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Regulation of a Novel *Acidithiobacillus caldus* Gene Cluster Involved in Metabolism of Reduced Inorganic Sulfur Compounds▽

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***Acidithiobacillus caldus* has been proposed to play a role in the oxidation of reduced inorganic sulfur compounds (RISCs) produced in industrial biomining of sulfidic minerals. Here, we describe the regulation of a new cluster containing the gene encoding tetrathionate hydrolase (*tetH*), a key enzyme in the RISC metabolism of this bacterium. The cluster contains five cotranscribed genes, IS*ac1*, *rsrR*, *rsrS*, *tetH*, and *doxD*, coding for a transposase, a two-component response regulator (RsrR and RsrS), tetrathionate hydrolase, and DoxD, respectively. As shown by quantitative PCR, *rsrR*, *tetH*, and *doxD* are upregulated to different degrees in the presence of tetrathionate. Western blot analysis also indicates upregulation of TetH in the presence of tetrathionate, thiosulfate, and pyrite. The *tetH* cluster is predicted to have two promoters, both of which are functional in *Escherichia coli* and one of which was mapped by primer extension. A pyrrolo-quinoline quinone binding domain in TetH was predicted by bioinformatic analysis, and the presence of an *o*-quinone moiety was experimentally verified, suggesting a mechanism for tetrathionate oxidation.**

It is well established that consortia of bacteria and archaea play a pivotal role in the recovery of metals in industrial "biomining" operations (25, 27, 30, 32). Biomining refers to both "bioleaching," whereby target metals are solubilized from the sulfide mineral, and "biooxidation," where the target metal is exposed by oxidation of the sulfide mineral surrounding it. In biomining operations, the principal function of the microbial consortium is the oxidation of Fe^{2+} to Fe^{3+}, whereby the microorganisms gain energy for metabolic functions and the resulting Fe^{3+} carries out mineral oxidation. A major limitation to the use of biomining has been the accumulation of solid elemental sulfur (S^0) compounds on the surfaces of minerals (e.g., see references 14 and 42). It has been suggested that these compounds contribute to a decrease in mineral dissolution by limiting Fe^{3+} access to the metal sulfide bond, termed passivation (12). However, in the absence of an iron-oxidizing catalyst, the presence of S^0 did not reduce leaching rates from marcasite (FeS_2) and arsenopyrite (FeAsS). Possibly this was because areas of the mineral were "clean" of sulfur compounds or because the sulfur layer was permeable to the metal sulfide oxidant at the thickness observed in the study (14).

Microorganisms that use reduced inorganic sulfur compounds (RISCs) as a source of energy include archaea and bacteria and comprise acidophilic or neutrophilic photo- and chemolithotrophs that often use sulfur oxygenase (*sox* gene cluster) and sulfur oxygenase reductase, coded by the *sor* gene (16, 19, 37). However, only a few RISC-metabolizing enzymes have been characterized from industrially important acidophilic microorganisms; these include *Acidithiobacillus ferrooxidans* sulfur dioxygenase, which yields sulfite from S^0 (33); sulfite oxidoreductase, which oxidizes sulfite to sulfate (40); a sulfide:quinone oxidoreductase which oxidizes sulfide to sulfur (41); thiosulfate oxidase, which catalyzes the oxidation of thiosulfate to tetrathionate (36); and tetrathionate hydrolase, which hydrolyzes tetrathionate to thiosulfate, sulfur, and sulfate (8). A number of enzymes and enzymatic activities have also been identified in *Acidithiobacillus thiooxidans*, including thiosulfate dehydrogenase (24), sulfite:ubiquinone oxidoreductase activity (38), and tetrathionate hydrolase (39).

Acidithiobacillus caldus is one of the most abundant microorganisms in industrial biomining (26, 31), where it is suggested to oxidize RISCs formed during sulfide mineral breakdown (12, 13). Elemental sulfur and tetrathionate are key intermediates in *A. caldus* metabolism, and tetrathionate hydrolysis yields thiosulfate, pentathionate, and eventually sulfate (6), while S^0 is oxidized to sulfate via sulfite (17). The protein responsible for *A. caldus* tetrathionate decomposition is a periplasmic homodimer with a maximum activity at pH 3 (6). Sulfur-grown *A. caldus* lacks a tetrathionate-metabolizing activity, suggesting that the expression of tetrathionate hydrolase is substrate dependent and regulated on either the transcriptional or translational level (6).

