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Bacterial CS_2 Hydrolases from *Acidithiobacillus thiooxidans* Strains Are Homologous to the Archaeal Catenane CS_2 Hydrolase

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Carbon disulfide (CS_2) and carbonyl sulfide (COS) are important in the global sulfur cycle, and CS_2 is used as a solvent in the viscose industry. These compounds can be converted by sulfur-oxidizing bacteria, such as *Acidithiobacillus thiooxidans* species, to carbon dioxide (CO_2) and hydrogen sulfide (H_2S), a property used in industrial biofiltration of CS_2-polluted airstreams. We report on the mechanism of bacterial CS_2 conversion in the extremely acidophilic *A. thiooxidans* strains S1p and G8. The bacterial CS_2 hydrolases were highly abundant. They were purified and found to be homologous to the only other described (archaeal) CS_2 hydrolase from *Acidianus* strain A1-3, which forms a catenane of two interlocked rings. The enzymes cluster in a group of β-carbonic anhydrase (β-CA) homologues that may comprise a subclass of CS_2 hydrolases within the β-CA family. Unlike CAs, the CS_2 hydrolases did not hydrate CO_2 but converted CS_2 and COS with H_2O to H_2S and CO_2. The CS_2 hydrolases of *A. thiooxidans* strains G8, 2Bp, Sts 4-3, and BBW1, like the CS_2 hydrolase of *Acidianus* strain A1-3, exist as both octamers and hexadecamers in solution. The CS_2 hydrolase of *A. thiooxidans* strain S1p forms only octamers. Structure models of the *A. thiooxidans* CS_2 hydrolases based on the structure of *Acidianus* strain A1-3 CS_2 hydrolase suggest that the *A. thiooxidans* strain G8 CS_2 hydrolase may also form a catenane. In the *A. thiooxidans* strain S1p enzyme, two insertions (positions 26 and 27 [PD] and positions 56 to 61 [TPAGGG]) and a nine-amino-acid-longer C-terminal tail may prevent catenane formation.

The sulfur compounds carbon disulfide (CS_2) and carbonyl sulfide (COS) play an important role in the earth's sulfur cycle. CS_2 and COS are released as breakdown products from organic matter, notably S-containing amino acids in soils (1) and dimethyl sulfide (DMS) in marine, mainly coastal and estuarine, environments (2). CS_2 is chemically and biologically converted to COS, which is highly stable when not in solution and is the most abundant sulfur species in the atmosphere (3). Anthropogenic CS_2 emissions account for approximately one-half of the total global emissions (2). This is due for a great part to the use of CS_2 as an organic solvent in the viscose and rayon industry, which brings with it a number of problems. First, CS_2 is toxic, causing vascular and coronary heart disease and affecting the central nervous system (4). Second, due to its low boiling point, large CS_2-polluted airstreams are created in factory plants and require treatment before release to the atmosphere.

Biofiltration is an effective and sustainable method to remove CS_2 from the contaminated airstreams (5–7). Several bacterial species that can grow chemolithoautotrophically on CS_2 at neutral pH have been identified from soil, sludge, and freshwater habitats (8–12). At acidic pH, so far only some (*Acidi*)*thiobacillus* strains, isolated from hot springs and volcanic areas, were shown to be able to use CS_2 (8, 13). Acidophilic *Acidithiobacillus thiooxidans* strains are currently in use in biotrickling filters (6, 14–16), due to the inherent acidification of the trickling filter upon growth on CS_2. The microorganisms convert CS_2 via a 2-step hydrolysis reaction (12, 17): $CS_2 + H_2O \rightarrow COS + H_2S$ and $COS + H_2O \rightarrow CO_2 + H_2S$. The H_2S is subsequently oxidized, ultimately to sulfuric acid (18), yielding the energy required for growth, but also acidifying the biofilter trickling water. Large quantities of water are used to maintain the pH at levels tolerated by the CS_2-removing microorganisms. The use of new, more extreme acidophiles would reduce water usage as well as operational costs, making biofiltration more sustainable and effective (6, 19).

Biofiltration technologies can benefit from more in-depth knowledge about the molecular mechanism of bacterial CS_2 conversion. Although several bacterial CS_2-converting species are now known (9–13, 17, 20), their CS_2-converting enzymes have not been characterized at all. However, we previously purified the CS_2 hydrolase from the CS_2-converting hyperthermophilic archaeon *Acidianus* strain A1-3 and showed that it could convert CS_2 to COS, H_2S, and CO_2 via the hydrolysis reaction as described above (21). The CS_2 hydrolase appeared to be homologous to β-carbonic anhydrases (β-CAs), which catalyze the reversible hydration of $CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$. The crystal structure of the CS_2 hydrolase revealed that the enzyme occurs as an octameric ring like the β-CA from the garden pea *Pisum sativum* (22). However, in the case of *Acidianus* CS_2 hydrolase, two of these rings interlock, forming a highly unusual hexadecameric catenane structure, both in the crystal form and in solution (21, 23). Intriguingly, despite the high homology with CAs, the *Acidianus* CS_2 hydrolase could not use CO_2 as a substrate, and CAs have not been found to use CS_2 as a substrate, although the conversion is theoretically possible (24–26). Recently, a COS hydrolase enzyme was purified from *Thiobacillus thioparus* strain THI15, which is also a β-CA homologue. It does not form a catenane structure. Instead,

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a weakly associated tetrameric ring is formed (27). Its physiological role is COS conversion, not CS_2 conversion (27). The COS hydrolase is not closely related to the *Acidianus* CS_2 hydrolase in the β-CA clade D cluster.

With the unusual catenane structure of the archaeal CS_2 hydrolase in mind, we set out to study bacterial CS_2 hydrolase enzymes from new strains of CS_2-degrading *Acidithiobacillus thiooxidans* isolated from sulfur-rich and highly acidic environments. Here, we report on the purification and characterization of two bacterial CS_2 hydrolases. We show that archaeal and bacterial CS_2 hydrolases are closely related and that the bacterial homologues also form catenane structures.

MATERIALS AND METHODS

Media and culture conditions. New *Acidithiobacillus thiooxidans* strains were isolated from sulfur-rich and highly acidic ecosystems including mixed hot spring samples (strain 2Bp), Solfatara, Naples (strain Sts 4-3), and Solfarata, Rome (strain S1p), or from samples from CS_2-removing biotrickling filters (strains G8 and BBW1). The strains were enriched and isolated with CS_2 as the sole energy source. Physiological details of the new strains will be described in a future article. Strains were grown in minichemostats, set up using 250-ml Schott bottles, with a culture volume of 150 ml. The medium was a basal salt mineral medium (MM) consisting of (in $g \cdot liter^{-1}$) KH_2PO_4 (0.2), NH_4Cl (0.5), $MgSO_4 \cdot 7\ H_2O$ (0.75), $CaCl_2 \cdot 2\ H_2O$ (0.1), and 1 ml/liter trace element solution and acidified with 1% (vol/vol) H_2SO_4 (pH 0.75). Trace elements consisted of the following (in $g \cdot liter^{-1}$): EDTA-Na $\cdot$ 2 H_2O (10.0), $ZnSO_4 \cdot 7\ H_2O$ (2.2), $MnCl_2 \cdot 4\ H_2O$ (1.02), $FeSO_4 \cdot 7\ H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ (0.22), $CuSO_4 \cdot 5\ H_2O$ (0.32), and $CoCl_2 \cdot 6\ H_2O$ (0.32). To maintain the extremely acidophilic strains, the sulfuric acid concentration was kept at 1%. The medium flow was 2.7 $ml \cdot h^{-1}$ ($D = 0.023\ h^{-1}$). The reactor temperature was 22°C. CS_2 gas (8.6 ± 0.05 μM) was bubbled through the reactors with a flow rate of 41.5 ± 0.1 $ml \cdot min^{-1}$ and mixed into the reactor with a stir bar, stirring at 1,000 rpm. *A. thiooxidans* strains S1p and G8 were also grown in batch reactors, in MM acidified with sulfuric acid to pH 2. The reactor temperature was 25°C. CS_2-containing air was bubbled through the reactor with a flow rate varying between 132 and 140 $ml \cdot min^{-1}$ and dispersed through the culture by stirring at 500 to 1,000 rpm. The concentration of CS_2 gas was varied between 10 $nmol \cdot ml^{-1}$ for low-density cultures and 125 $nmol \cdot ml^{-1}$ for high-density cultures. The μ_{max} values were determined by increasing the medium flow and simultaneously increasing the [CS_2] supplied to the reactors up to and beyond the point where S^0 formation started becoming visible in the reactors.

