tac-tetH, was obtained from the solid 2:2 medium with streptomycin. The existence of plasmid pMSD1-tac-tetH in the overexpression strain was identified by the successful amplification of part of the *mob* gene, which exists only in plasmid pMSD1-tac-tetH (data not shown).

**Characterization of the *tetH* knockout mutant and *tetH* overexpression strain.** The growth properties of the wild type,  $\Delta tetH$  mutant, and *tetH* overexpression strain of *A. ferrooxidans* ATCC 23270 were investigated and compared when grown in 9K-S<sub>4</sub>O<sub>6</sub><sup>2-</sup>, 9K-S<sup>0</sup>, and 9K-Fe<sup>2+</sup> media (Fig. 4). As streptomycin (300  $\mu$ g/ml) was added to the medium for cultivation of the *tetH* overexpression strain, *A. ferrooxidans* ATCC 23270 containing plasmid pMSD1 was used as its control in the experiments. As shown in Fig. 4A, no growth was detected with the  $\Delta tetH$  mutant when it was cultivated in S<sub>4</sub>O<sub>6</sub><sup>2-</sup> medium, whereas a significant growth increment was observed with the *tetH* overexpression strain, compared with the growth not only of the wild type carrying plasmid pMSD1 but also of the wild type without the plasmid (Fig. 4A). The complete inhibition of the cell growth of the  $\Delta tetH$  mutant with tetrathionate as the sole energy source suggested that TetH is the unique enzyme that decomposes tetrathionate in *A. ferrooxidans*. The remarkable stimulation of cell growth with the *tetH* overexpression strain may also prove the effect of TetH on the utilization of tetrathionate. However, the cell yields of all strains were obviously lowered by using S<sub>4</sub>O<sub>6</sub><sup>2-</sup> rather than S<sup>0</sup> as the energy source under the conditions used (Fig. 4).

As the decrease of S<sub>4</sub>O<sub>6</sub><sup>2-</sup> in the medium may reflect the growth capacity of the strains, the concentrations of S<sub>4</sub>O<sub>6</sub><sup>2-</sup> during cell growth were monitored and are shown in Fig. 4B. Almost no consumption of S<sub>4</sub>O<sub>6</sub><sup>2-</sup> was detected with the  $\Delta tetH$  mutant



**FIG 5** Specific activities of TetH in the cell extracts of different strains grown in 9K- $S_4O_6^{2-}$  medium. WT,  $\Delta tetH$  mutant, WT (pMSD1), and WT (pMSD1-tac-tetH) represent the wild type,  $\Delta tetH$  mutant, wild type carrying plasmid pMSD1, and *tetH* overexpression strain of *A. ferrooxidans* ATCC 23270, respectively.

during the whole cultivation process. As expected, the highest hydrolysis of  $S_4O_6^{2-}$  occurred in the *tetH* overexpression strain, whose cell yield was the highest as well. The decreases in the  $S_4O_6^{2-}$  concentration (Fig. 4B) were in accordance with the increased cell yields (Fig. 4A) for all strains.

When grown in  $S^0$  medium, the  $\Delta tetH$  mutant showed a relatively reduced growth capacity compared with that of the wild type (Fig. 4C). The addition of streptomycin greatly decreased cell growth, and the wild type containing plasmid pMSD1 grew poorest; however, the *tetH* overexpression strain grew best among all strains, though streptomycin was also added to its medium (Fig. 4C). The results indicated that the deletion of the *tetH* gene, which was predicted to play a key role in tetrathionate metabolism, could not completely impede cell growth with  $S^0$  as the energy source but could result in a reduced growth capacity. The overexpression of the *tetH* gene obviously stimulated cell growth of the *tetH* overexpression strain compared with that of the wild type harboring pMSD1, but only a slight increase in cell yield could be detected compared with that of the wild type without the plasmid. It indi-

cated that the stimulation of cell growth by the overexpression of one gene involved in sulfur metabolism could not greatly enhance the efficiency of sulfur utilization.

The oxidation of  $Fe^{2+}$  by all strains grown in 9K- $Fe^{2+}$  medium was evaluated, and levels were compared (Fig. 4D). The  $Fe^{2+}$  oxidation rates of the  $\Delta tetH$  mutant and the wild type were similar, while the lag time to the initiation of  $Fe^{2+}$  oxidation was shorter for the mutant. For the *tetH* overexpression strain, the  $Fe^{2+}$  oxidation rate was relatively higher than that of the wild type, but the lag time was slightly longer. TetH is a key enzyme involved in sulfur metabolism, and its influence on ferrous oxidation needs further study and discussion.