This report presents a novel gene cluster containing the gene coding for the *A. caldus* tetrathionate hydrolase, shows its differential expression by using quantitative PCR (Q-PCR) and Western blot analysis, maps the promoter regions, and demonstrates the presence of an *o*-quinone cofactor in tetrathionate hydrolase (TetH). Knowledge of RISC oxidation regula-

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TABLE 1. PCR, RT-PCR, and Q-PCR oligonucleotides used in this study

Name	Sequence (5′ to 3′)	Orientation	Application
a	AAGAATTCCGTGACCATAGCTTTTGCCAT	Forward	P0 promoter/*lac* operon fusions
b	AAGGATCCCCGTTGCTGATCAGATCCAA	Reverse	P0 promoter/*lac* operon fusions
c	TTTGAATTCAACGCACGTAAATTGTA	Forward	P1 promoter/*lac* operon fusions
d	TTGGATCCAGGGTTGCGGTAACGGCAA	Reverse	P1 promoter/*lac* operon fusions
e	ACAGATTGCCAGGCACCTTGTA	Forward	RT-PCR
f	GATCCATCCATATGCGAGCAGAT	Reverse	RT-PCR, primer extension
g	CCAAAATGTGTCTCTTGGTCAT	Forward	RT-PCR
h	TTCATACATATTTCTGGCTCATCG	Reverse	RT-PCR, Q-PCR
i	CGCCGACGATTACCTATCAAA	Forward	RT-PCR, Q-PCR
j	GTCATCGCATCATGGCATTGCACA	Forward	RT-PCR
k	TGGGGTAGGTATAAACGCGCAA	Reverse	RT-PCR, primer extension
l	TTGCGCGTTTATACCTACCCCA	Forward	RT-PCR
m	CGTATATGAATCGCCTGGATCC	Reverse	RT-PCR
n	TTGCGCGTTTGTACCTACC	Forward	Q-PCR
o	GCCGTCTACTTGAGCTCC	Reverse	Q-PCR
p	CTCTAATTTCGCTATGGGGATCAC	Forward	Q-PCR

tion in this industrially important bacterium could contribute to the development of new approaches in biomining.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. caldus* strain KU (ATCC 51756 and DSM 8584) was cultivated in mineral salts medium (MSM) (acidified to pH 2.5 with H_2SO_4) containing trace elements (12). The MSM was heated at 121°C for 15 min before filter-sterilized (0.22-μm PES membrane filter; Millipore) trace elements were added, and either sterile-filtered tetrathionate (5 mM) or 0.5% (wt/vol) S^0 (sterilized at 105°C for 24 h) was added as the energy source. Cultivation with 10 mM thiosulfate as the substrate was performed in a 500-ml continuous culture vessel (dilution rate = 0.006 h^{-1}; Watson Marlow 205S pump) to minimize acid degradation of the thiosulfate. The culture vessel was stirred (300 rpm) and aerated with 300 ml CO_2-enriched air min^{-1} (2%, vol/vol) at 45°C. Cultivation of "*Ferroplasma acidarmanus*" (proposed name) Fer1 (10, 15) was carried out in MSM (pH 2) with 0.02% (wt/vol) yeast extract (autoclaved at 121°C for 15 min) at 37°C with sparging with 300 ml air min^{-1} and 3% (wt/vol) pyrite. The pyrite concentrate has been previously described (11). The mixed culture of "*F. acidarmanus*" and *A. caldus* was grown as described for "*F. acidarmanus*" except that the yeast extract was omitted. *Escherichia coli* strains DH5α and BL21(DE3) were grown in Luria-Bertani medium with the addition of an appropriate antibiotic.

