Diversity and Ecophysiology of New Isolates of Extremely Acidophilic CS₂-Converting Acidithiobacillus Strains

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Biofiltration of industrial carbon disulfide (CS₂)-contaminated waste air streams results in the acidification of biofilters and therefore reduced performance, high water use, and increased costs. To address these issues, we isolated 16 extremely acidophilic CS₂-converting Acidithiobacillus thiiooxidans strains that tolerated up to 6% (vol/vol) sulfuric acid. The ecophysiological properties of five selected strains (2Bp, Sts 4-3, S1p, G8, and BBW1) were compared. These five strains had pH optima between 1 (2Bp) and 2 (S1p). Their affinities for CS₂ ranged between 80 (G8) and 130 (2Bp) μM. Strains S1p, G8, and BBW1 had more hydrophobic cell surfaces and produced less extracellular polymeric substance than did strains 2Bp and Sts 4-3. All five strains converted about 80% of the S added as CS₂ to S⁰ when CS₂ was supplied in excess. The rate of S⁰ consumption varied between 7 (Sts 4-3) and 63 (S1p) nmol O₂ min⁻¹ ml culture⁻¹. Low S⁰ consumption rates correlated partly with low levels of cell attachment to externally produced S⁰ globules. During chemostat growth, the relative amount of CS₂ hydrolase in the cell increased with decreasing growth rates. This resulted in more S⁰ accumulation during CS₂ overloads at low growth rates. Intermittent interruptions of the CS₂ supply affected all five strains. Strains S1p, G8, and BBW1 recovered from 24 h of starvation within 4 h, and strains 2Bp and Sts 4-3 recovered within 24 h after CS₂ was resupplied. We recommend the use of mixtures of Acidithiobacillus strains in industrial biofilters.

Carbon disulfide (CS₂) is a toxic, volatile, flammable, and explosive solvent widely used in, e.g., the viscose rayon industry. Because of its toxicity and the increasingly stringent rules governing the emission of harmful gases, it is necessary to treat CS₂-containing industrial waste gases. Biological treatment of CS₂, governing the emission of harmful gases, requires treatment of the toxic substance. Because of its toxicity and the increasingly stringent rules governing the emission of harmful gases, it is necessary to treat CS₂-containing industrial waste gases. Biological treatment of CS₂, governing the emission of harmful gases, requires treatment of the toxic substance. However, these archaea are not able to grow at the extremely low pH values (4) because the sulfur-oxidizing bacteria provide an alternative to conventional treatment systems (e.g., active carbon, incineration, caustic scrubbing) (2, 3). Typical concentrations of CS₂ in contaminated air from viscose industries are around 4 to 20 nmol ml⁻¹ (100 to 500 ppm) (2).

The number of microorganisms known to be able to grow chemolithoautotrophically on CS₂ is limited to some Thiobacillus species, Thiothrix ramosa, Paracoccus denitrificans, and a Thiomonas sp. (4–8). All of these CS₂-utilizing bacteria grow at neutral pH. Thus far, only one CS₂-utilizing species (Thiobacillus sp. strain TJ330, DSM8985) capable of growth under acidic conditions (as low as pH 0.5) has been described (9). The only reported screening of 10 (Acidi)thiobacillus strains showed that CS₂ conversion is not a general trait of (acid)thiobacilli (4). In that screening, only one strain, Thiobacillus thioparus TK-m, was found to be capable of CS₂ conversion. We recently discovered that CS₂ conversion is not limited to the domain Bacteria; the hyperthermophilic archaea Aciditium sp. strain A1-3 and Sulfolobus solfataricus P2 can also grow on CS₂ as a main carbon and energy source (10). However, these archaea are not able to grow at the extremely low pH values that acidithiobacilli can cope with.

CS₂-converting sulfur oxidizers in operating biofilters are acidophilic bacteria (2, 11–13). They convert CS₂ via the two-hydrolysis reaction steps CS₂ + H₂O → COS + H₂S and COS + H₂O → CO₂ + H₂S and obtain their energy from the oxidation of H₂S via S⁰ and SO₂⁻ to SO₄²⁻ as follows: H₂S + SO₂⁻ → SO₄²⁻ + 2H⁺ (14). Therefore, an inherent result of CS₂ conversion is acidification of the biofilters, which can be limited only by flushing the trickling filters with fresh water. Operating at a pH as low as possible will considerably reduce the volume of fresh water used for neutralization. Water use would be further reduced if the H₂SO₄ produced could be reused in the viscose-rayon industry. This becomes economically feasible when the H₂SO₄ concentration in the reactor effluent is at least 10% (wt/vol) (5.6% [vol/vol]). However, the performance of biotrickling filters is compromised by severe acidification and buildup of elemental sulfur (S⁰) that can clog the filters. Therefore, we set out to isolate new CS₂-converting bacterial strains able to tolerate extremely low pH values with variable CS₂ loads without loss of CS₂ conversion efficiency and without the production of large amounts of elemental sulfur.

MATERIALS AND METHODS

Media and culture conditions. Strains were enriched and cultured in basal salt mineral medium (MM) with CS₂ as the sole carbon and energy source as described previously (15). Bacteria were grown at room temperature (RT, 22°C) in 120-ml bottles containing 20 ml MM acidified with sulfuric acid. Alternatively, bacteria were grown on MM plates solidified with 1% (vol/vol) Gelrite (16) and acidified with 0.1% (vol/vol) sulfuric acid. This was the maximum [H₂SO₄] at which plates could still be poured without the Gelrite solution immediately solidifying when brought into contact with the H₂SO₄-containing MM solution. In the case of Gelrite, H₂SO₄ increases solidification while the opposite occurs with agar(ose). Plates were incubated in an airtight jar. Unless stated differently, sulfuric acid concentrations are reported as percentages (vol/vol) and neutralized.
equivalent to 1.8% [wt/vol] and 0.18 M sulfuric acid). Strains were also grown in minichromosmat reactors as described previously (15).