Methylomicrobium alcaliphilum DSM19304 was grown at 28°C, with shaking at 200 rpm, as 10-ml cultures in 120-ml bottles closed with butyl rubber stoppers, on DSMZ medium 1180, and containing 15 ml CH_4. *Halopiger xanaduensis* DSM18323 was grown in Erlenmeyer flasks at 37°C and shaking at 200 rpm in the medium described in reference 28. The pH of the medium was adjusted to pH 8.0 before and after autoclaving with sterile 1 M NaOH. *Mycobacterium marinum* strain M was grown statically in 7H9 medium at 30°C.

Preparation of cell extracts. Batch reactors with a culture optical density at 600 nm (OD_{600}) of 1.7 were harvested by centrifugation at 9,000 × *g* for 15 min. The pellets (about 4 g, wet weight) were washed with 20 mM Tris, pH 8, to raise the pH to 8.0. Pellets were resuspended in 15 ml 20 mM Tris, pH 8, containing EDTA-free protease inhibitors (Roche Diagnostics). Cells were broken by a French press (21), DNase I was added, and the suspension was centrifuged at 48,000 × *g* for 60 min. Supernatants were frozen at −20°C. Protein concentrations were determined using the Bio-Rad protein microassay. Using dry weight measurements of purified CS_2 hydrolase, we determined that bovine serum albumin (BSA) as a standard rather than IgG produced more accurate protein determinations for CS_2 hydrolase. Cell extracts from steady-state reactor-grown bacterial cells were prepared as follows: 30 to 50 ml was removed from the reactors and centrifuged at 4°C for 30 min at 12,000 × *g*. The cell pellets were washed with 15 ml sterile distilled water (sdH_2O) and resuspended in 0.5 ml 20 mM KP_i, pH 7. Approximately 350 μl glass beads (size, 80 to 110 μm) were added, and the cells were broken by bead beating for 2 times 2 min at 30 Hz (Retsch, Germany) with intermittent cooling on ice. The broken cell mixtures were centrifuged for 5 min at 16,000 × *g*, and the supernatants were either used directly for protein assays and kinetic measurements or stored at −20°C with a final concentration of 10% glycerol.

Purification of CS_2 hydrolases. CS_2 hydrolases from *A. thiooxidans* strain G8 and S1p were purified by ammonium sulfate precipitation, followed by hydrophobic interaction chromatography (HIC) and anion-exchange chromatography. Chemicals used for chromatography were of ultrapure grade. Prior to ammonium sulfate precipitation, the Tris concentration was raised from 20 to 50 mM by adding an appropriate volume of 1 M Tris-HCl, pH 8. Extracts were stirred continuously and kept on ice, while finely ground $(NH_4)_2SO_4$ was added slowly to 30%, wt/vol. The extract was left on ice for 1 h and centrifuged for 45 min at 3,000 × *g*, and another aliquot of $(NH_4)_2SO_4$ was added until 60% saturation was reached.

For strain G8, $(NH_4)_2SO_4$ was added to 60%, wt/vol, followed by incubation and centrifugation as described above. The pellet was dissolved in 3 ml 50 mM Tris-HCl, pH 8, and centrifuged for 5 min at 13,500 × *g*. The resulting supernatant was filtered through a 0.45-μm filter unit (Millex-HV polyvinylidene difluoride [PVDF] Durapore). $(NH_4)_2SO_4$ was added from a 3.4 M stock to raise the concentration to 1 M, and the whole fraction was loaded onto a 1-ml HIC Hitrap Phenyl Sepharose 6 Fast Flow (High sub) column (GE Healthcare) equilibrated with 1.7 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 8), using the Åkta system (GE Healthcare). Proteins were separated using a linear gradient from 1.7 to 0 M $(NH_4)_2SO_4$ over 10 column volumes at a flow rate of 2 $ml \cdot min^{-1}$. Two-milliliter fractions were collected and assayed for CS_2 hydrolase activity (see below). The CS_2 hydrolase eluted at the very end of the gradient at 122 mM $(NH_4)_2SO_4$ together with at least 2 other dominant proteins. Active fractions were pooled and concentrated 3 times, and the buffer was exchanged to 20 mM Tris-HCl (pH 8) by centrifugation at room temperature (RT) through a Vivaspin 30-kDa cutoff spin column (Sartorius). Two milliliters was loaded onto a 1-ml Hitrap ANX-Fast Flow (High sub) column (GE Healthcare), and proteins were separated at RT using a linear gradient from 0 to 1 M NaCl over 25 column volumes and a flow rate of 1 $ml \cdot min^{-1}$. The CS_2 hydrolase eluted at 117 mM (11.7%) NaCl.

For strain S1p, the 30% $(NH_4)_2SO_4$ supernatant fraction was used to separate the proteins on the HIC column equilibrated with 1.3 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 8) described above. The S1p CS_2 hydrolase eluted in a broad peak between 1.1 M and 179 mM $(NH_4)_2SO_4$, but the highest activity was seen at the lower salt concentrations, perhaps due to inhibition by $(NH_4)_2SO_4$. Pooled active fractions were concentrated 13 times, and the buffer was exchanged as described above. The ANX column was loaded with 100-μl samples of the concentrated fractions, and proteins were separated at RT using a linear gradient from 0 to 1 M NaCl over 18 column volumes and a flow rate of 1 $ml \cdot min^{-1}$. The CS_2 hydrolase eluted in a well-separated peak at 202 mM NaCl. Fractions containing the purified enzyme were stored on ice or, if not used within 1 week, frozen at −20°C.

Protein gel electrophoresis. SDS-PAGE (10 or 12% polyacrylamide, pH 8.3) and native PAGE (6 or 8% polyacrylamide, pH 8.3) was performed using a Mini-Protean III Cell (Bio-Rad) at RT. Fermentas unstained or prestained and the PageRuler Plus prestained molecular weight standards were used for SDS-PAGE. Invitrogen P/N 57030 markers were used for native PAGE. Ten micrograms of protein was loaded per lane. Proteins were visualized by Coomassie brilliant blue (CBB) G 250.

CS_2 and COS hydrolase activity measurements. CS_2 hydrolase activity of purified fractions and on native PAGE gels was measured qualitatively as described previously (21) but at 30°C. CS_2 and COS hydrolase activities of purified CS_2 hydrolases were quantified by gas chromatography (29). To 120-ml bottles, 500 μl 20 mM HEPES (pH 7) was added. As

COS was unstable in the buffer and its breakdown rate depended on the buffer volume, the latter was minimized and kept at 500 μl. This small volume simultaneously maximized the gas to liquid transfer of CS_2. Experiments were run against abiotic controls to check that substrate conversion was due to enzyme activity rather than chemical breakdown. Bottles were sealed with a gray butyl rubber stopper, and between 0.25 and 10 ml of CS_2-saturated air (final concentration in the buffer, between 8 and 800 μM CS_2) or between 0.125 and 4 ml COS (final concentrations in the buffer, between 5 and 473 μM COS) was injected. CS_2-saturated air was obtained by incubating an airtight 1-liter bottle containing 50 ml CS_2 for several hours. COS was injected from a COS gas bottle. The bottles were incubated at 30°C with shaking at 400 rpm for 15 min to ensure good gas transfer into the buffer. Purified CS_2 hydrolase (0.3 to 0.7 μg) in 100 μl 20 mM HEPES (pH 7) was injected. The formation of hydrolysis products from CS_2 (COS and H_2S) and COS (H_2S) was measured by gas chromatography, and production rates over the first 3 min were used to calculate enzyme activity. CS_2 and COS concentrations in the buffer were determined by measuring the concentration in the headspace of the bottles at the end of each experiment and converting it to liquid concentrations using the solubility of COS and CS_2 in 20 mM HEPES buffer (pH 7) at 30°C. The solubility of CS_2 or COS was determined by adding known amounts to a 120-ml bottle containing 60 ml buffer, incubating them for 30 min at 30°C with shaking at 400 rpm, and measuring the remaining CS_2 or COS in the headspace as well as the liquid phase by gas chromatography. The liquid/gas ratio at 30°C was 0.45:1 for COS and 0.94:1 for CS_2.

CS_2 hydrolase activity was also quantified using an H_2S Clark-type microsensor (Unisense A/S, Denmark), calibrated using an anaerobic Na_2S stock diluted in 20 mM HEPES (pH 7). A 6 mM CS_2 stock was prepared by mixing 200 μl CS_2 with 500 ml dH_2O in a 500-ml serum bottle by vigorous shaking for at least 30 min. Up to 0.1 μg cell extract in 20 mM HEPES (pH 7), 0.1 μg purified CS_2 hydrolase, or 100 μg bovine carbonic anhydrase was added to a 1-ml glass vessel containing 1 ml of 20 mM HEPES (pH 7) stirred at 500 to 1,000 rpm, in a 22°C water bath. The H_2S sensor was lowered into the vessel, CS_2 was added from the stock bottle to 600 μM CS_2, and the formation of H_2S was followed for approximately 30 s. Initial H_2S production rates were calculated to determine the V_{max} values for each cell extract.

