An extracellular tetranionate hydrolase from the thermoacidophilic archaeon *Acidianus ambivalens* with an activity optimum at pH 1

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**Background:** The thermoacidophilic and chemolithotrophic archaeon *Acidianus ambivalens* is routinely grown with sulfur and CO₂-enriched air. We had described a membrane-bound, tetranionate (TT) forming thiosulfate:quinone oxidoreductase. Here we describe the first TT hydrolase (TTH) from Archaea. **Results:** *A. ambivalens* cells grown aerobically with TT as sole sulfur source showed doubling times of 9 h and final cell densities of up to 8 x 10⁹/ml. TTH activity (~0.28 U/mg protein) was found in cell-free extracts of TT-grown but not of sulfur-grown cells. Differential fractionation of freshly harvested cells involving a pH shock showed that about 92% of the TTH activity was located in the pseudo-periplasmatic fraction associated with the surface layer, while 7.3% and 0.3% were present in the soluble and membrane fractions, respectively. The enzyme was enriched 54-fold from the cytoplasmic fraction and 2.1-fold from the pseudo-periplasmatic fraction. The molecular mass of the single subunit was 54 kDa. The optimal activity was at or above 95°C at pH 1. Neither PQQ nor divalent cations had a significant effect on activity. The gene (*tth1*) was identified following N-terminal sequencing of the protein. Northern hybridization showed that *tth1* was transcribed in TT-grown cells in contrast to a second paralogous *tth2* gene. The deduced amino acid sequences showed similarity to the TTH from *Acidithiobacillus* and other proteins from the PQQ dehydrogenase superfamily. It displayed a β-propeller structure when being modeled, however, important residues from the PQQ-binding site were absent. **Conclusion:** The soluble, extracellular, and acidophilic TTH identified in TT-grown *A. ambivalens* cells is essential for TT metabolism during growth but not for the downstream processing of the TTQ reaction products in S⁴-grown cells. The liberation of TTH by pH shock from otherwise intact cells strongly supports the pseudo-periplasm hypothesis of the S-layer of Archaea.

**Keywords:** Archaea, sulfur metabolism, tetranionate hydrolase, S⁴ intermediate pathway, surface layer, disproportionation

**INTRODUCTION**

The dissimilatory oxidation of inorganic sulfur compounds (ISC) is one of the most important sources of metabolic energy for CO₂ fixation in light-independent ecosystems like solfataras and other volcanic hot springs (reviewed for example by Kelly et al., 1997; Friedrich et al., 2005; Stetter, 2006; Kletzin, 2007; Ghosh and Dam, 2009). In addition, ISC oxidation is essential for bioleaching of precious and/or toxic metals from low-grade ores and slag heaps, regardless whether this effect is desired (metal extraction) or undesired (acid mine drainage; Sand et al., 2001; Gadd, 2010). Sulfur oxidation pathways and especially the SOX complex from neutrophilic chemo- or phototrophic bacteria are fairly well understood (for review, see Friedrich et al., 2005, 2008; Frigaard and Dahl, 2009; Sakurai et al., 2010). In contrast, there are many gaps in our knowledge concerning the mechanisms of ISC oxidation in acidophilic Bacteria and Archaea, the dominant microorganisms in volcanic hot springs and bioleaching environments (Kletzin, 2007, 2008; Ghosh and Dam, 2009).

Our model organism *Acidianus ambivalens* belongs to the Sulfolobales order within the archaean domain (Zillig et al., 1986; Fuchs et al., 1996). *A. ambivalens* is a chemolithotrophic acidophile growing optimally at 80°C and pH 2.5. It oxidizes S⁴ to sulfuric acid under aerobic conditions and uses hydrogen as the electron donor for S⁴ reduction under anaerobic conditions. A soluble sulfur oxygenase reductase (SOR) mediates the initial step in the S⁴ oxidation pathway of aerobically grown cells, a unique enzyme catalyzing the simultaneous S⁴ oxidation and disproportionation to sulfite, thiosulfate, and sulfide (Kletzin, 1989; Urich et al., 2004; Veith et al., 2011; this volume). It is not known whether thiosulfate is a primary product of the enzyme or whether it originates from a rapid non-enzymatic reaction between S⁴ and sulfite under the assay conditions (Kletzin, 1989; Müller et al., 2004). Regardless of that, S⁴-grown *A. ambivalens* cells contain significant amounts of a membrane-bound thiosulfate:quinone oxidoreductase (TQO) oxidizing thiosulfate to tetranionate (TT) and reducing quinones (Müller et al., 2004). This type of thiosulfate oxidation is known...
as the $S_2$ intermediate pathway (Ghosh and Dam, 2009). Two observations suggest that the active site of the TQO is facing the cytoplasm. First, the SOR, which delivers thiosulfate, is a soluble and cytoplasmic enzyme. Secondly, thiosulfate is not stable at the growth conditions of A. ambivalens (acidic pH and high temperature; Johnston and McAmish, 1973; Moll and Schäfer, 1988) so that it rapidly decomposes, when being added to the medium forming sulfur and sulfite. In contrast, TT is not very stable at the near-neutral pH (6.5; Johnston and McAmish, 1973) so that it rapidly decomposes, and the reducing contrast, TT is not very stable at the near-neutral pH (6.5; Johnston and McAmish, 1973) so that it rapidly decomposes, and the reducing conditions in the cytoplasm. We had hypothesized that the TQO and a non-enzymatic TT reduction to thiosulfate mediated by hydrogen sulfide and sulfite in the cytoplasm ultimately feeds electrons into the respiratory chain (Müller et al., 2004; Kletzin, 2008).