Bioinformatic analysis of the *tetH* gene cluster. Candidate protein-coding genes of the partial genome sequence of *A. caldus* KU were predicted using GLIMMER (9) and CRITICA (3). The gene prediction results were combined, and the corresponding amino acid sequences were compared against GenBank's nonredundant database using BLASTP (1). The alignments of the N terminus of each gene model versus the best match were used to select the preferred gene model. The revised genes were compared against the GenBank nonredundant database and the Swissprot, Pfam, Tigrfam, PROSITE, PRINTS, and COGS databases. The annotated sequences were displayed in Artemis (5) to facilitate further functional curation. Promoter prediction was performed using programs available at www.fruitfly.org/seq_tools/promoter.html and www.softberry.com and a combined Hidden Markov Model/Neural network program developed by David Holmes's laboratory (unpublished). The TetH alignment and construction of the phylogenetic tree were carried out using MEGA version 3.1 (20). Three separate phylogenetic trees were created by distance and neighbor joining, parsimony, and minimum evolution methods, and the neighbor joining tree is presented (see Fig. 2). Those nodes supported by all three trees and by two trees have been indicated.

N-terminal sequencing of the tetrathionate hydrolase. To ensure that the *tetH* gene sequence coded for the purified tetrathionate hydrolase protein (6), N-terminal and internal amino acid sequences were determined by Edman degradation and in-gel trypsin digestion (carried out by the Protein Analysis Center, Karolinska Institute, Sweden).

DNA manipulation and sequencing. *A. caldus* cells were cultured as described above and harvested at 10,000 × *g* for 10 min, washed twice in 10 mM Tris HCl buffer (pH 8), and lysed by the addition of 100 mg lysozyme ml^{-1} for 10 min at room temperature followed by a final concentration of 0.5% (wt/vol) sodium dodecyl sulfate (SDS) for 30 min. Genomic DNA was prepared from the cell lysate using phenol:chloroform:isoamyl alcohol extraction and isopropanol precipitation (34). Established methods were used for restriction enzyme digestion, ligation, transformation, and plasmid purification.

RNA preparation, RT-PCR, and Q-PCR. RNA was prepared from exponential-phase cells grown on tetrathionate or S^0 by using an RNeasy mini kit (QIAGEN) with subsequent treatment with a DNA-*free* kit (Ambion), according to the manufacturers' recommendations. The RNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Saveen Werner). Reverse transcription-PCR (RT-PCR) was performed in two steps. First, cDNA was produced from total RNA by using a RevertAid first strand cDNA synthesis kit (Fermentas) and random hexamer primers. In the second step, PCR was carried out with sequence-specific primers (RT-PCR oligonucleotides shown in Table 1) and PuRe *Taq* ready-to-go PCR beads (GE Healthcare). To ensure that the RT-PCR was functioning correctly, positive controls using convergent primers within *tetH* and *doxD* were also carried out. Controls using the same primers to test for DNA contamination in the RNA preparations were all negative. Q-PCR was also prepared in a two-step manner with the cDNA produced from RNA using a RevertAid first strand cDNA synthesis kit (Fermentas) and random hexamer primers. The second step was performed with sequence-specific primers (Q-PCR primers shown in Table 1) and iTaq SYBR green supermix (Bio-Rad) using a Bio-Rad iCycler iQ multicolor real-time PCR detection system. The expression of the analyzed genes was calculated using iCycler iQ optical system software version 3.1 (Bio-Rad). All Q-PCR experiments were carried out in triplicate from two independent experiments.

Protein purification, antibody preparation, and Western blotting. TetH was purified from *A. caldus* cells according to the method of Bugaytsova and Lindström (6), with an additional purification step of polyacrylamide gel electrophoresis for the enzyme prior to use for antibody generation. Polyclonal antibodies against TetH were raised using a series of four immunizations in a rabbit (AgriSera AB, Vännäs, Sweden). Western blotting detection of TetH (34) was carried out using anti-tetrathionate hydrolase primary and horseradish peroxidase-labeled secondary antibodies. The Western blot was visualized by using an ECL-PLUS detection kit (Amersham Pharmacia), and images were captured with a GelDoc XR system equipped with a charge-coupled-device camera and Quantity One software version 4.6.1 (Bio-Rad). The Western blot image was processed using Adobe Photoshop.