The CS_2 hydrolase activity of bacterial strains was also tested by gas chromatography. A CS_2 stock bottle was prepared by adding 400 μl CS_2 to an empty 500-ml bottle sealed with a rubber seal and incubating it for 30 min at RT for the CS_2 to evaporate. Five- or 10-ml samples from late-logarithmic or early-stationary-phase bacterial cultures were added to 120-ml bottles, which were sealed with gray butyl rubber stoppers, and 0.25 ml CS_2-containing air was added from the stock bottle to obtain a CS_2 concentration of about 10 to 15 nmol · ml^{-1} CS_2. The cultures were incubated at the temperature used for growth and shaken at 300 rpm. Headspace samples were taken at intervals for up to 24 h and analyzed for COS, H_2S, and CS_2 as described previously (29). The *M. marinum* strain M cultures were incubated with CS_2 statically and at RT. To make sure this strain could adapt to CS_2 conversion, fresh medium was added to the grown culture in addition to CS_2, and CS_2 conversion was followed for 24 h.

Genome sequencing. Genomic DNA from *A. thiooxidans* strains G8 and S1p was isolated (30) and sequenced using next-generation sequencing. Strain G8 was sequenced by a combination of 454 titanium technology (31) (about 50 Mb in 450-nucleotide [nt] reads) and Illumina technology (32) (about 1.5 Gb in 75-nt reads) at the genome sequencing facilities of the departments of Human Genetics and Molecular Biology (Radboud University Nijmegen). The reads of both methods were combined using CLC bio software (Aarhus, Denmark) and assembled in about 200 contigs of >1,000 nt, including several large genome fragments of 100 kb. *A. thiooxidans* strain S1p was sequenced by Illumina sequencing only, resulting in a 1.7-Gb sequence in 75-nt reads. Sequence reads were trimmed to remove low-quality reads and ambiguous nucleotides, selecting those with a minimum read length of 30 nt. The resulting sequences (total, 0.8 Gb) were used for *de novo* assembly, yielding 265 contigs with an average length of 11.758 nt. Putative G8 and S1p CS_2 hydrolase genes were identified in both assemblies using the sequences of the CS_2 hydrolases from *Acidianus* strain A1-3 (GenBank accession number HM805096) and *Sulfolobus solfataricus* P2 (GenBank accession number AE006641.1) in BLASTP and motif searches.

MALDI-TOF MS. Purified CS_2 hydrolase from pieces excised from SDS-PAGE gels was extracted, digested with trypsin, and analyzed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) as described previously (33).

Phylogenetic analysis. The carbonic anhydrase(-like) protein sequences most homologous to the CS_2 hydrolase of *Acidianus* strain A1-3 and *A. thiooxidans* strains G8 and S1p were retrieved via Blast. They were aligned using MUSCLE (34), and phylogenetic analysis was performed in MEGA 5.05 (35).

Stopped-flow spectrometry. Carbonic anhydrase activity was measured by stopped-flow spectrometry (21). Per assay, up to 30 μg purified CS_2 hydrolase was used. The positive control consisted of up to 63 ng bovine carbonic anhydrase (Sigma) per assay.

Analytical ultracentrifugation. The oligomeric state of the CS_2 hydrolases in solution was investigated by analytical ultracentrifugation at $A_{280}^{1\ cm}$ of 0.2 (S1p) and 0.67 (G8) as described in reference 21.

Structure modeling. The hexadecameric structure of the *Acidianus* A1-3 CS_2 hydrolase (Protein Data Bank accession number 3TEO) was used as a template to form the hexadecameric Zn-containing CS_2 hydrolase from 3TEN (which is an octamer). The YASARA & WHAT IF Twinset (36) was used for homology modeling and subsequent analysis of the *A. thiooxidans* G8 and S1p CS_2 hydrolases. Sequence identity between the models and the *Acidianus* A1-3 CS_2 hydrolase template was 49% for *A. thiooxidans* G8 CS_2 hydrolase and 46% for the CS_2 hydrolase from strain S1p. Access routes from outside the enzymes to the active sites were calculated using MOLE 2.0 online (37).

Nucleotide sequence accession numbers. The sequences of the bacterial CS2 hydrolases were deposited in the GenBank database under accession numbers KC902814 and KC902815.

RESULTS

Purification of the CS_2 hydrolases from *A. thiooxidans* strains G8 and S1p. From a variety of sulfur-rich and highly acidic ecosystems, we isolated five new extremely acidophilic CS_2-converting *A. thiooxidans* strains (2Bp, Sts 4-3, S1p, G8, and BBW1). SDS-PAGE analysis of the soluble proteins in cell extracts from cells grown in chemostats with CS_2 as the sole energy source indicated that in all strains there was one hugely dominant protein present, of approximately 24 kDa (Fig. 1A). This size corresponds to the size of the monomeric CS_2 hydrolase from *Acidianus* A1-3, which is also present in high abundance in CS_2-grown cells (21). Therefore, we postulated that these highly abundant proteins in the *A. thiooxidans* extracts might be the bacterial CS_2 hydrolases.

The highly abundant protein of strain S1p was approximately 1 kDa higher in mass than those of the other 4 strains as well as the *Acidianus* A1-3 CS_2 hydrolase. To investigate potential differences between the CS_2 hydrolases, we purified the CS_2 hydrolase from both strain G8 and strain S1p by ammonium sulfate precipitation followed by HIC and anion-exchange chromatography (Fig. 1B). The purified CS_2 hydrolases both corresponded to the highly abundant protein present in the *A. thiooxidans* cell extracts.

Identification of the CS_2 hydrolase genes of *A. thiooxidans* strains G8 and S1p. In order to identify the genes encoding the purified CS_2 hydrolase enzymes, genomic DNA of *A. thiooxidans* strain G8 and S1p was sequenced by next-generation sequencing platforms and assembled. Using the sequences of the *Acidianus* A1-3 and *S. solfataricus* P2 CS_2 hydrolase genes, homologues were

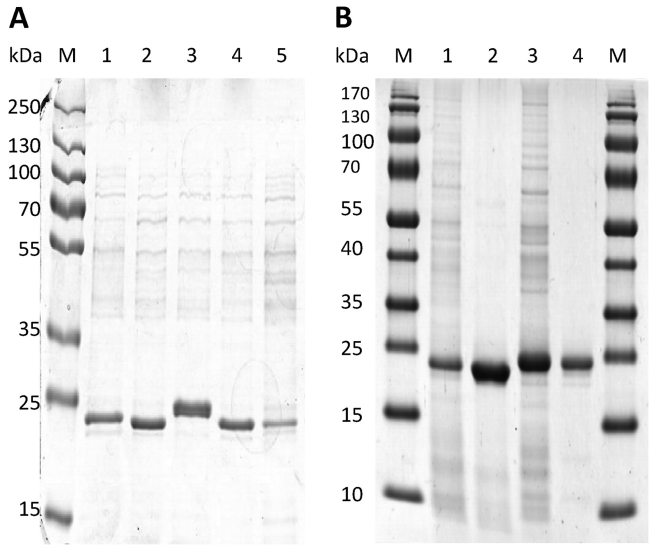

FIG 1 Purification of CS_2 hydrolases from CS_2-converting *Acidithiobacillus thiooxidans* strains. (A) SDS-PAGE (12% polyacrylamide) of cell extracts from CS_2-converting *Acidithiobacillus thiooxidans* strains 2Bp (lane 1), Sts 4-3 (lane 2), S1p (lane 3), G8 (lane 4), and BBW1 (lane 5) grown in minichemostats, with 10 nmol · ml^{-1} CS_2 as the energy source and at a *D* of 0.02. (B) Cell extract from *A. thiooxidans* strains G8 (lane 1) and S1p (lane 3), and purified CS_2 hydrolases of strains G8 (lane 2) and S1p (lane 4). Ten micrograms of protein was loaded per lane.

identified in the assemblies of both *A. thiooxidans* strains and aligned with the most homologous genes identified in the NCBI databases. The putative G8 CS_2 hydrolase (predicted mass, 22.6 kDa) had 49% amino acid identity and the S1p CS_2 hydrolase (predicted mass, 24.3 kDa) 46% amino acid identity to the *Acidianus* A1-3 enzyme, and they had 50% amino acid identity with each other. The active-site residues that are characteristic of β-CAs are perfectly conserved (Fig. 2). The translated S1p gene was 18 amino acids longer than the translated G8 gene and 13 amino acids longer than the *Acidianus* A1-3 CS_2 hydrolase, corresponding to the larger mass of the S1p enzyme observed on SDS-PAGE (see above). MALDI-TOF MS confirmed that the purified CS_2 hydrolases were encoded by the identified *Acidianus* A1-3 CS_2 hydrolase homologous genes: for the purified CS_2 hydrolase of strain G8, peptides from the trypsin digest covered 60% of the identified (translated) gene. For strain S1p, the coverage was 90%.