As the fate of the TT produced during the TQO reaction is not clear, we initiated a search for TT-metabolizing enzymes in A. ambivalens. Three ways of TT metabolism are known: (1) the reduction by either multiheme or molybdenum-containing reductases enabling mesophilic proteobacteria like Salmonella enterica to grow anaerobically with TT as a terminal electron acceptor (Hensel et al., 1999; Price-Carter et al., 2001; Mowat et al., 2004); (2) the oxidation by still unknown subunits of SOX multienzyme complexes (Mukhopadhyay et al., 2000; Lahiri et al., 2006); or (3) a disproportionation by tetrathionate hydrolases (TTH) forming sulfate, thiosulfate or sulfite, and sulfur or pentathionate (De Jong et al., 1997a; Bugaytsova and Lindström, 2004; Kanao et al., 2007; Rzhepishevskaya et al., 2007). TTHs isolated from various acidophilic Acidithiobacillus species and Acidiphilium acidophilum (formerly Thiobacillus acidophilus) are usually monomeric and/or homodimeric, membrane-associated, or soluble enzymes with pH optima around 2.5–4 (De Jong et al., 1997a; Bugaytsova and Lindström, 2004; Egorova et al., 2004; Kanao et al., 2007; Mangold et al., 2011; this volume). Sequence comparisons predicted that the TTHs belong to a pyrroloquinoline quinone (PQQ) containing protein family. Kanao et al. (2010) however showed that recombinant Acidithiobacillus ferroxidans TTH purified from E. coli inclusion bodies could be refolded into the active state without PQQ and under acidic conditions.

In this contribution, we demonstrate that A. ambivalens grows well with TT as the sole sulfur source thereby producing a highly active TTH with an optimum at pH 1. We also show that the protein is extracellular and associated with the S-layer of the archaeon.

**MATERIALS AND METHODS**

**ORGANISM AND GROWTH CONDITIONS**

_Acidimnus ambivalens_ DSM 3772 was grown as described (Zillig et al., 1986) with slight modifications. The medium contained per liter: KH$_2$PO$_4$, 2.8 g; (NH$_4$)$_2$SO$_4$, 1.5 g; MgSO$_4$ x 7 H$_2$O, 0.25 g; CaCl$_2$, 2 H$_2$O, 70 mg; FeSO$_4$ x 7 H$_2$O, 28 mg; Na$_2$B$_4$O$_7$, 10 H$_2$O, 9 mg; MnCl$_2$, 4 H$_2$O, 3.6 mg; ZnSO$_4$, 7 H$_2$O, 0.44 mg; CuCl$_2$, 2 H$_2$O, 0.1 mg; Na$_2$SO$_4$, 15 g; Mg$_2$S$_3$O$_7$, 0.07 mg; Na$_2$MoO$_4$, 2 H$_2$O, 0.06 mg; CoSO$_4$, 7 H$_2$O, 0.02 mg. The pH was adjusted to 2.5 with sterile 50% H$_2$SO$_4$. About 10 ml/l of freshly prepared and filter-sterilized 1 M K$_2$S$_4$O$_6$ solution (Merck, Darmstadt, Germany) was added as sulfur source. Additionally, the same amounts were added 24 and 48 h post-inoculation. Alternatively, elemental sulfur was used (sulfur flour, 10 g/l).

Cultures of 13–14 l were grown at 80°C in a 15-l glass bottle heated in a silicon oil bath and stirred with a magnetic stirrer at a speed of 500/min. The culture was continuously bubbled with CO$_2$-enriched air. The fermenter was inoculated with 500 ml of a late-exponential culture and harvested by centrifugation (10,000 × g) 72 h after inoculation. Cell densities were estimated by measuring both the optical density at 600 nm and the cell counts with a Hemocytometer of 10 μm depth.

**ENZYME ASSAY**

Tetrathionate hydrolase (TTH) activity was routinely measured following the decrease of TT concentration in the assay mixture by cyanalysis (Kelly et al., 1969). The mixture (1 ml, pH 1) contained 100 mM maleic acid, 1 mM MgCl$_2$, 1.5 or 1.8 mM K$_2$S$_4$O$_6$, and 25–190 μl of protein extracts. About 4 μM Pyrroloquinoline quinone (PQQ, methoxatin; Sigma, München, Germany), 2 mM Mg$^{2+}$, or 2 mM Ca$^{2+}$ were added if appropriate. The assay mixture including the protein extracts was preheated to 80°C for 5 min prior to TT addition. Aliquots (50 μl) were taken at appropriate time points, chilled on ice water, and kept at 4°C pending cyanalysis. One unit is defined as the conversion of 1 μmol TT in 1 min.

**PROTEIN PURIFICATION FROM WHOLE CELLS**

Washed cells were resuspended in 40 mM KPi pH 7.0 (10 ml/g of cells) and homogenized in a potter after addition of 1 mM MgCl$_2$, 25 μg/ml RNase A, and 10 μg/ml DNase I. Cells were disrupted by two passages through a high-pressure homogenizer at 175 MPa (Constant Cell Disruption Systems, Daventry, UK). Undisrupted cells and cell debris were removed by centrifugation at 10,000 × g for 30 min at 4°C. Particle fractions were removed by a second centrifugation step at 135,000 × g and 4°C for 60 min. The supernatant (i.e., soluble extract) was dialyzed against 40 mM KPi, 1 mM MgCl$_2$, 1 nM PQQ, pH 7.0 for 16 h.