Promoter fusion and β-galactosidase assays. DNA fragments containing predicted promoters P0 and P1 (Fig. 1) were amplified using primer pairs a and b and c and d (Table 1), digested with EcoRI and BamHI, and cloned upstream from the promoterless *lac* operon in the multiple cloning site on the broad-host-range plasmid pRW2 (21). β-Galactosidase activity measurements were performed in *E. coli* strain DH5α (22). β-Galactosidase measurements were carried out in triplicate, and the means ± standard deviations are presented below.

Primer extension. Primer extension was carried out according to Balsalobre et al. (4), except that Moloney murine leukemia virus reverse transcriptase (SuperScript II reverse transcriptase; Invitrogen) instead of avian myeloblastosis virus reverse transcriptase was used with the supplied buffer in the extension reaction mixture. Primers f and k were used for P0 and P1, respectively (Table 1). The primer extension image was processed using Adobe Photoshop.

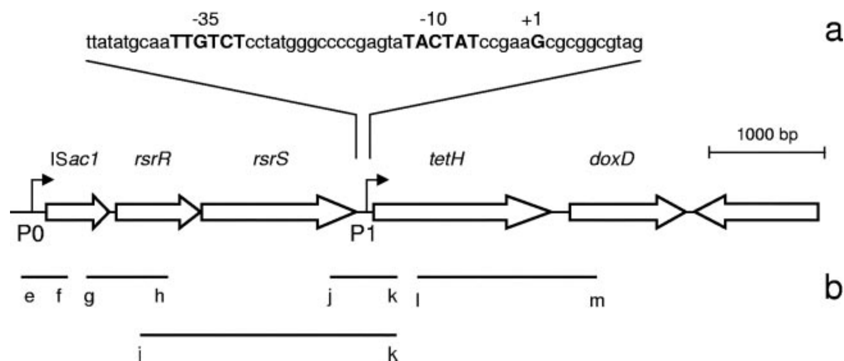

FIG. 1. (a) *A. caldus tetH* gene cluster with the expanded portion describing the P1 promoter region showing the −10 and −35 sequences and transcription start site (all in bold caps). (b) Positive transcripts detected by RT-PCR represented by lines under the corresponding genes (to scale; see Table 1 for primers). The second transposase was not labeled as it is coded on the opposite strand.

Measurement of the TetH PQQ content. Purified TetH (1.6 mg) was lyophilized, and the dried pellet extracted with 2 ml methanol. After incubation at 37°C for 30 min, the suspension was centrifuged at 10,000 × *g* for 10 min and the supernatant evaporated. The dry material was resuspended in 5 mM Tris-HCl buffer (pH 8.0) and centrifuged at 10,000 × *g* for 10 min, and the supernatant used as an extract. The pyrrolo-quinoline quinone (PQQ) content was measured enzymatically using the recombinant soluble apoenzyme from *Acinetobacter calcoaceticus* LMD 79.41 (28). Fifty microliters of the extract and 10 μl of 0.4 M $CaCl_2$ were added to 10 μl of apo-glucose dehydrogenase (5 mg/ml). The mixture was incubated at 30°C for 30 min (5 mM Tris-HCl buffer [pH 8.0] was used instead of extract in the control experiments). The glucose dehydrogenase activity was assayed by using a dye-linked system containing 20 mM glucose, 50 μM phenazine methosulfate, and 100 μM 2,6-dichlorophenol indophenol (DCPIP; total volume 1 ml) at 30°C. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol DCPIP min^{-1}. Experiments were carried out in triplicate, and the means ± standard deviations are presented below.

Staining for quinoproteins. Quinoproteins were detected by staining with nitro blue tetrazolium (NBT; 0.24 mM in 2 M potassium glycinate, pH 10) (29). The protein samples were applied to the nitrocellulose filter, dried at room temperature, immersed in the glycinate-NBT solution for 45 min in the dark, and then dipped in 0.1 M sodium borate, pH 10. Quinoproteins were specifically stained as purple-blue bands due to NBT reduction to formazan.