Phylogenetic analysis of CS_2 hydrolases and homologous enzymes. The newly identified CS_2 hydrolases cluster within a group of β-CA homologues that contain the two confirmed CS_2 hydrolases from *Acidianus* strain A1-3 and *S. solfataricus* P2. This group is divided into archaeal and bacterial enzymes, with three cyanobacterial exceptions: three enzymes, from *Calothrix* sp., *Cyanothece* sp., and *Nostoc punctiforme*, fall in the archaeal instead of the bacterial group (Fig. 3). Previously, we identified two phenylalanine residues, F77 and F78, in the *Acidianus* A1-3 sequence that were present in the other confirmed CS_2 hydrolases. These residues form part of a long hydrophobic tunnel, the only access to and from the active center. Amino acid F78 was shown to be crucial for activity of the CS_2 hydrolase of *Acidianus* A1-3 (21). As the F77 and F78 residues were not conserved in β-CA homologues outside the cluster that contained the CS_2 hydrolases, we proposed that they may be signature residues for CS_2 hydrolase enzymes. In support of this hypothesis, this FF motif was also conserved in the G8 and S1p CS_2 hydrolases. In fact, in all the enzymes of both the bacterial and the archaeal branches of the putative CS_2 hydrolase cluster within the β-CAs, the FF motif is conserved. There are two exceptions: the enzyme from *Nostoc punctiforme* has a VF motif, and that of *Haloquadratum walsbyi* DSM16790 has a YF motif instead, but the F78 residue, important for activity in the *Acidianus* A1-3 enzyme, is still conserved.

CS_2 conversion by microorganisms present in the CS_2 hydrolase phylogenetic tree. Three strains present in the putative CS_2 hydrolase cluster were grown and tested for CS_2 conversion by adding 10 nmol · ml^{-1} CS_2 gas to the headspace of a 10-ml grown culture. Of the three strains, *Methylomicrobium alcaliphilum* 20Z (DSM19304) converted CS_2 immediately to H_2S with COS as an intermediate. After 1 h, all CS_2 was depleted. Although CS_2 conversion was immediate and effective, we did not observe growth of *M. alcaliphilum* on CS_2 instead of CH_4, even after incubation periods of more than 1 month. *Halopiger xanaduensis* SH-6 (DSM18323), on the other hand, was not able to convert CS_2 over

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Acidianus A1-3   1  --MVSEYIDS ELKRLEDYAL RRVKG--IPN NRRLWVLTCM DERVHIEQSL GIQPD----- -DAHIYRNAG     60
S.isl HEV10/4    1  --MVSEYIDS EIKRLEDYAL RRVKG--IPN NRRLWVLTCM DERVHIEQTL GIQPD----- -DAHIYRNAG     60
S.isl LS215      1  --MISEYVDE EIKRREDYSL RRLRG--IPN DRRLWILTCM DERIHVEEAL GIKPE----- -DAHIYRNAG     60
S.solf P2        1  --MISEYVDE EIKRREDYSL RRLKG--IPN DRRLWILTCM DERVHVEEAL GIRPE----- -DAHIYRNAG     60
A.thio G8        1  -MSLKQQLES DFEGHKRWAL RRQMG--IPN NRRLWVCACM DERLPVDDAL GIRGDR---- GDAHVFRNAG     63
A.caldus         1  -MSLKQQLES DFEGHKRWAL RRQMG--IPN NRRLWVCACM DERLPVDEAL GIRGDQ---- GDAHVFRNAG     63
A.thio S1p       1  MSTLKEQLTA HVASYDHWAQ RRRYGPDGHN NRSLWVLACM DERLPVDEAL GIHVDTPAGG GDAHCFRNAG     70

Acidianus A1-3  61  GIVTDDAIRS ASLTTNFFGT KEIIVVTHTD CGMLRFTGEE VAKYFISKGI KPTEVQLDPL LPAFRISSEE    130
S.isl HEV10/4   61  GIVTDDAIRS ASLTTNFFGT KEIIVVTHTD CGMLRFTGEE VAKYFESKGI KPTEIQLDPL LPAFRISTEE    130
S.isl LS215     61  GIVTDDAIRS ASLTTNFFGT KEIIVITHTD CGMLRFTGDE VARYFIEKGV KVKELQIDPL LPSLKLENEQ    130
S.solf P2       61  GIVTDDAIRS ASLTTNFFGT KEIIVITHTD CGMIRFTGDE VAKYFLDKGV KVNELQIDPL LPSLRLQSTE    130
A.thio G8       64  GLITDDAIRS AMLTCNFFGT EEIVIINHTE CGMMSAQTDT IVKALKDKGI DLDNLQLDPD LPELTLKAG-    132
A.caldus        64  GLITDDAIRS AMLTCNFFGT EEIVIINHTE CGMMSAHTDT IVKALKDKGI DLDNIQLDPD LPELTIKAG-    132
A.thio S1p      71  GIVTDDAIRS AMLTCNFFGT KEIVIVQHTQ CGMLSGNANE MEKVLREKGM DTDNITLDPT LPELQLAKG-    139

Acidia A1-3    131  DFIKWFKFYE DLGVKSPDEM ALKGVEILRN HPLIPKDVRI TGYVYEVETH RLRKPNQIIY NETSKFEHGT IVKE------ --- 204
S.isl HEV10/4  131  DFIKWFKFYE DLGMKSPDEM ALKGVEILRN HPLIPKDVRI TGYVYEVETH RLRKPNQFIY NETSKFEHGS VVKE------ --- 204
S.isl LS215    131  DFVKWFKFFR DLGANTPDEI ALKNVEILKN HPLIPKHVSI SAYVYEVETH KLRKPNQRLY ELTSRFEHGT VVKD------ --- 204
S.solf P2      131  DFTKWFKFFR DLGANSPDDI ALKNAEILKN HPLIPKNVTI SAYVYEVETH KLRKPHQRLY ELTSRFEHGT VVKE------ --- 204
A.thio G8      133  MFGKWVKMYQ DV-----DET CARQVEYMRN HPLIPKHVTI SGWIWEVETG HLRPPHFRIG EKVNTNKAMG AK-------- --- 199
A.caldus       133  AFGKWIKMYS DV-----DET CARQVEYVRN HPLIPKHVTV SGWIWEVETG HLRPPHIRIG EKVNTNKAMG AK-------- --- 199
A.thio S1p     140  AFAKWIGMMD DV-----DET CMKTINAFKN HPLIPKDIVV SGWVWEVENR RLRAPTLDKE KRARTDCTPT PYGVKGNQPP RWK 217
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FIG 2 Amino acid alignment of CS_2 hydrolases from *Acidianus* strain A1-3, *Sulfolobus islandicus* strains HEV 10/4 and LS 2.15, *S. solfataricus* P2, *A. thiooxidans* strain G8, *Acidithiobacillus caldus* strain SM-1, and *A. thiooxidans* strain S1p. Underlining indicates residues forming the catalytic center of the enzymes. Boldface indicates perfectly conserved amino acids. Gray box, FF motif.