The soluble extract was applied onto a DEAE Sepharose fast-flow column [36 ml column volume (CV); GE Healthcare Europe, Munich, Germany] equilibrated with 40 mM KPi, pH 7.0. Proteins were eluted with a linear gradient from 0 to 0.15 M NaCl in 6 CV followed by a linear gradient from 0.15 to 0.6 M NaCl in 6 CV. Fractions showing TTH activity were pooled and dialyzed against 50 mM Tris/HCl, pH 8.5 for 16 h at 4°C prior to addition of 2 M ammonium sulfate followed by centrifugation for 1 h at 40,000 × g and 4°C. The supernatant was applied onto a Phenyl-Sepharose Fast-Flow column (bed volume 24 ml; GE Healthcare). Proteins were eluted with a linear gradient from 0 to 2 M ammonium sulfate. Fractions showing TTH activity were pooled, concentrated with Centricon YM-30 Devices (Millipore, Bedford, MA, USA), and applied on a Superdex 200 size exclusion column (30 mm x 600 mm; GE Healthcare). Elution was performed with 50 mM Tris/HCl, 150 mM NaCl, pH 8.5, and a constant flow rate of 1 ml/min. The fraction with the highest specific activity of the TTH was dialyzed against 50 mM Tris/HCl, 5 mM NaCl, pH 7.5 for 16 h at 4°C, and applied on a fast-flow Q-Sepharose column (GE Healthcare; bed volume 3.5 ml). Proteins were eluted with a linear gradient from 187.5 to 675 mM NaCl.

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PROTEIN PURIFICATION FROM SPHEROBLASTS

Freshly harvested cells (wet mass 8.6 g) of a late-exponential and TT-grown *A. ambivalens* culture were split into 10 equal parts of 0.86 g and each resuspended in 10 ml spheroblast buffer of various pH values or sterile culture supernatant, followed by overnight incubation (12 h) at 4°C and centrifugation at 1000 × g for 15 min. The buffers were composed of each 500 mM sucrose, 100 mM NaCl, and the buffering substance, i.e., 50 mM KH₂PO₄/K₂HPO₄ (pH 5–6.5), 50 mM formic acid/NaOH (pH 3–4.5), or 50 mM maleic acid (pH 2). The supernatants containing the pseudo-plasmidic fraction were used for TTH activity assays and gel electrophoresis (Figure 1C).

For protein purification, the pseudo-plasmidic fraction of 6 g of *A. ambivalens* cells obtained by incubation in 500 mM sucrose, 100 mM NaCl, and 50 mM KH₂PO₄/K₂HPO₄, pH 6.5 was dialyzed against 20 mM Tris/HCl, 5 mM NaCl, pH 7.5, and applied to a fast-flow Q-Sepharose column (bed volume 3.5 ml; GE Healthcare). Proteins were eluted with a linear gradient from 187 to 675 mM NaCl. Fractions showing TTH activity were pooled and the salt concentration was adjusted to 500 mM NaCl, 1 mM MnCl₂, and 1 mM CaCl₂. This protein solution was applied to a concanavalin A column (Hi-Trap Con A 4B, GE Healthcare; bed volume 1 ml) at a flow rate of 0.1 ml/min. Proteins were eluted with 20 mM Tris/HCl, 500 mM NaCl, 500 mM methyl-d-glucoside (Acros, Fisher Scientific, Bonn, Germany) pH 7.5 with a linear gradient of 0–500 mM methyl-d-glucoside in 2.5 CV. Fractions showing TTH activity were pooled, concentrated with Centriprep YM-30 Devices (Millipore, Bedford, MA, USA), and applied to a Superdex 200 size exclusion column (30 mm × 600 mm; Amershams GE Healthcare). The elution was performed with 50 mM Tris/HCl, 150 mM NaCl, pH 8.5, and a constant flow rate of 1 ml/min.

SEQUENCE ANALYSIS

The amino acid sequence of *A. ferrooxidans* TTH was used as probe in searches of a partial genomic database of *A. ambivalens*, which resulted in the identification of two homologs (*tth1* and *tth2*). The dedicated amino acid sequences were used in standard BLASTP searches conducted against the non-redundant protein database. The deduced amino acid sequences were used in standard BLASTP searches conducted against the non-redundant protein database. The deduced amino acid sequences were used in standard BLASTP searches conducted against the non-redundant protein database. The deduced amino acid sequences were used in standard BLASTP searches conducted against the non-redundant protein database. The deduced amino acid sequences were used in standard BLASTP searches conducted against the non-redundant protein database.

Three-dimensional models of TTH1 and TTH2 were predicted using the Phyre server (Kelley and Sternberg, 2009). Energy minimization of the PHYRE models was performed using UCSF Chimera (Pettersen et al., 2004). The figures were generated with PyMol (DeLano, 2002).

The nucleotide sequences of the *tth1* and *tth2* genes were submitted to EMBL database; they were assigned the accession numbers FR734215 and FR734216, respectively.

ISOLATION OF RNA AND NORTHERN ANALYSIS

*Acidianus ambivalens* cells were grown on tetrathionate or elemental sulfur. Samples (21 each) were taken at 24, 48, and 72 h after inoculation and harvested by centrifugation (10 min, 6000 × g). RNA was prepared from these samples by the guanidinium isothiocyanide extraction method (Chomczynski and Sacchi, 1987) and quantified by UV absorption measurements. Five micrograms of each RNA preparation were separated on a denaturing agarose gel and subsequently transferred to a nylon membrane (Biodyne A; Pall Filtron; Seldin, 1988). RNA ladder mix (Invitrogen, Darmstadt, Germany) was used as a size marker.