Nucleotide sequence accession number. The DNA sequence of the *tetH* gene cluster is available under the GenBank accession number EF460464.

RESULTS AND DISCUSSION

TetH sequencing and bioinformatic analysis of the gene cluster containing *tetH*. Trypsin digestion and N-terminal sequencing of the tetrathionate hydrolase protein provided three peptide sequences (SITPVLQPGNPFDPDSPFARLYLPQNA, GVQWNFP, and GEIPGAVNTG) that were used to confirm the corresponding gene in the genome sequence of *A. caldus*. TetH (GenBank accession number ABP38225) is predicted to be a 503-amino-acid-long protein containing a 24-amino-acid Sec signal peptide with a signal peptide cleavage site. It is predicted to be a periplasmic exposed protein with four transmembrane spanning loops, two nonoverlapping WD-40 repeats, and a quinone binding domain (Table 2). *tetH* was linked to the upstream genes *rsrR* and *rsrS*, predicted to encode a two-component response regulator belonging to the osmoregulatory family, and to a downstream gene, *doxD*, predicted to encode a subunit of thiosulfate: quinol oxidoreductase. The cluster is flanked by two predicted transposases (Table 2; Fig. 1a).

The predicted PQQ binding domain is a propeller structure

TABLE 2. Cluster of genes containing *tetH* and flanked by two insertion sequences

Gene name	Suggested function	Best hit in NCBI database	Score[d]	E value[d]	% S[a]	No. of TM[b]	Domain(s) and motif(s)[c]
IS*ac1*	Transposase (IS family 256)	Transposase (*Azoarcus* sp. strain EbN1)	131	$3e^{-29}$	72	None	PF00872
rsrR	Two-component transcriptional regulator system	Response regulator receiver (*Acidovorax* sp. strain JS42)	195	$1e^{-48}$	67	None	PD000039, PF00072, D000329, PF00486, SSF 52172, S50110, SM00448
rsrS	Two-component transcriptional regulator system	Periplasmic sensor signal transduction histidine kinase (*Shewanella baltica* OS195)	119	$4e^{-25}$	46	4	PF02518, SM00387, SSF55874, PF00672, PS50885, PF00512
tetH	PQQ binding dehydrogenase or kinase	Tetrathionate hydrolase (*A. ferrooxidans*)	506	$1e^{-141}$	71	4	PF01011, SM00564, SSf50998
doxD	Quinol oxidase subunit (DoxD)	DoxD subunit (*Gluconobacter oxydans* 621H)	307	$5e^{-82}$	67	5	PF04173
IS*ac2*	Transposase (IS family 630)	Transposase (*Burkholderia vietnamiensis*)	413	$7e^{-114}$	76	None	SSF46689, SSF53098

[a] S, similarity of the alignment between the gene and the top hit from the NCBI database using BLASTP.
[b] TM, transmembrane domain; predicted by the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html).
[c] Domains were identified in the proteins by using InterProScan (http://www.ebi.ac.uk/InterProScan/).
[d] Statistics derived from BLASTP (NCBI database).