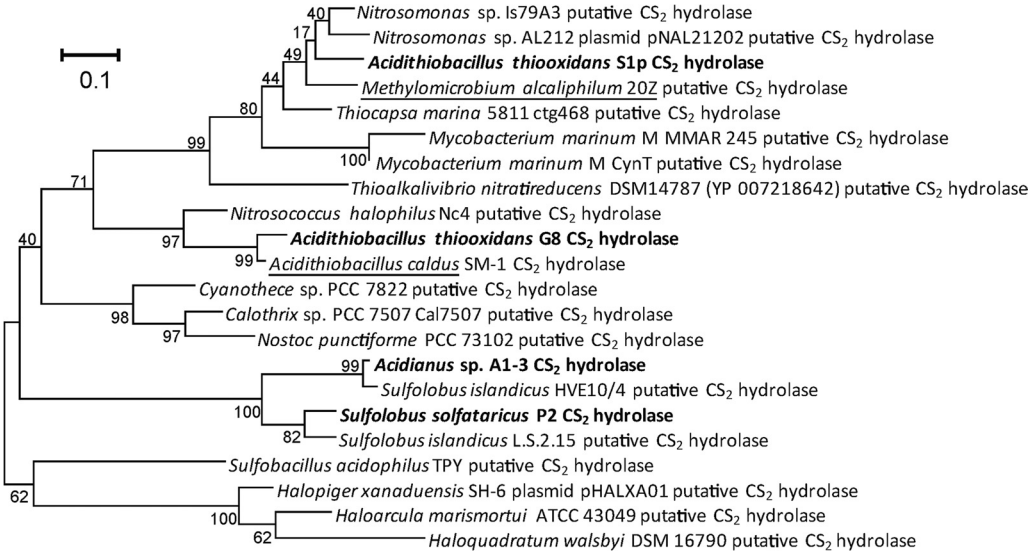

FIG 3 Phylogenetic analysis of putative and confirmed CS_2 hydrolases. Amino acid sequences were retrieved from GenBank and through BLAST and aligned using the MUSCLE aligner as implemented in Mega5.0. All putative CS_2 hydrolases are β-CA homologues and are usually annotated as such in GenBank. The evolutionary history was inferred by using the maximum likelihood method based on the Dayhoff matrix-based model. The tree with the highest log likelihood (−3645.9759) is shown. The percentage of trees (500 replicates) in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 183 positions in the final data set. A phylogenetic tree based on DNA sequences showed comparable clustering. Underlining indicates putative CS_2 hydrolases from strains with confirmed CS_2 conversion activity. Boldface indicates confirmed CS_2 hydrolases.

a period of at least 3 h. A small, nontransient increase in COS was seen over time, but this occurred also in cultures that had been inhibited or killed by heat treatment or addition of sodium azide or potassium cyanide, as well as in *Escherichia coli* cultures, and did not significantly reduce the concentration of CS_2 in the headspace. *Mycobacterium marinum* strain M was also tested for CS_2 conversion activity. However, under the conditions tested, this strain did not show CS_2 hydrolase activity, not even after 24 h of incubation of a growing culture with CS_2.

Substrate specificity of purified CS_2 hydrolases from *A. thiooxidans* strains G8 and S1p. As the CS_2 hydrolase enzymes are highly homologous to the β-CAs, it is possible that β-CAs may be able to hydrate CS_2 as well as CO_2. Although the possibility of this conversion was predicted quantum chemically (24–26), it has never been experimentally confirmed. In addition, we tested potential CS_2 hydrolase activity of bovine erythrocyte α-CA, the γ-CAs Cam from *Methanosarcina thermophila* (38), Cab from *Methanobacterium thermoautotrophicum* (39), and Cam from *Pelobacter carbinolicus* (kindly made available by R. Siva Sai Kumar and James G. Ferry) and the β-CA from *Streptococcus pneumoniae* (40, 41), but none of these could convert CS_2. Reversely, CS_2 hydrolases may be able to hydrate CO_2 instead of CS_2. We have already shown that for the CS_2 hydrolase from *Acidianus* strain A1-3 this was not the case (21). Here, we tested the substrate specificity of the *A. thiooxidans* CS_2 hydrolases by stopped-flow spectrophotometry. Like the *Acidianus* CS_2 hydrolase, the *A. thiooxidans* enzymes could not convert CO_2 to HCO_3^- (Fig. 4).

The newly purified CS_2 hydrolases did convert CS_2 and COS to H_2S (Table 1). With CS_2 as the substrate, the *A. thiooxidans* G8 CS_2 hydrolase had a nearly 10-fold-higher catalytic efficiency (K_{cat}/K_m) than the *Acidianus* A1-3 and *A. thiooxidans* S1p CS_2 hydrolases. This was due to both a higher V_{max} (131 versus 32 nmol product · min^{-1} · μg enzyme) as well as a higher affinity (lower K_m of 46 versus 93 μM CS_2). The difference in catalytic efficiency between the strain G8 CS_2 hydrolase and the strain S1p CS_2 hydrolase was even more pronounced with COS as the substrate, due to the much higher affinity of the strain G8 CS_2 hydrolase for COS.

We compared the kinetic properties of the purified enzymes with those of cell extracts from the five new CS_2-converting *A.*

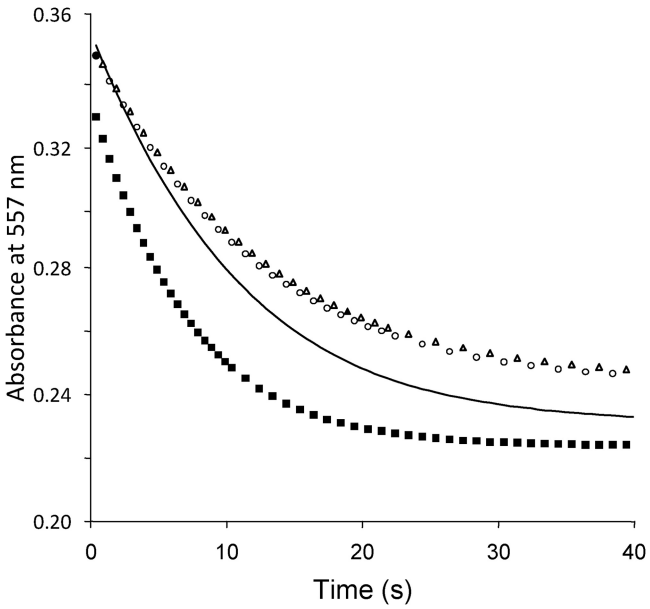

FIG 4 Stopped-flow spectrophotometry to measure carbonic anhydrase activity of purified CS_2 hydrolases from *A. thiooxidans* strains G8 and S1p compared with carbonic anhydrase from bovine erythrocytes. Symbols: open circles, strain G8; open triangles, strain S1p; solid squares, bovine carbonic anhydrase; solid line, chemical control.

TABLE 1 Comparison of kinetic constants[a] of the conversion of CS_2 (to H_2S and COS) and COS (to H_2S) by purified CS_2 hydrolases from *Acidithiobacillus thiooxidans* strains S1p and G8 with those of *Acidianus* A1-3 CS_2 hydrolase

Strain	CS_2			COS		
	K_m	V_{max}	K_{cat}/K_m	K_m	V_{max}	K_{cat}/K_m
A. thiooxidans S1p	93 ± 21	32 ± 2	8.4×10^6	74 ± 11	34 ± 2	1.1×10^7
A. thiooxidans G8	46 ± 6	131 ± 4	6.4×10^7	14 ± 2	97 ± 3	1.6×10^8
Acidianus A1-3[b]	130 ± 3	40 ± 0	7.3×10^6	22 ± 3	74 ± 3	8.0×10^7

[a] K_m values are expressed in μM substrate in the buffer ± standard error of the mean (SEM); V_{max} is given in nmol product · min^{-1} · μg enzyme ± SEM. K_m, V_{max}, and K_{cat}/K_m values (in $S^{-1} \cdot M^{-1}$) were determined by nonlinear regression using GraphPad Prism 5.04 ($n \geq 9$).
[b] Data from reference 21.

thiooxidans strains growing in chemostats supplied with 10 nmol · ml^{-1} CS_2. Under these conditions, these strains all produced large amounts of CS_2 hydrolase (Fig. 1). The K_m values varied between 81 μM for strain G8 and 130 μM CS_2 for strain 2Bp. The V_{max} of cell extracts from strain S1p was higher than those of cell extracts from the 4 other strains tested, which is in contrast to the results obtained with the purified CS_2 hydrolases. However, due to the lower affinity of the purified CS_2 hydrolase to CS_2, strain S1p would require larger amounts of CS_2 hydrolase when growing on the low CS_2 concentration supplied to the reactor to be able to gain as much energy for growth as strain G8. Indeed, strain S1p had relatively more CS_2 hydrolase present in cell extracts than strain G8 (Fig. 1).