The TetH enzyme activity of the cell extract from all strains grown in 9K- $S_4O_6^{2-}$  medium was measured, and the highest activity was detected with the *tetH* overexpression strain (Fig. 5). The result for each strain was in accordance with its growth and tetrathionate consumption (Fig. 4). Due to the nondetectable growth of the  $\Delta tetH$  mutant, its enzyme activity was presumed to be zero. However, by introducing plasmid pMSD1-tac-tetH into the  $\Delta tetH$  mutant to complement the *tetH* gene by the same conjugation method used with wild-type *A. ferrooxidans*, the  $\Delta tetH$  knockout mutant harboring pMSD1-tac-tetH could grow in 9K- $S_4O_6^{2-}$  and possessed a TetH activity of  $17.6 \pm 1.1$  U/mg in the cell extract. All the results indicated that *tetH* is the very gene that encodes the TetH responsible for the decomposition of tetrathionate. The TetH activity of the cell extract from all strains grown in 9K- $S^0$  medium was also determined, and no positive result could be obtained. The absent activity might be due to the too-small amount of TetH in cells when elemental sulfur was used as the energy source.

**Transcriptional analysis of the *tetH* knockout mutant and *tetH* overexpression strain.** The transcriptional responses to the knockout or overexpression of the *tetH* gene were further investigated when sulfur ( $S^0$ ), tetrathionate ( $S_4O_6^{2-}$ ), or ferrous iron ( $Fe^{2+}$ ) was used as the energy source. A subset of genes involved in sulfur metabolism in *A. ferrooxidans* ATCC 23270 was selected (listed in Table 2), and the relative mRNA levels of these genes were determined by qRT-PCR analysis against the wild type (Table 2). As shown in Table 2, when cultivated in 9K- $S^0$  and

**TABLE 2** Relative mRNA levels of a subset of selected genes involved in sulfur metabolism in response to the knockout of *tetH* and *tetH* overexpression<sup>a</sup>

Gene	Locus	Gene description	$\log_2$ ratio median <sup>b</sup>					
			9K- $S^0$		9K- $K_2S_4O_6$		9K- $FeSO_4$	
			A	B	A	B	A	B
<i>tetH</i>	AFE_0029	Tetrathionate hydrolase	—*	3*	—	12.7*	—	2.3*
<i>doxD2</i>	AFE_0044	DoxD-like family protein	-1.8*	2.6*	—	10.4*	-1.6*	1.4
<i>doxD1</i>	AFE_0048	DoxD-like family protein	-0.6	0.9	—	1.5*	-0.9	0.3
<i>sqr</i>	AFE_0267	Sulfide quinone reductase, putative	-1.4	2.3*	—	1.3	-0.6	-1.2
<i>sdo</i>	AFE_0269	Metallo-beta-lactamase family protein	1.2	-0.2	—	-0.5	-1.1	-0.4
<i>sat</i>	AFE_0539	Sulfate adenylyltransferase, putative adenylylsulfate kinase	1.3	-0.6	—	1.6*	-0.2	-0.6
<i>sqr</i>	AFE_1792	Sulfide quinone reductase, putative	-1.0	1.9*	—	0.9	-0.5	-0.7
<i>hdrB</i>	AFE_2554	Heterodisulfide reductase subunit B, homolog	-1.1	1.7*	—	1.1	-1.1	-0.9
<i>rhd</i>	AFE_2558	Rhodanase-like domain protein	-1.4	1.5*	—	1.5*	-0.9	-0.5

<sup>a</sup> Determined by qRT-PCR analysis against the wild-type *A. ferrooxidans* ATCC 23270 cultivated in different media.

<sup>b</sup> A, the  $\Delta tetH$  mutant of *A. ferrooxidans*; B, the *tetH* overexpression strain of *A. ferrooxidans*; —, no mRNA of the relevant genes was detected in the  $\Delta tetH$  mutant. Genes for which the  $\log_2$  ratio of the median was larger than |1.5| (corresponding to genes induced more than 2.8-fold) are considered differentially expressed (indicated with \*). The locus of a relevant gene and its description are those of the GenBank genome annotation of NC\_011761. The negative numbers represent downregulation, while positive numbers represent upregulation. The qRT-PCRs were performed in triplicate, with at least three independent biological replicates.

9K-Fe<sup>2+</sup> media, the mRNA of *tetH* was not detectable in the  $\Delta tetH$  mutant, which is in accordance with the previous genetic determination. However, in the *tetH* overexpression strain, the higher level of *tetH* expression than in the wild type, whatever the growth conditions, was expected due to a higher copy number of the *tetH* gene and the *tetH* gene under the control of *Ptac* in the plasmid.