After heat-fixation for 2 h at 80°C, the membrane was stained with methylene blue (Herrin and Schmidt, 1988). The DNA fragments used for probe synthesis were amplified with the primers TTH1_sig_fwd (ggaat ctgca acctaa aatcattcggg) and TTH1_1018_rev (cttcc tcggag atagat tgtacc agga) for the *tth1* gene and TTH2_sig2_fwd (tccatt ccttaga aagag agag) and TTH2_442_rev (tggcgt cagttac aagatcg ggtaa) for the *tth2* gene. The primersBorn fwd and sorc_rev (Urich et al., 2005) were used for the amplification of the sor gene. All primer sets amplified the entire open reading frames (ORF). The PCR products were Digoxigenin-labeled using the random hexamer labeling kit (No. 11175033910; Roche, Mannheim, Germany). The hybridization solution contained 50% formamide, 10% (w/v) dextran sulfate (Sigma, Germany), 1% (w/v) SDS, and 0.5% (w/v) skim milk powder. The detection reaction was performed using the DIG luminescence detection kit (11363514910; Roche) and Amersham hyperfilm ECL (GE Healthcare, Munich, Germany) as described by the manufacturers.

ANALYTICAL PROCEDURES

Protein concentration was determined by the bicinchoninic acid method using the BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL, USA). The molecular masses and subunit composition were determined by size exclusion chromatography in the course of the purification (see Protein Purification from Spheroplasts). The monomer, dimer, and trimer peaks of the buffering substance, i.e., 50 mM KH₂PO₄/K₂HPO₄ (pH 5–6.5), cells obtained by incubation in 500 mM sucrose, 100 mM NaCl, and 50 mM KH₂PO₄/K₂HPO₄, pH 6.5 was dialyzed against 20 mM Tris/HCl, 5 mM NaCl, pH 7.5, and applied to a fast-flow Q-Sepharose column (bed volume 3.5 ml; GE Healthcare). Proteins were eluted with a linear gradient from 187 to 675 mM NaCl. Fractions showing TTH activity were pooled, concentrated with Centriprep YM-30 Devices (Millipore, Bedford, MA, USA), and applied to a Superdex 200 size exclusion column (30 mm × 600 mm; Amershams GE Healthcare). The elution was performed with 50 mM Tris/HCl, 150 mM NaCl, pH 8.5, and a constant flow rate of 1 ml/min.

Secondary structure predictions were done using PSIPRED and PROSITE. Predictions of topology and signal sequences were done with TMHMM and SignalP and MEMSAT (at the Pispred server).

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1. www.ncbi.nih.gov
2. http://mafft.cbrc.jp/alignment/server/index.html
3. http://bioinf.cs.ucl.ac.uk/psipred/
4. http://www.cbs.dtu.dk/services/
Purification of the tetrathionate hydrolase from whole cell extracts and identification of the gene

A specific TTH activity of 40 mU/mg protein was found in total cell extracts of *A. ambivalens* grown on TT. The particle-free extract showed a specific activity of 280 mU/mg protein (Table 1). After four chromatographic purification steps, the specific TTH activity was increased to 15 U/mg protein equaling a 54-fold purification and 0.1% recovery.

In the SDS/PAGE (Figure 1A) of different purification steps, a clear relation is visible between the increase of the specific TTH activity and a protein band at 54 kDa. N-terminal sequencing of this band gave the amino acids Gly-Pro-Ile-Val-Tyr-Thr-Thr-Glu-Tyr-Asn*-Gly-Thr-Tyr with Asn* being glycosylated. These residues correspond to the amino acid sequence encoded by the *tth1* gene (Gly40-Tyr53) previously identified by similarity searches in a database of partial *A. ambivalens* genome sequences (5 MBp) using the amino acid sequence of the *A. ferrooxidans* TTH as a query. Residues 1–39 obviously form a signal peptide that was also predicted by the SignalP web server7. Another paralogous gene termed *tth2* was identified in the genome sequences as well. The smaller band with a molecular mass of ≈20 kDa seen in the SDS gel was identified as a pyrophosphatase (not shown), the larger weak protein band (approximate molecular mass of 90–100 kDa) did not give an N-terminal sequence. Specific pyrophosphatase activities (Richter and Schäfer, 1992) were up to 107 mU/mg in enrichment fractions.

Based on the TTH liberation by pH shock (see below), an alternative purification protocol was developed. The pseudo-periplasmic fraction of 6 g *A. ambivalens* cells (after incubation in pH 6.5 buffer) displayed a

![FIGURE 1 | Silver-stained SDS gels and activities of TTH fractions from *A. ambivalens*.](http://www.cbs.dtu.dk/services/)

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RESULTS

**ACIDIANUS AMBIVALENS IS GROWING WITH TETRATHIONATE AS SOLE SULFUR SOURCE**

*Acidianus ambivalens* grown aerobically with 10 mM tetrathionate (TT) as sole sulfur and energy source reached cell densities of 5.9–8.0 × 10⁸/ml after 72 h. Doubling times were 9 h and the final pH of the culture was 0.8–1.0. The cell yields with TT were 0.26–0.36 g/l of culture volume (wet mass). Cells grown with elemental sulfur as previously reported (Zillig et al., 1986; Teixeira et al., 1995) reached cell densities of 9.9 × 10⁸/ml at doubling times of 8.4 h. Cell yields were 0.35–0.47 g/l, however, cell pellets still contained elemental sulfur. When cells were grown at TT concentrations of 20 mM or more, the cultures showed a whitish turbidity after 12 h from transiently formed hydrophilic and finely dispersed sulfur droplets. The turbidity decreased and vanished after 36–48 h due to elemental sulfur oxidation. TT concentrations of more than 40 mM were inhibitory (data not shown).