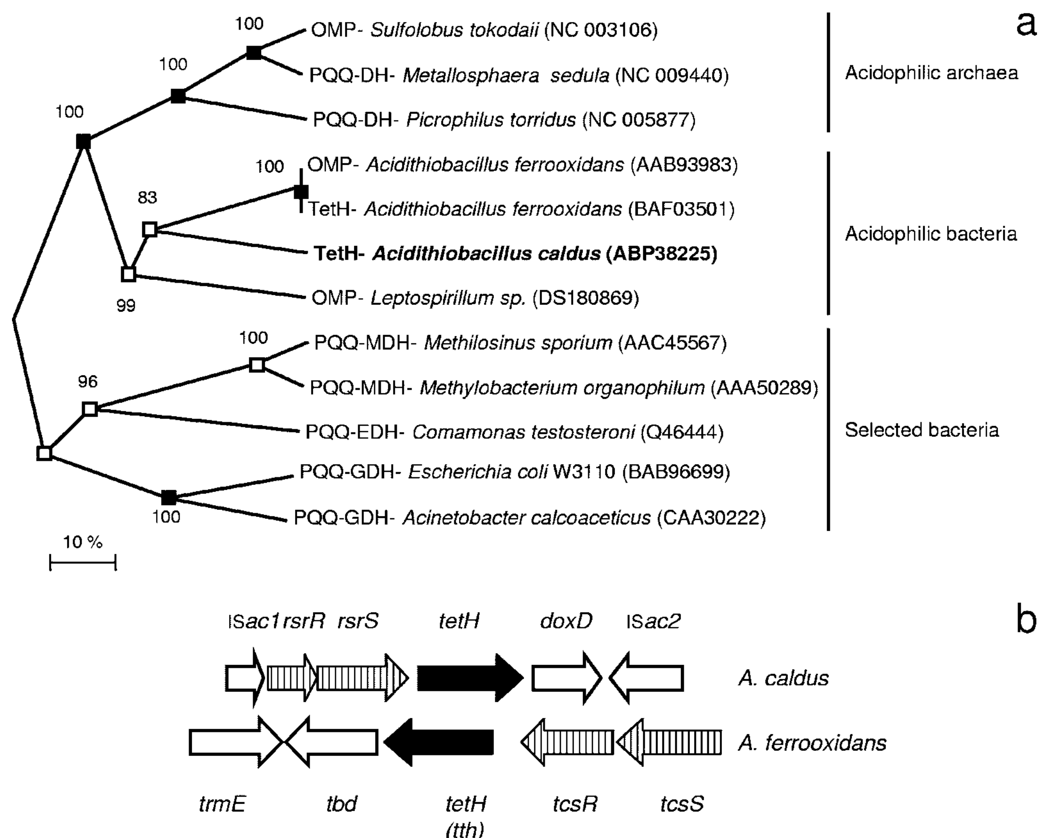

FIG. 2. (a) Unrooted neighbor joining tree of the *A. caldus tetH* (in bold) alignment with closest relatives from the NCBI database containing a PQQ binding domain and selected neutrophilic dehydrogenases also containing a PQQ binding domain. Phylogenetic analysis was carried out by the minimum evolution, distance neighbor joining, and maximum parsimony methods in MEGA; the nodes supported by all three trees (filled boxes) and by two trees (open boxes) have been marked, and the values by the nodes are bootstrap values of 1,000 runs. Accession numbers are given in parentheses. The scale bar represents 10% sequence similarity. (b) Gene block comparison of the *A. caldus* and *A. ferrooxidans* gene clusters. Vertical lines represent the two-component regulation system, and solid black denotes the tetrathionate hydrolase gene (termed *tth* in *A. ferrooxidans*).

found in dehydrogenases that use PQQ as a cofactor (2). Bioinformatic analysis showed that TetH was similar to several other membrane-bound PQQ dehydrogenases, and the TetH amino acid sequence formed a clade with *A. ferrooxidans* tetrathionate hydrolase and other PQQ binding domain-containing proteins from acidophilic bacteria and archaea that were distinct from neutrophilic quinone dehydrogenases (Fig. 2a). The proteins from acidophilic microorganisms were suggested to be membrane-bound dehydrogenases that agreed with the suggested localization of *A. caldus* TetH. The abundance of this gene group among acidophilic bacteria and archaea suggests its specificity and importance at low pH. The *A. caldus tetH* gene cluster context was compared to that found in *A. ferrooxidans* (Fig. 2b), and both clusters contain a two-component regulatory system. The *A. ferrooxidans* TcsRS system was most similar to the σ^{54}-dependent ZraRS-like two-component systems, whereas the *A. caldus* RsrRS was similar to OmpRS-like systems.

RT-PCR analysis of the *tetH* cluster. Amplification products were obtained by RT-PCR experiments between primer pairs g and h, i and k, j and k, and l and m, indicating that IS*ac1*, *rsrR*, *rsrS*, *tetH*, and *doxD* are cotranscribed. IS*ac2* is not part of the operon as it is oriented in the opposite direction (Fig. 1b).