When cultured in 9K-K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> medium, the mRNAs of all selected genes, including *tetH*, were not detected in the  $\Delta tetH$  mutant due to no growth (Table 2). In the *tetH* overexpression strain cultivated in 9K-K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> medium, the genes *tetH*, *doxD2*, *doxD1*, *sat*, and *rhd* were upregulated, and among them, we detected much higher mRNA levels of *tetH* and *doxD2*. The relative mRNA levels of the two genes were also much higher than those in 9K-S<sup>0</sup> medium. Tetrathionate can be hydrolyzed by TetH to thiosulfate, sulfate, and sulfur. The *doxD* genes, encoding thiosulfate:quinone oxidoreductase, were predicted to be involved in thiosulfate decomposition (5). So, the results indicated that the transcription of *tetH* and *doxD2* could be induced by tetrathionate and thiosulfate. The transcription levels of the genes predicted to function in elemental sulfur metabolism, such as *sqr* and *hdrB*, did not show obvious changes in 9K-K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> medium compared with that in 9K-S<sup>0</sup> medium, which might suggest that these genes do not play crucial roles in tetrathionate metabolism.

For the *tetH* overexpression strain cultured in 9K-S<sup>0</sup> medium (Table 2), the transcription of *tetH*, *doxD2*, the two *sqr* genes, *hdrB*, and *rhd* were upregulated, among which *tetH* and *doxD2* might be driven by the overexpression of the *tetH* gene from plasmid pMSD1-tac-tetH, resulting in the speedup of the hydrolysis of tetrathionate and the production of thiosulfate and sulfur. The lesser extent of increased expression of *tetH* and *doxD2* compared with that in 9K-K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> medium could be explained by the lower concentration of thiosulfate produced during the metabolism of sulfur. Accordingly, transcription of *doxD2* was downregulated in the  $\Delta tetH$  mutant. Of the two *doxD* genes, *doxD2* showed positive signals, consistently with the knockout or overexpression of the *tetH* gene, while no apparent changes were detected in *doxD1* expression. This indicated that the *doxD2* gene might play a main part in thiosulfate decomposition. In addition, the upregulation of the transcription of the two *sqr* genes, *hdrB*, and *rhd* in the *tetH* overexpression strain cultured in 9K-S<sup>0</sup> medium demonstrated that TetH might play an indirect role in the transcriptional regulation of the genes involved in RISC oxidation. As for the two *sqr* genes, they behaved the same way but with slightly larger changes for AFE\_0267 than for AFE\_1792. However, most of the research on sulfide metabolism has been focused on AFE\_1792 (31–34). In our study, the qRT-PCR data indicated that AFE\_0267 might also play a key role in H<sub>2</sub>S metabolism after elemental sulfur enters the cells.

The relative mRNA levels of the selected genes of the tested strains cultivated in 9K-Fe<sup>2+</sup> medium were also investigated (Table 2). As expected, no mRNA of *tetH* was detected in the  $\Delta tetH$  mutant, and upregulated transcription of *tetH* was detected in the *tetH* overexpression strain. Most of the genes did not show obvious changes in transcriptional level, suggesting that the two energy source-metabolic systems of sulfur and ferrous iron in *A. ferrooxidans* ATCC 23270 are relatively independent.

## DISCUSSION

It is well established that *A. ferrooxidans* can obtain energy by the oxidation of ferrous iron or RISCs for their chemolithoau-

trophic growth in acidic environments. The study on oxidation of RISCs by *A. ferrooxidans* has proved particularly challenging, and the proposed models of sulfur metabolism were built based merely on the bioinformatic predictions of its genome sequence, coupled with analyses of its transcriptomic or proteomic experimental data (4, 5). The functions of most genes involved in the oxidation of RISCs remain uncertain, though some genes have been characterized by cloning and expressing them in *Escherichia coli* (12, 13, 32, 36). Considering the marked differences in the growth requirements between the genera *Acidithiobacillus* and *Escherichia*, the periplasmic or outer membrane proteins of *A. ferrooxidans*, such as some enzymes and electron carriers related to sulfur oxidation, might find it hard to function in *E. coli*. So, the most effective method relies mainly on direct proof of functional identification by the knockout and overexpression of a certain gene in *A. ferrooxidans* itself. Unfortunately, to the best of our knowledge, no progress on this problem has been reported until now due to the difficulties of genetic manipulation in *A. ferrooxidans*.

The markerless gene deletion method which was newly established for the extremely acidophilic organism *A. ferrooxidans* (22) was used in the construction of the *tetH* knockout mutant. The potential single recombinants of *A. ferrooxidans* were screened with little effort after the first transfer of a suicide plasmid due to the existence of a selectable marker of kanamycin integrated into the chromosome. However, the double-recombination events may occur randomly during cell growth, which makes it really laborious to obtain potential double-recombination mutants among the transconjugants without any selectable markers. Considering the difficulties of making replicate plates of *A. ferrooxidans* colonies, the negative-selection method was incapable of convenient employment in *A. ferrooxidans*. An improved method for screening double-recombination mutants based on colony color has been established lately in our lab and will be applied in the knockout of other genes of *A. ferrooxidans*.