Catenane formation in the *A. thiooxidans* CS_2 hydrolases. To investigate whether the bacterial CS_2 hydrolases form the highly unusual catenanes observed for the *Acidianus* A1-3 CS_2 hydrolase (21), we compared cell extracts from five CS_2-converting *A. thiooxidans* strains and two purified CS_2 hydrolases with the *Acidianus* enzyme by native PAGE. Four of the five *A. thiooxidans* strains had CS_2 hydrolases of a size similar to that of the *Acidianus* 16-mer form of the native enzyme. This suggests that, like the archaeon *Acidianus*, these *A. thiooxidans* strains contain the interlocked, double-ring catenane form of the enzyme rather than the single octameric ring. However, strain S1p CS_2 hydrolase migrated faster than the other CS_2 hydrolases, both in cell extract and in purified form (Fig. 5), suggesting that the S1p CS_2 hydrolase may not form hexadecameric catenanes. To confirm this finding, purified CS_2 hydrolases from strains S1p and G8 were analyzed by analytical ultracentrifugation (AUC) (Fig. 6). For the CS_2 hydrolase of strain G8, a small peak with sedimentation coefficient at 8.4 S and a large peak at 13.8 S were identified, corresponding to an 8-mer and 16-mer, respectively. The CS_2 hydrolase of strain S1p yielded only one peak, at 7.8 S. From these data we conclude that the CS_2 hydrolases from *A. thiooxidans* strains G8, 2Bp, Sts 4-3, and BBW1 share the unique hexadecameric catenane structure with the CS_2 hydrolase from *Acidianus* A1-3. However, the *A. thiooxidans* S1p CS_2 hydrolase forms only the single octameric ring.

Structure models of the *A. thiooxidans* CS_2 hydrolases. To compare the structural homology between the three CS_2 hydrolases and to find a structural basis for the differences in oligomerization between the *A. thiooxidans* CS_2 hydrolases, we used YASARA to create structural models of the *A. thiooxidans* G8 and S1p enzymes using the hexadecameric *Acidianus* A1-3 CS_2 hydrolase as a template. The CS_2 hydrolases of both enzymes could be modeled as catenane hexadecamers (Fig. 7A, B, and C). The models show a highly conserved structural homology in the active sites of the enzymes (Fig. 8). In addition, the long and narrow hydrophobic tunnel that forms the only access to the active site in the *Acidianus* A1-3 CS_2 hydrolase is also predicted to be the only available access route in the modeled structures of *A. thiooxidans* strains G8 and S1p CS_2 hydrolases (Fig. 7D, E, and F). The residues that form these tunnels are partly conserved: the F77F78 motif critical for activity in the *Acidianus* A1-3 enzyme is perfectly conserved, and some of the residues at the outer entrance of the tunnel are still hydrophobic but smaller, potentially creating a slightly wider tunnel at those positions (Fig. 7G, H, and I). The tunnel in the *Acidianus* A1-3 enzyme is formed by residues from 3 monomers, with the third monomer contributing one isoleucine residue (I201) from the C-terminal extending arm. However, in both the *A. thiooxidans* strain G8 and the strain S1p CS_2 hydrolase models, the C-terminal domain of the third monomer is positioned away from the tunnel. As the C-terminal domain of the enzyme from strain G8 is shorter and that of the strain S1p enzyme is longer than the C terminus of the *Acidianus* A1-3 CS_2 hydrolase, the model may not be accurate in this position. It therefore remains to be seen if a third monomer contributes to the tunnel formation in the *A. thiooxidans* CS_2 hydrolase.

From the structure of the *Acidianus* A1-3 CS_2 hydrolase as well as in the models of the *A. thiooxidans* strain G8 and strain S1p enzymes, it appears that the N-terminal α-helical extending arms

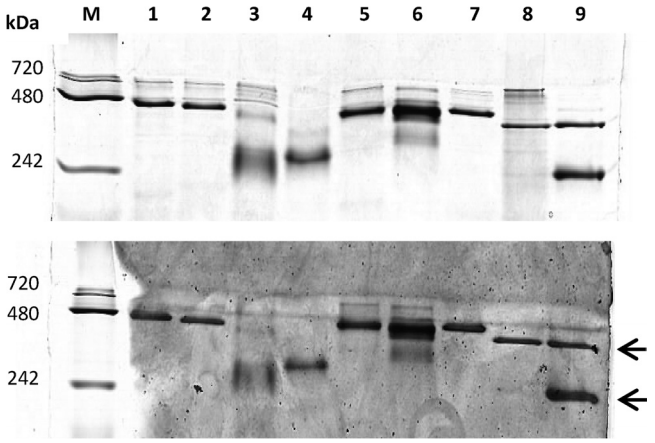

FIG 5 Native PAGE (8% polyacrylamide) of three purified CS_2 hydrolases and of cell extracts from five CS_2-converting *A. thiooxidans* strains and of *Acidianus* A1-3, stained for protein (top) or CS_2 hydrolase activity (bottom). Ten micrograms of protein was loaded per lane. Lanes: M, marker; lane 1, cell extracts of strain 2Bp; lane 2, cell extracts of strain Sts 4-3; lane 3, cell extracts of strain S1p; lane 4, purified CS_2 hydrolase of strain S1p; lane 5, strain G8 cell extract; lane 6, strain G8 purified CS_2 hydrolase; lane 7, strain BBW1 cell extract; lane 8, *Acidianus* A1-3 cell extract; lane 9, *Acidianus* A1-3 purified CS_2 hydrolase. Arrows indicate the expected positions of 8-mer (about 192-kDa) and 16-mer (about 384-kDa) configurations.

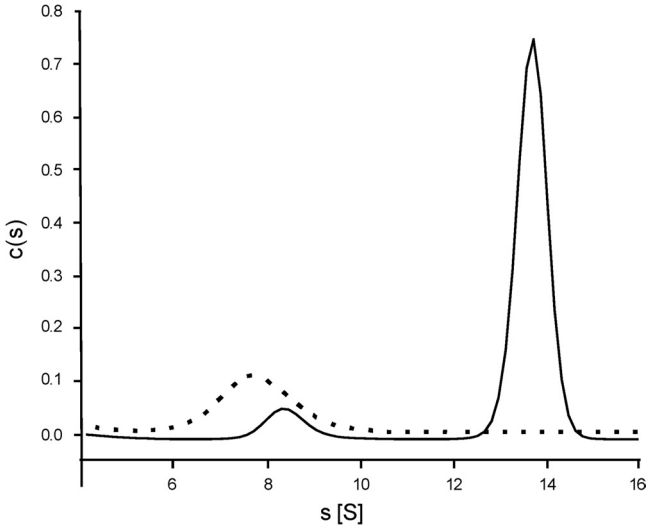

FIG 6 Analytical ultracentrifugation results for purified CS_2 hydrolases from *A. thiooxidans* strains S1p and G8, fitted as the concentration distribution (c) (arbitrary units) of sedimentation coefficients (s) in Svedberg units (S). For strain G8 (solid line), two species are observed, corresponding to octamers (at 8.3 S) and hexadecamers (13 S). For strain S1p (dashed line), only one peak was observed, corresponding to an octameric form of the enzyme.

of the monomers are responsible for most of the interaction between the 2 rings in the catenane structure (Fig. 9). In particular, in the *Acidianus* structure the residue K22 just beyond the N-terminal helix of one octamer is in close proximity to the Q49 and P50 on a small 4-residue loop (residues 49 to 52, QPDD) of the other octamer. In the model of the *A. thiooxidans* G8 CS_2 hydrolase, this small loop is one residue larger (residues 50 to 55, RGDRD) but points in a different direction from that of the *Acidianus* A1-3 loop. Although the CS_2 hydrolase of *A. thiooxidans* S1p could also be modeled as a catenane double ring, there is a much larger loop corresponding to the sequence insertion at residues 56 to 61, TPAGGG, that is not present in the *Acidianus* A1-3 or *A. thiooxidans* G8 CS_2 hydrolase (Fig. 2). The *A. thiooxidans* S1p CS_2 hydrolase has a second 2-residue insertion (26P-27D [Fig. 2]) that is part of a loop in the other octamer (residues 21 to 28, RRRYGPD [Fig. 9]). This loop protrudes into the space close to where the TPAGGG loop is positioned. It is therefore possible that these insertions in the *A. thiooxidans* S1p CS_2 hydrolase protein are interfering with catenane formation.