Q-PCR analysis of *tetH* expression. Two-step Q-PCR was performed with RNA samples prepared from *A. caldus* cultures grown utilizing either S^0 or tetrathionate as the growth substrate. All three tested genes (*rsrR*, *tetH*, and *doxD*) were upregulated with tetrathionate as the substrate in comparison to their levels with growth on S^0, by 6.5-fold ± 5.6-fold, 233.5-fold ± 134.0-fold, and 25.3-fold ± 20.2-fold, respectively (for all, *n* [number of replicate experiments] = 6). This suggests that internal promoters may be present within the gene cluster, that posttranscriptional processing of mRNA takes place, or that there are different levels of primer binding due to mRNA structure.

Western blotting shows high levels of TetH in tetrathionate-grown *A. caldus*. Western blots identified bands corresponding to TetH in cells grown on tetrathionate, thiosulfate, and a mixture of tetrathionate and S^0 (Fig. 3). The positive result with cells grown on thiosulfate may be explained by the fact that the products of thiosulfate oxidation include tetrathionate (17). A weaker band was found in *A. caldus* and "*F. acidarmanus*" cells grown in mixed culture on pyrite that is oxidized to Fe^{2+} and thiosulfate (35) but not in "*F. acidarmanus*" protein alone as it does not oxidize RISCs (10). The weak band may be explained by a standard concentration of protein being loaded

FIG. 3. Western spot immunodetection of *A. caldus* TetH. Lane 1, *A. caldus* cells grown in mixed culture with "*F. acidarmanus*" on pyrite; lane 2, negative control with "*F. acidarmanus*" grown on pyrite; lane 3, *A. caldus* grown on a mixture of S^0 and tetrathionate; lane 4, *A. caldus* grown on S^0; lane 5, *A. caldus* grown on tetrathionate; and lane 6, *A. caldus* grown on thiosulfate. All lanes except lane 6 (100 μg) were loaded with 50 μg total protein. The dashed line indicates where the image of the single gel was cropped.

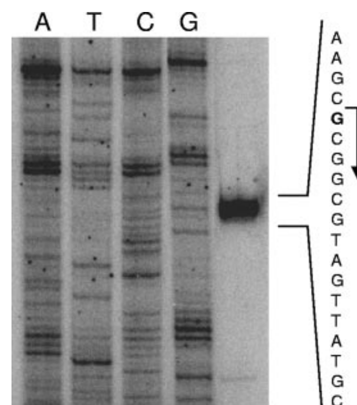

FIG. 4. Primer extension analysis of the *A. caldus* P1 promoter during growth on tetrathionate. The predicted transcription start site is marked in bold with a bent arrow.

onto the gel that would have been derived from both species. The expression of TetH during bioleaching suggests its potential importance, as the end point of RISC metabolism is sulfuric acid and this produces the acidic environment necessary for the growth of *A. caldus* (and other biomining microorganisms). An industrial aim within bioleaching is the ability to control the oxidation of RISCs, and this study is the first step in understanding its regulation. The TetH enzyme was not observed when 50 μg total cell protein from S^0-grown *A. caldus* was loaded on the gel. However, with 500 μg total protein, a faint band was observed (data not shown). This result correlates with the Q-PCR data, where it was shown that the expression of the *tetH* gene was strongly upregulated during cultivation on tetrathionate compared to its expression during cultivation on S^0, and with previous data demonstrating lack of TetH activity in *A. caldus* cultured with S^0 (6). The expression of *tetH* in cells cultured on tetrathionate and S^0 suggests that its expression was induced by the presence of tetrathionate rather than repression by S^0 and that it was regulated at the transcriptional level, depending on the growth conditions.

Investigation of the *tetH* operon promoters. The two predicted promoters within the *tetH* cluster (P0 and P1) (Fig. 1) were cloned upstream of the pRW2 promoterless *lac* operon in *E. coli* strain DH5α, and the β-galactosidase activities were measured. The β-galactosidase activities were 42 ± 15, 103 ± 29, and 33,011 ± 4,675 Miller units for the vector control, P0, and P1 promoters, respectively (for all, $n = 3$). This gives 2.4- and 786-fold higher β-galactosidase expression from the P0 and P1 promoters, respectively, than from the vector control, suggesting that the promoters were active.