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Proteins were stained with colloidal Coomassie blue (Roti Blue, Roth, Karlsruhe, Germany) or with silver nitrate (Blum et al., 1987). For N-terminal sequence analysis, the concentrated protein was run on 12% SDS gel and transferred to a PVDF (polyvinylidene difluoride) membrane using a Multiphor NovaBlot system (GE Healthcare) according to the method of Kyhse-Andersen (1984). The protein bands were visualized with conventional Coomassie brilliant blue R-250 [0.1% (w/v), 1% (v/v) acetic acid, 40% (v/v) methanol] followed by N-terminal sequence analysis by Edman degradation using a 492-cLC protein sequencer (Lifetech-Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s instructions.

The results of the TTH liberation by pH shock are shown in Figure 1C. The supernatants were obtained by incubation of whole cells in buffers of the pH values given above. The supernatant fraction obtained after incubation in buffer pH 6.5 contained a dimer peak of the size exclusion chromatography, GF D12, and a monomer peak (lane from a different SDS gel). The supernatant fraction obtained after incubation of whole cells in buffers of the pH values given above was used for the determination of the TTH activity per milliliter of supernatant (total: 10 ml from 0.86 g of cells each).
specific activity of 9 U/mg and a total activity of 56 U. When being fractionated with a fast-flow Q-Sepharose column (GE Healthcare Europe, Freiburg, Germany) the TTH activity eluted at about 320 mM NaCl. A pool of the active fractions showed a specific activity of 4 U/mg and a total activity of 23 U. Lectin affinity chromatography was carried out for the removal of non-glycosylated proteins. The TTH activity eluted at a concentration of 325 mM methyl-β-glucoside. Active fractions were pooled and concentrated prior to size exclusion chromatography with a Superdex 200 column. The TTH eluted in two major 280 nm absorption peaks (not shown) with specific TTH activities of 19 U/mg ($V_r = 165$ ml; corresponding to a molecular mass of 105 kDa) and 10 U/mg ($V_r = 190$ ml; 54 kDa), respectively (Figure 1B). The elution pattern points to equilibrium formation between monomer and homodimer. The alternative three-step purification scheme from the pseudo-periplasmic fraction resulted in a 2.1-fold higher specific TTH activity – despite an intermediate drop – and a 62-fold higher yield of enzyme recovery compared to the purification from total soluble extracts (Table 1). The fractions did not have pyrophosphatase activity and the 20-kDa band was not seen in SDS gels. The 54-kDa TTH band was predominant in the dimer peak and almost homogeneous in the monomer peak (Figure 1B).

TTH HAS AN EXTRACELLULAR LOCATION
TTH activity was not detectable in cell-free culture supernatants (pH 1) immediately after harvesting. This changed when cells were resuspended in a 20-fold volume of spent supernatant after harvesting and incubated overnight at 4°C followed by another round of centrifugation: the TTH activity was 7.5 mU/ml (0.3 U/mg protein) in the secondary supernatant suggesting a slow release of the protein (Table 2). After overnight incubation of cells in pH 6.5 phosphate buffer, 92% of the TTH activity was found in the supernatant. 7.3% and 0.3% were found in the cytoplasmic and membrane fractions, respectively. In order to determine the effect of pH on the solubilization of the TTH from the cells, buffers of different pH values were used for overnight incubation after harvesting. The TTH activities in the supernatants increased significantly between pH 4 and 4.5, while the highest amount of TTH activity and the highest protein concentration were found in the pH 6.5 supernatant (Table 2; Figure 1C).

TTH IS AN EXTREMELY THERMOPHILIC AND ACIDOPHILIC ENZYME
Decrease of TT catalyzed by the TTH showed a sigmoidal behavior and an activation period whose duration is depended on the amount of enzyme in the assay mixture. The slope of the regression

### Table 1 | Purification of the tetrathionate hydrolase from A. ambivalens cell extracts.

| Fraction | Total protein (mg) | Specific activity (U/mg) | Total activity (U) | Yield (%) | Fold |
|----------|--------------------|--------------------------|--------------------|------------|------|
| **A. PURIFICATION FROM SOLUBLE CELL EXTRACTS** | | | | | |
| Cell extract | 1405.7 | 0.28 | 388.0 | 100 | 1.0 |
| DEAE | 414.0 | 0.94 | 3872 | 99.8 | 3.4 |
| HIC | 179 | 1.77 | 31.8 | 8.2 | 6.3 |
| Size exclusion | 1.4 | 3.83 | 5.3 | 1.4 | 13.7 |
| Q-sepharose | 0.02 | 14.99 | 0.3 | 0.1 | 53.5 |
| **B. PURIFICATION FROM THE PSEUDO-PERIPLASMIC FRACTION** | | | | | |
| Pseudo-periplasm | 6.23 | 8.99 | 55.98 | 100 | 1.0 |
| Q-sepharose | 5.57 | 4.17 | 23.2 | 41.5 | 0.5 |
| Con A | 0.71 | 4.65 | 3.30 | 5.9 | 0.5 |
| Size exclusion GF C9 | 0.18 | 18.94 | 3.44 | 6.2 | 2.1 |