Although it was generally considered very difficult to return a certain gene to the extremely acidophilic organism *A. ferrooxidans* (6, 37, 38), the *tetH* gene was successfully introduced into *A. ferrooxidans* ATCC 23270 by conjugation, and an engineering strain overexpressing the *tetH* gene under the control of the powerful *tac* promoter from a broad-host-range plasmid, pMSD1-tac-tetH, was constructed. Therefore, except for the elimination in function by being knocked out, the *tetH* gene could also be elucidated by its increase in function on account of its overexpression. In this study, the significant growth increment of the *tetH* overexpression strain, the lack of growth of the  $\Delta tetH$  mutant, and the restored growth of the  $\Delta tetH$  mutant complemented with the *tetH* gene were detected by using tetrathionate (S<sub>4</sub>O<sub>6</sub><sup>2-</sup>) as the sole energy source, which provided sufficient proof that *tetH* acts as the sole gene to encode the tetrathionate hydrolase (TetH) for the decomposition of tetrathionate in *A. ferrooxidans* ATCC 23270.

Since TetH was thought to be an induced enzyme involved in RISC oxidation (12), little research has focused on its role in Fe<sup>2+</sup> oxidation. It was reported that TetH could show a tetrathionate-dependent Fe<sup>2+</sup>-producing activity due to the enzymatic catalysis of tetrathionate hydrolysis followed by a chemical Fe<sup>3+</sup> reduction, to give Fe<sup>2+</sup> by the produced thiosulfate (14). Although the evidence that TetH could be purified from Fe<sup>2+</sup>-grown cells proved the expression of *tetH* in Fe<sup>2+</sup> medium (14), the relation between TetH and Fe<sup>2+</sup> oxidation is still not clear. Therefore, the influence



on Fe<sup>2+</sup> oxidation by knocking out and overexpressing the *tetH* gene in *A. ferrooxidans* needs further study.

It is well established that multiple genes are involved in the sulfur-metabolic pathways of *A. ferrooxidans*, among which the *tetH* gene takes part just in the dissimilation of tetrathionate, an intermediate during sulfur oxidation (4–6, 38). The corresponding reduced and increased growth obtained, respectively, by the  $\Delta tetH$  mutant and the *tetH* overexpression strain when grown in 9K-S<sup>0</sup> medium clearly indicated that TetH influenced the whole metabolism of sulfur to some extent. The existence of TetH in sulfur-grown *A. ferrooxidans* cells has been proved, but the procedures resulted in approximately 93-fold purification (14), indicating a quite small amount of TetH in sulfur-grown cells. This might be the reason why we failed to detect the TetH activities of strains grown on sulfur. A similar negative result was also reported for *Acidithiobacillus caldus*, in which TetH activity was not detected in the crude enzyme extract when the organism was cultivated in liquid medium with elemental sulfur as the energy source (10). As TetH is thought to be responsible for the hydrolysis of tetrathionate to thiosulfate, sulfur, and sulfate, the upregulation of *doxD2* in the *tetH* overexpression strain and its downregulation in the  $\Delta tetH$  mutant according to qRT-PCR analysis indicated that the predicted *doxD2* operon (5, 36) might play an important role in thiosulfate metabolism in *A. ferrooxidans*. This could be supported by the identification of the *doxD2* cotranscribed gene AFE\_0042, which encodes the thiosulfate dehydrogenase responsible for the enzymatic metabolism of thiosulfate (36). With regard to the two *sqr* genes, AFE\_0267 seemed to play as important a role in sulfide oxidation as AFE\_1792 according to qRT-PCR analysis, while only AFE\_1792 was reported to be upregulated among sulfur-induced genes (5). Moreover, AFE\_0267 is much closer to AFE\_0269, a newly identified *sdo* gene encoding sulfur dioxygenase (35) in the genome of *A. ferrooxidans* ATCC 23270 in our studies (unpublished data). So, the function of AFE\_0267 needs further elucidation. In any case, the genes selected by qRT-PCR analysis were quite limited, and the influence on the whole metabolic network by deletion of the *tetH* gene still needs further transcriptomic analysis.

In conclusion, this is the first report on the characterization of the *tetH* gene by construction of a *tetH* overexpression strain and a *tetH* knockout mutant of *A. ferrooxidans*. The successful construction of the  $\Delta tetH$  mutant of *A. ferrooxidans* demonstrates that the markerless gene deletion technology is no doubt a useful and stable tool for clarifying the functions of certain genes by knocking them out in *A. ferrooxidans*. This study on the *tetH* gene could be considered a model for investigating other candidate genes involved in the whole sulfur oxidation network, including genes that might completely block sulfur oxidation through application of ferrous iron as an energy source, and relevant research is under way in our lab.

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