In addition, the C-terminal tail of the *A. thiooxidans* S1p CS_2 hydrolase is nine amino acids longer and its sequence differs from that of the *Acidianus* A1-3 CS_2 hydrolase C-terminal tail, making it

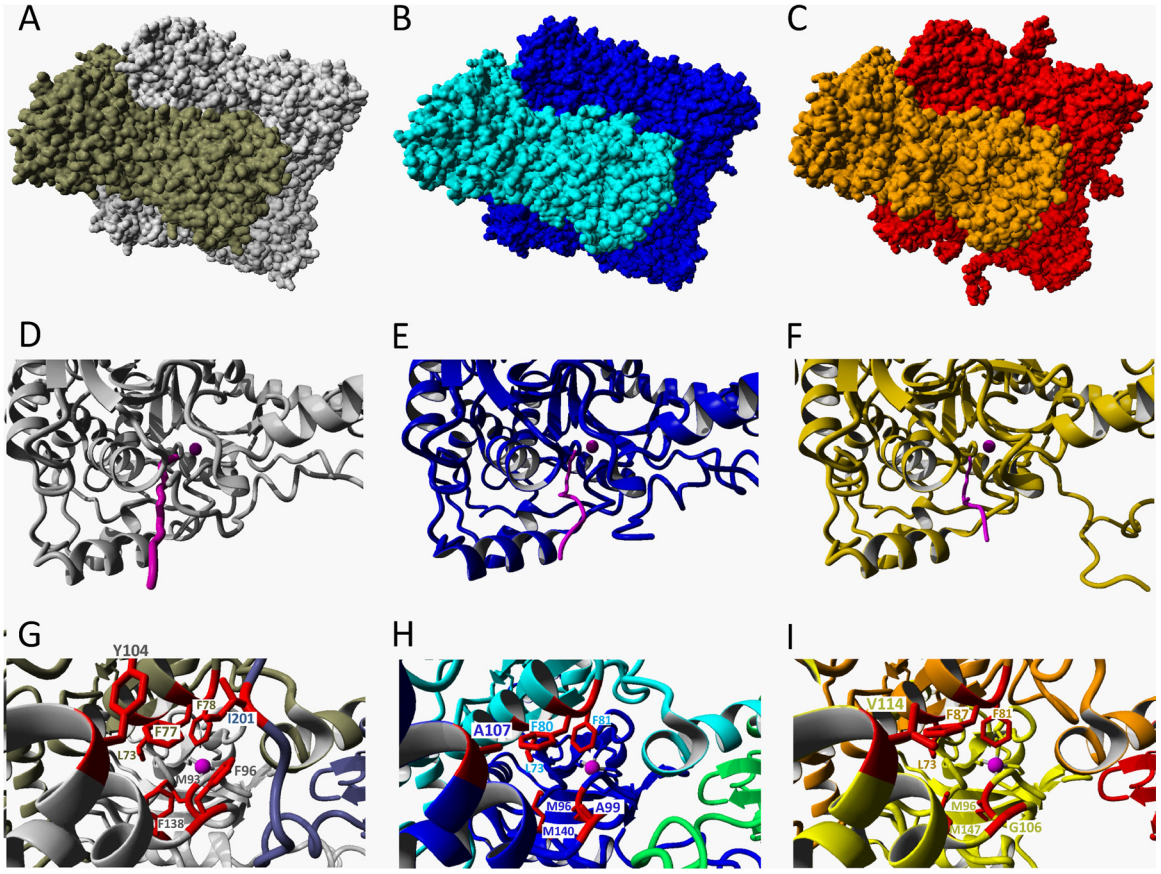

FIG 7 Crystal structure and models of CS_2 hydrolases. (A) Resolved crystal structure of the CS_2 hydrolase from *Acidianus* strain A1-3 and structural models of the CS_2 hydrolases from *A. thiooxidans* strain G8 (B) and strain S1p (C), modeled using the hexadecameric structure of the *Acidianus* A1-3 CS_2 hydrolase as a template. The two octameric interlocking rings forming the hexadecamer are shown in different colors. (D, E, F) Details of octamers showing the narrow hydrophobic entrance tunnels (pink) to the Zn-containing active site in the *Acidianus* CS_2 hydrolase (D) and in the models of the G8 (E) and S1p (F) CS_2 hydrolases. The Zn atoms are indicated by pink spheres. (G, H, I) Views of the tunnels looking in from the entrances to the active sites, showing the three monomers (two shades of gray and blue) and the residues (red) forming the tunnel in the *Acidianus* CS_2 hydrolase (G) and in the models of the G8 hydrolases (blue, cyan, and green monomers) (H) and the S1p CS_2 hydrolases (yellow, orange, and red monomers) (I).

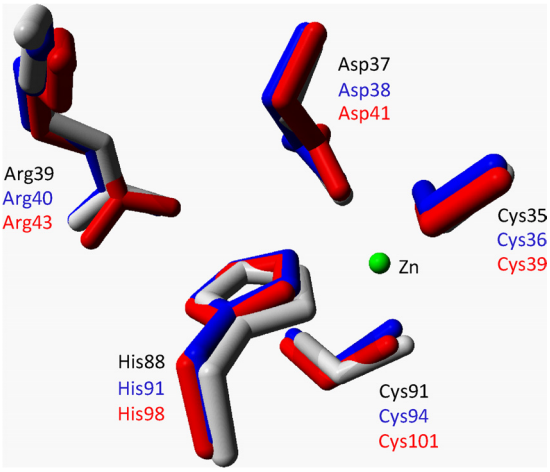

FIG 8 Superposition of active-site residues of the resolved structure of the *Acidianus* A1-3 CS_2 hydrolase (gray) and the models of the *A. thiooxidans* G8 (blue) and S1p CS_2 hydrolases (red). The Zn residue is shown as a green sphere.

difficult to model (Fig. 7C). The C-terminal tails of the *Acidianus* A1-3 CS_2 hydrolase as well as the *A. thiooxidans* G8 modeled CS_2 hydrolase contain several residues that make stabilizing interactions with the core of the structure, where potential interactions between the 2 octamers take place, and therefore it seems likely that the C-terminal tail is not very flexible. Whether the C-terminal tail of the *A. thiooxidans* S1p CS_2 hydrolase adopts a similar conformation remains unclear. Because of the differences in length and sequence, it is possible that this tail can also interfere with hexadecameric catenane formation.

DISCUSSION

In this study, we identified the bacterial CS_2 hydrolase enzymes from five *A. thiooxidans* strains, purified the CS_2 hydrolases from *A. thiooxidans* strains G8 and S1p, and identified the encoding genes. The enzymes were highly homologous to each other and to the CS_2 hydrolase from *Acidianus* strain A1-3, both on DNA and on protein levels. All putative CS_2 hydrolases enzymes are phylogenetically most related to a group of β-CAs that contain mostly bacterial rather than archaeal enzymes (21). This suggests that the gene evolved in bacteria and spread via lateral gene transfer to archaeal species. Indeed, two of the putative CS_2 hydrolases were plasmid encoded. Also, transposon-related sequences surrounding the (putative) CS_2 hydrolase were identified in the available genome sequences of both archaeal species (*S. solfataricus* P2, *Sulfolobus islandicus* strains L.S.2.15 and HVE10/4, and *Haloquadratum walsbyi*) and bacterial species (*Nitrosomonas* sp. AL212 and *Mycobacterium marinum* M). *Acidithiobacillus* and the archaeal *Sulfolobus* and *Acidianus* species occupy the same sulfur-rich acidic environments such as hot springs and solfataras (42, 43). Gene transfer between these species from the two domains of life is possible and likely (44–46).

In addition to the acidophilic species that are represented in the (putative) CS_2 hydrolase phylogenetic tree, most other species were isolated from saline and/or alkaline environments. Saline marshes and estuaries are rich sources of CS_2 (47, 48). Also, the haloalkaline soda lakes harbor an active sulfur cycle with a high biodiversity of sulfur-oxidizing bacteria (SOB) that respire mainly on sulfide, thiosulfate ($S_2O_3^{2-}$), and polysulfide (49). *Thioalkalivibrio paradoxus*, isolated from a Kenyan soda lake, was shown to be able to respire on CS_2 when grown on thiocyanate (SCN^-) (50). We could not find a CS_2 hydrolase homologue in the available genome sequence of this species (which at the time of writing is still wrongly labeled as the *Thioalkalivibrio thiocyanoxidans* genome [D. Sorokin, personal communication]). However, the *Thioalkalivibrio paradoxus* genome does contain a homologue of the recently described COS hydrolase from *T. thioparus* strain THI 115, one of only three close homologues currently in the databases. *T. thioparus* strain THI 115 converts SCN^- to COS and NH_3. COS is subsequently converted to H_2S by COS hydrolase. This enzyme can also convert CS_2 at a lower rate (27). It is possible that the CS_2 hydrolase activity observed for *Thioalkalivibrio para-*