### Table 2 | TTH activities in the cell-free supernatants following incubation of 0.86 g each of A. ambivalens cells in 10 ml of buffer of different pH values; average values from two to three enzyme assays.

| pH | 6.5 | 6.0 | 5.5 | 5.0 | 4.5 | 4.0 | 3.5 | 3.0 | 2.0 | CS^1 |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Protein conc. in supernatant (µg/ml) | 177 ± 7 | 121 ± 4 | 63 ± 2 | 117 ± 15 | 49 ± 4 | 32 ± 1 | 29 ± 10 | 65 ± 11 | 48 ± 2 | 25 ± 5 |
| Activity/ml in supernatant (U/ml) | 12 ± 0.02 | 0.8 ± 0.02 | 0.7 ± 0.08 | 0.8 ± 0.02 | 0.5 ± 0.12 | 0.08 ± 0.10 | 0.12 ± 0.16 | 0.04 ± 0.03 | 0.13 ± 0.03 | 0.01 ± 0.002 |
| Specific activity in supernatant (U/mg) | 6.7 ± 0.10 | 6.7 ± 0.17 | 11.5 ± 1.31 | 6.5 ± 0.14 | 11 ± 2.41 | 2.6 ± 3.11 | 4.1 ± 5.36 | 0.6 ± 0.52 | 2.7 ± 0.68 | 0.3 ± 0.07 |
| Total activity in supernatant (U) | 11.9 ± 0.18 | 8.1 ± 0.21 | 72 ± 0.82 | 76 ± 0.17 | 5.4 ± 1.17 | 0.8 ± 0.99 | 1.2 ± 1.57 | 0.4 ± 0.34 | 1.3 ± 0.33 | 0.06 ± 0.02 |
| Total activity in cellular fraction (U) | 1 ± 0.41 | 4.9 ± 0.24 | 5.7 ± 0.68 | 5.1 ± 0.23 | 76 ± 0.98 | 12.3 ± 1.21 | 12.1 ± 0.73 | 12.1 ± 0.42 | 11.7 ± 0.13 | 11.4 ± 0.46 |
| Fraction of total activity in supernatant (%) | 92 ± 1.4 | 63 ± 1.6 | 56 ± 6.4 | 59 ± 1.3 | 41 ± 8.8 | 6 ± 7.4 | 9 ± 11.8 | 3 ± 2.6 | 10 ± 2.5 | 2 ± 0.7 |

^1Incubation in 10 ml of culture supernatant (pH = 1).
The specific activity increased in an almost linear way with the temperature with no activity at 40°C, modest activity at 50°C, and maximal activity at 95°C (Figure 3). The optimal and maximal temperatures were not determined for lack of suitable assay equipment. The highest enzymatic activity was recorded at pH 1. From pH 2–5, the TTH showed a specific activity of 1–0.75 U/mg (Figure 3). No enzyme activity was detected at pH 6. Addition of PQ and/or Mg²⁺ or Ca²⁺ did not have a significant effect on the TTH activity (not shown). The Kₘ was recorded using a different TTH preparation of higher purity. At 80°C and pH 1, the Kₘ of the TTH was 0.8 mM TT when assayed using 2.7 μg of protein. The observed Vₘₐₓ was 43.8 nmol/min corresponding to a specific activity of 16 U/mg.

**ONLY THE tth1 GENE IS EXPRESSED IN TT-GROWN A. AMBIVALENS CELLS**

In order to analyze expression of the two tth genes, total RNA was isolated from *A. ambivalens* grown aerobically with sulfur or TT at different stages of the growth curve. Northern hybridization was carried out with probes derived from the *tth1* and *tth2* genes and, as a control, with a sor probe from the gene encoding the sulfur oxygenase reductase (Kletzin, 1992). Hybridization signals appeared at a size of about 1500 nt with the *tth1* gene pointing toward a monocistronic transcription (Figure 4). Signals developed in hybridization reactions with RNA from cells grown under aerobic but not under anaerobic conditions (not shown). Signals were stronger in RNA from TT-grown cells as compared to sulfur-grown cells at late-exponential phase. The *tth2* gene did not give hybridization signals under any condition tested. Signals were stronger in S°-grown cells (Figure 4). These results showed that the sor gene is expressed in TT-grown cells presumably due to transient formation of elemental sulfur.

**TETRATHIONATE HYDROLASE IS SIMILAR TO PQQ-CONTAINING DEHYDROGENASES**

The *tth1* gene encodes an ORF of 538 codons. The deduced amino acid sequence includes a signal sequence of 39 residues, so that the mature protein has a length of 499 aa. The calculated molecular mass of 53,642 without glycosylation corresponds well with the apparent molecular masses in SDS gels and gel filtration (54 kDa). The *tth2* gene encoded an ORF of 449 aa including a predicted signal sequence of 23–32 aa in length. Both proteins are 29–32% identical (depending on alignment parameters), which is less than the pairwise identity of TTH1 with the orthologous enzyme from *A. ferrooxidans* (40–42%).