Primer extensions using total RNA from *A. caldus* identified a transcription start site located between *rsrR* and *tetH* for promoter P1 (Fig. 4). However, no band was detected in association with the P0 promoter (data not shown). This was probably due to the low expression level from this promoter, as shown by Q-PCR with *A. caldus* and by *lac* promoter fusion experiments with *E. coli*. Therefore, *tetH* regulation may occur via the *rsrRS* system at the P1 promoter, and future work will aim to elucidate possible transcriptional regulation by several predicted transcriptional factor (Fnr, ArcA, OmpR, and GcvA) binding sites. Interestingly, the *A. ferrooxidans* tetrathionate hydrolase gene (*tth*) also has a two-component regulatory system directly upstream, although, unlike the *A. caldus rsrRS*, the *A. ferrooxidans* system belongs to the σ^{54}-specific family.

Identification of a TetH-bound quinoid compound. The prediction of a putative PQQ binding domain in TetH was surprising, as previous analyses had not suggested a dehydrogenase activity (6, 17). Therefore, it was tested whether a TetH methanol extract restored the function of an apo form of the recombinant PQQ-dependent glucose dehydrogenase. The negative controls (lacking TetH extract, apoenzyme, or glucose; for all, $n = 3$) all gave a glucose dehydrogenase activity of $\leq$0.09 U ml^{-1} ± 0.03 U ml^{-1}, whereas the positive control with added PQQ gave 86.2 U ml^{-1} ± 0.7 U ml^{-1}. In the presence of TetH methanol extract, the activity was 0.09 U ml^{-1} ± 0.03 U ml^{-1}, suggesting that TetH does not contain PQQ (data not shown). However, native TetH was suggested to contain a quinoid compound by NBT-glycinate staining after direct blotting on a nitrocellulose filter (Fig. 5) and sodium dodecyl sulfate electrophoresis with subsequent blotting (data not shown). However, it was not possible to identify the cofactor, as the staining does not discriminate between different quinoid compounds. The predicted quinone binding domain and the positive *o*-quinone staining suggest that TetH is involved in quinone turnover. However, the fact that the TetH extract did not restore the function of the apo form of glucose dehydrogenase suggests that this cofactor is not PQQ.

Conclusions. RT-PCR results show that *tetH* is cotranscribed with *doxD*. Although the role of *doxD* in *A. caldus* has not been defined, it is similar to a gene from the acidophilic thermophile *Acidianus ambivalens* that encodes a subunit of thiosulfate:caldariella quinone oxidoreductase and to a gene encoding one of the components of a predicted thiosulfate:quinol oxidoreductase in *A. ferrooxidans*. However, *A. ferrooxidans* contains further copies of *doxDA*, possibly used for thiosulfate metabolism (23). It has been previously reported that uncouplers and inhibitors of

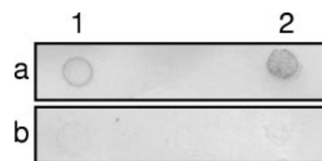

FIG. 5. *o*-Quinone staining of two different amounts of native TetH (a) and TetH boiled and precipitated with trichloroacetic acid before blotting and staining (b). The amounts of TetH were 15 and 80 μg protein in lanes 1 and 2, respectively.

electron transport, such as carbonyl cyanide *m*-chlorophenyl-hydrazone and 2,4-dinitrophenol suppress tetrathionate hydrolysis of *A. caldus* (17). Moreover, bioinformatic analysis suggests that *tetH* is similar to PQQ binding dehydrogenases that normally transfer electrons to the quinol oxidase or bc_1 complex via ubiquinone as a primary electron acceptor (2, 7). Based on this data, we propose that TetH is connected to the respiratory chain through an *o*-quinone cofactor. Although the *A. caldus* and *A. ferrooxidans* tetrathionate hydrolases had 71% similarity, the latter is not inhibited by 2,4-dinitrophenol (18) and *doxD* genes are not located in the neighborhood of *tetH* on the *A. ferrooxidans* chromosome (Fig. 2b), suggesting that the proteins may have different mechanisms for transferring electrons to the respiratory chain.

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