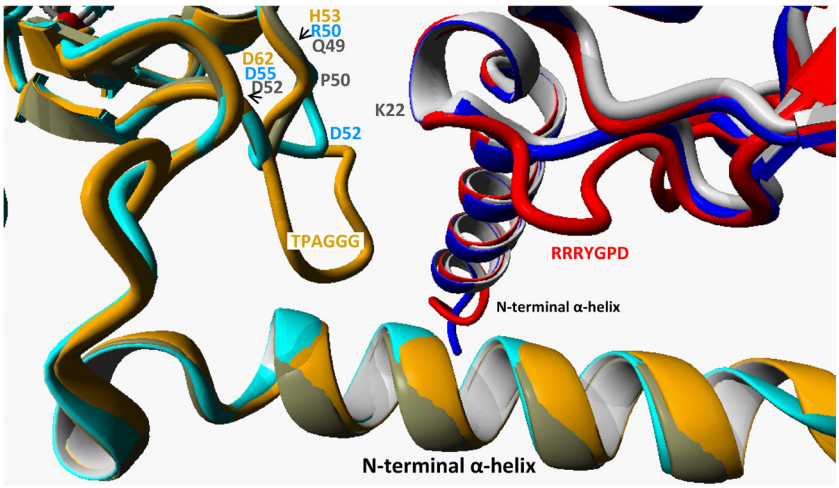

FIG 9 Structure detail of the proximity of two octamers in the hexadecameric form of the CS_2 hydrolase, shown as an overlay of the models of the *A. thiooxidans* strains G8 (two shades of blue, one for each octamer) and S1p (red and ochre) onto the *Acidianus* strain A1-3 resolved structure (two shades of gray). In the *Acidianus* CS_2 hydrolase, the smallest distance between the 2 octamers is between a small loop of 4 residues between Gln49 and Asp52 (QPPD) and the Lys22 on the other octamer (residues in gray). In the model of the G8 CS_2 hydrolase, the G8 loop is 6 residues long (RGDRGD, in blue). In the model of the S1p, the loop is 10 residues long (HVDTPAGGGD, in ochre). The S1p enzyme is modeled to have an additional loop in the second octamer (RRRYGPD).

doxus is due to activity of the COS hydrolase homologue. Interestingly, the closely related *Thioalkalivibrio nitratireducens*, isolated from the Egyptian soda lake Lake Fazda (51), contains both a CS_2 hydrolase and a close COS hydrolase homologue. *Thioalkalivibrio paradoxus* and *Thioalkalivibrio nitratireducens* both convert SCN^-, but they do not produce COS or CS_2 as intermediates (D. Sorokin, personal communication). Therefore, the function of the CS_2 hydrolase homologues in these strains remains unclear at present.

Of the three species that were tested for CS_2 conversion, only *M. alcaliphilum* was able to convert CS_2. This methanotroph was isolated from the highly alkaline soda lake Shara-Nur, in Central Asia (52, 53). CS_2 conversion by methane-grown cultures was immediate, suggesting that the enzyme was not induced but already present in the cell. Why this organism requires the presence of CS_2 hydrolase activity is not clear at present. However, another *Methylomicrobium* species, *M. kenyense* strain AMO1 isolated from a Kenyan soda lake, was also found to have CS_2 conversion activity (54). These authors suggested that CS_2 could be a suicide substrate for methane monooxygenase (MMO), the enzyme that converts methane to methanol as the first step in the energy-generating pathway of methanotrophs. MMO is mechanistically and evolutionary homologous to the ammonium monoxygenase (AMO) from nitrifiers. Their substrates are often interchangeable, and they share alternative substrates and inhibitors (55). The nitrifier *Nitrosomonas europaea* was shown to have CS_2 conversion activity, but the mechanism of CS_2 conversion was not investigated (56). CS_2 is one of the oldest known inhibitors of nitrification (57). The inhibitory action has been attributed to the CS_2 molecule forming a complex with a nucleophilic amino acid close to the active center of AMO, thereby chelating the Cu cofactor from the active center (58, 59). If MMO is similarly inhibited by CS_2, then both methanotrophs and nitrifiers would benefit from an enzyme capable of CS_2 removal. Indeed, in addition to the methanotroph *M. alcaliphilum*, two *Nitrosomonas* strains and one *Nitrosococcus* strain were found to contain CS_2 hydrolase homologues in their genomes (Fig. 3). We therefore propose that these CS_2 hydrolases could function as a detoxification mechanism for environmental CS_2 in nitrifiers and methanotrophs.

COS is an ecologically relevant alternative substrate for CS_2 hydrolase, as it is the most abundant sulfur species in the atmosphere. The major sinks for COS are vegetation and soil (2). Biological conversion of COS by plants via their carbonic anhydrases has been described (60, 61), and COS-degrading soil microorganisms, including 4 *Mycobacterium* spp., were readily isolated from Japanese soils (62). It is therefore possible that (some of) the CS_2 hydrolase homologues identified in the phylogenetic tree convert mainly COS rather than CS_2 in their natural environments. Interestingly, neither the two CS_2 hydrolases from *A. thiooxidans* strains S1p and G8 nor the archaeal CS_2 hydrolase (21) or the *T. thioparus* COS hydrolase (27) converted CO_2. This suggests that their function evolved specifically for CS_2 and COS conversion and that they are not just broad-specificity CAs.

All (putative) CS_2 hydrolase proteins presented in Fig. 3 but two contain the conserved FF residues that are crucial in forming the hydrophobic access tunnel to the active center. As they are not conserved in CAs or in the *T. thioparus* COS hydrolase, the FF residues were proposed to be a CS_2 hydrolase-specific motif (21). However, *H. xanaduensis* and *M. marinum*, which have CS_2 hydrolase homologues containing the FF motif, do not convert CS_2 under the conditions that we tested. It is possible that the amino acids surrounding the FF motif are also important for CS_2 hydrolase activity: in all confirmed bacterial and archaeal CS_2 hydrolases as well as in *M. alcaliphilum*, the sequence stretch that is perfectly conserved is NFFGT, but in *H. xanaduensis* the sequence is NFFDT. In the structural model of the *Acidianus* CS_2 hydrolase, the N76 and G79T80 residues are positioned away from the hydrophobic tunnel that the FF residues are a part of, but it is possible that the larger D79 residue causes a conformational change that affects the positioning of the crucial F78 residue. Although both the CS_2 hydrolase homologues present in *M. marinum* strain M contain the NFFGT motif, they are also the only two CS_2 hydrolase homologues with an extended C terminus, 56 amino acids longer than that of the CS_2 hydrolase from *A. thiooxidans* strain S1p. This may have profound effects on oligomerization and therefore enzyme activity (see below).

The *Acidianus* A1-3 CS_2 hydrolase exists as a 16-mer interlocked ring (catenane) structure. Catenanes are extremely rare in biology. We are aware of only 3 reports: the gp5 capsid protein of bacteriophage HK97 (63), the bovine mitochondrial peroxiredoxin III (64), and a *Pyrobaculum aerophilum* citrate synthase in which the catenanes are formed by disulfide bridging between the N- and C-terminal ends of each of the homodimer chains (65). The molecular mechanism of the catenane assembly in the *Acidianus* A1-3 enzyme is not understood. The N- and C-terminal domains of the monomers, which are crucial for interlinking dimers into octamers (21), also reside in the field of interaction between the 2 octameric rings (Fig. 9). Interestingly, the bacterial CS_2 hydrolases of four of the five isolated *A. thiooxidans* strains also existed in the 16-mer catenane form. The reason why these enzymes adopt a catenane conformation is unclear at present. However, cells growing on low concentrations of CS_2 produce vast amounts of enzyme, probably due to the poor affinity of the CS_2 hydrolase to CS_2. This results in extremely high intracellular concentrations with very dense packing of the enzyme, similar to what has been reported for the most abundant enzyme on earth, RuBisCo, that can be expressed to about 40% of total protein in cells and is packed in cell structures called carboxysomes (66). Dense packing of CS_2 hydrolases can be obtained both with octameric rings and with 16-mer catenanes, but the exclusion volume is a little bit smaller for catenanes than for single rings. Also, highly densely packed *Acidianus* strain A1-3 CS_2 hydrolase in crystals consisted of pure 16-mer catenanes (21).

The *A. thiooxidans* S1p enzyme was found only in the 8-mer form, both by AUC and by native PAGE analysis. The S1p CS_2 hydrolase monomer is 11 amino acids longer at the C terminus than the monomer from strain G8 and 9 amino acids longer than the monomer from *Acidianus* A1-3. In addition, the S1p enzyme has an insertion at residues 56 to 61 that the other two CS_2 hydrolases lack. Modeling the G8 and S1p enzymes on the *Acidianus* A1-3 CS_2 hydrolase structure indicated that in the S1p CS_2 hydrolase, both the elongated C-terminal tail and the insertion may interfere with the formation of the 16-mer catenane by filling the space where the other octamer should reside. It is possible that the N- and C-terminal areas of the *Acidianus* A1-3 and G8 CS_2 hydrolases, which are situated in the interaction field between the two octameric rings, may play a role in the formation of the rare catenane structure. Whether this is indeed the case is the subject of future investigations.

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