The top hits in BLASTP searches of TTH1 at NCBI (pairwise identity >33%; e-value <1 × 10⁻⁵⁴) were restricted to close homologs from acidophilic Bacteria and Archaea (TTH cluster; Figure 5), which seem to be true tetrathionate hydrolases. Comparable results were obtained with the TTH2 protein. Both proteins and their homologs gave distinct clades in the dendrogram. These proteins belong to a large superfamily of β-propeller proteins, whose best-known members are pyrroloquinoline quinone (PQQ) containing dehydrogenases. Secondary structure predictions confirmed that TTH1 and TTH2 are all-beta proteins except for the transmembrane helices within the signal sequences (not shown). Six copies of a PQQ enzyme β-propeller repeat are present in each protein (PFAM family PF01011).8

8http://pfam.sanger.ac.uk
with the exception of SOX complexes from some but not all neutrophilic ISC-oxidizing bacteria (Mukhopadhyaya et al., 2000; Lahiri et al., 2006). We report here on the identification, enzyme characterization, and expression data of the first archaeal TTH described so far.

Growth of \textit{A. Ambivalens} on tth gene expression data, and enzyme properties

Acidianus ambivalens growth rates and yields were comparable with TT and elemental sulfur. An indication for the presence of a TTH came from the turbidity observed in cultures growing at high TT concentrations, which showed that elemental sulfur is transiently formed. TTH activity was measured in TT-grown but not in sulfur-grown cells suggesting that the protein is formed only in the presence of the substrate. This observation is supported by the Northern data, which showed a stronger signal of the \textit{tth} gene in TT-grown cells (Figure 4).

The TTH activity was initially localized in the soluble fraction. The enzyme was highly enriched during various chromatographic steps but the resulting preparation was not electrophoretically homogeneous. A band of approximately 54 kDa was co-enriched with the TTH activity, whose N-terminus was identical to a protein that was previously identified by sequence similarity search with the \textit{A. ferrooxidans} TTH. A pyrophosphatase and a protein of unknown identity were still present in the preparation (Figure 1A). The same 54 kDa band was enriched during purification from the pseudo-periplasmic fraction (Figure 1B).

Nine out of 10 top hits in homology modeling of TTH1 (and of TTH2) were PQQ-containing dehydrogenases with an eight-bladed \( \beta \)-propeller structure, a calcium site, and a cytochrome \( c \) domain (e.g., alcohol dehydrogenase from \textit{Pseudomonas putida} HK5; PDB accession 1kv9; e-value \( 1 \times 10^{-25} \); Figure 6). Homologs of the cytochrome \( c \) domain were missing in both TTHs. Closer examination showed that the \( \text{Ca}^{2+} \)-coordinating residues were absent as well. Likewise, a nearby disulfide from two adjacent cysteine residues was missing (Figure 7). The models predict that the glycosylated Asn of TTH1 is located in the center of the \( \beta \)-propeller.

**DISCUSSION**

Growth of \textit{(Acidi-)} \textit{Thiobacillus} species and other bacteria by TT oxidation is known for a long time. Likewise, TT-oxidizing Archaea were described previously, most of them acidophiles (Wood et al., 1987; Kurosawa et al., 2003). In contrast, most of the known TT-metabolizing enzymes come from neutrophilic proteobacteria and are molybdenum or heme-containing reductases allowing these organisms to grow anaerobically by TT respiration (Hensel et al., 1999; Price-Carter et al., 2001; Mowat et al., 2004). Although TT hydrolysis had been described already in the 1960s (Trudinger, 1964) and TT hydrolases in the 1990s (De Jong et al., 1997a), the link between purification and identification of the gene was established only recently by Kanao et al. (2007) and Rzhepishevska et al. (2007) for the TTHs from \textit{A. ferrooxidans} and \textit{Acidithiobacillus caldus}, respectively. Enzymes oxidizing TT to sulfate without intermediate(s) are not known, with the exception of SOX complexes from some but not all neutrophilic ISC-oxidizing bacteria (Mukhopadhyaya et al., 2000; Lahiri et al., 2006). We report here on the identification, enzyme characterization, and expression data of the first archaeal TTH described so far.

**GROWTH OF \textit{A. AMBIVALENS} ON TT, TTH GENE EXPRESSION DATA, AND ENZYME PROPERTIES**

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Again, two larger protein bands were visible in the gel but their
stoichiometries were not constant. Therefore, we concluded that the 54-kDa protein represents the sole subunit of the TTH. This conclusion is supported by the results from the gel permeation chromatography, which showed two peaks with TTH activity, representing the monomer and the homodimer.

As expected, the optimal reaction temperature of the TTH is higher (≥95°C; Figure 3) than for the enzymes from mesophilic species (optima 40–65°C; Sugio et al., 1996; Tano et al., 1996; De Jong et al., 1997a,b; Bugaytsova and Lindström, 2004; Kanao et al., 2007). In addition, the optimal pH was lower, being around pH 1. The protein was glycosylated at Asn50 and probably other residues as well. Glycosylation seems to be a common protection mechanism of membrane-bound and extracellular proteins with bacterial TTHs, which were localized in the periplasm or attached to the membrane. The localization was verified experimentally in the case of the A. caldus TTH (Bugaytsova and Lindström, 2004). In most other cases the localization was inferred by the acidic pH optima of the enzymes (pH 2.5–4; Bugaytsova and Lindström, 1996; Tano et al., 1996; De Jong et al., 1997a,b). We showed here that the TTH is liberated from the cells by pH shift alone. We deduced from these results that the shift in pH causes a rearrangement of the S-layer thereby liberating the TTH. Alternatively, the TTH might be attached to the S-layer by ionic and/or hydrophobic interactions, however, the total lack of TTH activity in the medium argues against this hypothesis. Therefore, we conclude that our results support the pseudo-periplasm hypothesis and that the TTH is one of few enzymes so far shown to be located in this compartment. This conclusion is consistent with the acidic pH optimum of the A. ambivalens TTH (around pH 1) corresponding to the media conditions.

**ACIDITHIOBACILLUS AND ACIDIANUS TTHs ARE SIMILAR ENZYMES**
The extracellular location of the A. ambivalens enzyme is consistent with bacterial TTHs, which were localized in the periplasm or attached to the membrane. The localization was verified experimentally in the case of the A. caldus TTH (Bugaytsova and Lindström, 2004). In most other cases the localization was inferred by the acidic pH optima of the enzymes (pH 2.5–4; Bugaytsova and Lindström, 1996; Tano et al., 1996; De Jong et al., 1997a,b). Comparison of the amino acid sequences showed that almost all of the TTHs...
genes. The gene products fall into two separate phylogenetic clades. The clade around the TTH1 encompasses true tetrathionate hydro-
lasers from their similarity and from the known enzyme activities. The TTH2 proteins are paralogous proteins with a different and 
unknown function, however, they also contain a signal sequence 
and should therefore be secreted (Figure 5).

The absence of tth genes in many organoheterotrophic Sulfolobales 
species is of particular interest as for example Sulfolobus acidocaldarius had originally been described as a facultatively chemoi-
thoautotrophic, sulfur-dependent species (Brock et al., 1972). 

including TTH2 contain a canonical signal sequence (not shown) 
thereby confirming the presumed or verified periplasmic or extra-
cellular location. The molecular masses of the TTHs purified so 
far are fairly similar and differ from the only known trithionate 
ydrolase, which is a homotrimeric enzyme with a subunit mass 
of 34 kDa (Meulenberg et al., 1992). Meulenberg’s results sug-
gest that trithionate hydrolases represent separate and unrelated 
entities, however, this cannot be confirmed because the genes are 
not known.

The sequence analysis and comparisons of the TTH genes 
showed that they are rare and present only in obligatory or fac-
culitative chemolithoautotrophs. In addition, most of the species 
represented in the dendrogram (Figure 5) harbor at least two tth 
genes. The gene products fall into two separate phylogenetic clades. 
The clade around the TTH1 encompasses true tetrathionate hydro-
lases from their similarity and from the known enzyme activities. 
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TTH IN LITHOAUTOTROPHIC AND ORGANOHETEROTROPHIC 
SULFOLOBALES SPECIES

The absence of tth genes in many organoheterotrophic Sulfolobales 
species is of particular interest as for example Sulfolobus acido-
caldarius had originally been described as a facultatively chemoi-
thoautotrophic, sulfur-dependent species (Brock et al., 1972).
Neither S. acidocaldarius nor Sulfolobus solfataricus/Sulfolobus islandicus species have tht genes suggesting that they are unable to grow at the expense of TT oxidation. An analogous observation was made with the sulfur oxygenase reductase (SOR): (facultatively) chemolithoautotrophs like Sulfolobus metallicus, Metallosphaera sedula, and Sulfolobus tokodaii possess sor genes, while S. acidocaldarius, S. solfataricus, and S. islandicus lack the gene and corresponding enzyme activities (Kletzin, 1989; Veith et al., 2011; this volume). Both observations suggest that the colony-isolated, heterotrophic Sulfolobales species are not sulfur-dependent at all and unable to grow oxidatively with sulfur or tetrathionate.

**TTH: β-PROPELLER STRUCTURE WITHOUT PQP**

It can be concluded from sequence analysis and structure prediction that the TTHs are all-beta and also β-propeller-forming proteins with a conserved 3D structure similar but not identical to PQP-containing dehydrogenases. These proteins, e.g., type II alcohol dehydrogenases and glucose dehydrogenases, consist of a PQP domain, composed of seven to eight conserved, propeller-forming PQP repeats (PFAM family PF01011; Figure 6; Oubrie et al., 1999; Chen et al., 2002). The alcohol dehydrogenases contain an additional heme c domain (Chen et al., 2002). The PQP repeats contain a conserved motif of amino acids (Ala-x3-Gly-x3-Trp) thought to stabilize the structure (Chen et al., 2002).

The model of the A. ambivalens TTH1 and the sequence alignments used for dendrogram calculation (Figure 5; alignment not shown) contain five to six of these tryptophane motifs, whereas TTH2 contains four (Figure 6). In contrast, the heme c domain and conserved PQP-binding site residues are missing including the disulfide-forming cysteines and the calcium-coordinating amino acids (Figure 7). The glycosylated Asn of TTH1 sits in the middle of the β-propeller and far from the region where the dehydrogenases bind PQP. The alignments did not show a conserved cysteine residue—the usual suspect for sulfur binding—so that the reaction mechanism remains enigmatic at present, especially in the absence of PQP and metal ions.

**CONCLUSION**

Some final conclusions for the A. ambivalens sulfur metabolism can be drawn: One of the initial questions was, how TT produced in the TQO reaction is metabolized (Müller et al., 2004). The TTH found here does not give an answer to this question because the protein was found only in TT-grown cells and the gene is poorly expressed in sulfur-grown cells. The localization and pH optimum of the TTH argue against a role in the oxidative metabolism of elemental sulfur. In contrast, the SOR does seem to play an important role in TT-grown cells, presumably by disproportionation of the sulfur generated by the TTH.

The TTH itself seems to be a monomeric or homodimeric enzyme with an overall β-propeller structure. The enzyme has a high temperature optimum and very low pH optimum, both of which are in accordance with the extracellular location shown by the pH shock-mediated enzyme liberation.

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