The headspaces of the bottles and jars used were continuously flushed with a CS2-containing air stream from a purpose-built distribution system (see the supplemental material).

**Enrichment and isolation.** Volumes of 0.5 to 1 ml of environmental or industrial samples were inoculated into 120-ml serum bottles with 20 ml acidified MM. The initial H2SO4 concentration was 0.5 to 1% (vol/vol) in the environmental samples and 2% in the samples from the biotrickling filters. CS2 was supplied as the sole energy source via the distribution system. When a visually dense culture was obtained, the enrichment was transferred to fresh MM. The maximum H2SO4 concentration at which growth occurred was determined by the subsequent transfer of enrichments to MM with higher H2SO4 concentrations. Pure cultures were obtained from enrichment cultures grown in 4 to 6% H2SO4 on Geltite plates. Single colonies were serially transferred three times to fresh plates and checked microscopically for purity. Fungal contamination (mainly in the enrichments from industrial samples) was eliminated by adding 150 μg ml−1 chlorothalonil either to the plates or to liquid cultures. The H2SO4 tolerance of each isolated strain was confirmed by repeated subculturing at least three times in liquid MM containing 4 to 5% H2SO4.

**Screening of known Acidithiobacillus strains for CS2 conversion capacity.** Five Acidithiobacillus strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and grown in batch cultures in the media suggested (DSMZ medium number in parentheses after the strain name) or as otherwise stated, i.e., A. ferrooxidans DSM14882 (882), A. caldus DSM8584 (150a), A. thiooxidans DSM14887 (71), A. thiooxidans DSM504 (MM plus 10 g liter−1 sterile S0), and A. albertensis DSM14366 (71). After growth for 1 week, the headspace of the cultures was supplemented with CS2 (20 to 30 nmol ml−1). The CS2 concentration and the presence of intermediates (H2S and COS) in the headspace were monitored over time by gas chromatography (17).

**PCR, cloning, and sequencing.** DNA was isolated from each strain by phenol extraction (18). The 16S rRNA gene and the 16S-23S intergenic spacer region (ISR) of 16 isolated CS2-hydrolyzing bacterial strains were amplified by hot-start PCR with the Go Taq Green buffer system (Fermentas) with 2.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates, 1 μl bovine serum albumin, 0.4 μM each primer (see Table S1 in the supplemental material), and 1 μl Taq polymerase (Fermentas). The PCR protocol consisted of 2 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 54 to 65°C, and 2 min at 72°C; and a final elongation step of 10 min at 72°C. PCR products were ligated into pGEM-T Easy (Promega) and transformed into Escherichia coli strain TOP10 (Invitrogen) according to the manufacturer’s instructions. Plasmids with a correct insert were Sanger sequenced by the following method (19) in MEGA 4.0 (20) with the following settings: maximum composite likelihood nucleotide substitution model, gaps and missing data eliminated, transitions and transversions included. Sequences of the ribosomal operon of A. thiooxidans ATCC 19377 and A. caldus ATCC 51756 were kindly made available by Jorge Valdés and David Holmes, CBGB, Santiago, Chile (21).

**Cryo-SEM.** Cryo-scanning electron microscopy (cryo-SEM) was used to study the morphology of colonies growing on 1% Geltite plates acidified with 0.018 M H2SO4 by a method similar to that described in reference 22. Blocks containing colonies were cut out of the Geltite, mounted on electroconductive aqueous colloidal graphite (DAG; Agar Scientific) on a mounting stub, and quickly frozen by submersion in degassed liquid N2 (−196°C). While under vacuum, the sample was transferred to the Gatan cryotransfer box. Sections through colonies were made with a razor blade at this stage when required. The temperature was subsequently increased to −100°C to sublime off water that had settled on top of the specimen by condensation for a period of 5 min. When all of the surface water had been removed, the temperature was reduced to between −100 and −150°C. The sample was then sputter coated for 45 s with a mixture of 60% gold and 40% palladium and transferred to a JEOL 6330F scanning electron microscope.

**Yield determinations.** For total carbon measurements, 3- to 4-ml reactor samples were centrifuged and the pellets were resuspended in 2 ml 1 mM HCl, pH 3. The washed cells were dried overnight under vacuum at 70°C. The C/N ratio of the dried material (0.3 to 0.4 mg) was determined by elemental analysis with a Thermo Fisher Scientific EA 1110 CHN element analyzer coupled to a Finnigan DELTAPlus mass spectrometer. For protein determinations, 2-ml reactor samples were centrifuged at 4°C for 30 min at 18,000 × g. The pellets were resuspended in 0.5 ml 1 M NaOH, boiled for 5 min, and neutralized with 0.5 ml 1 M HCl. Alternatively, 0.5 ml 1 M NaOH was added directly to 200-μl reactor samples, and after boiling, the mixture was neutralized with 0.3 ml 1 M HCl. Protein concentrations were determined with the Bio-Rad protein assay kit according to the manufacturer’s instructions. Culture density, determined by both cell density and the presence of S0, was measured spectrophotometrically as the optical density at 600 nm (OD600).

**Determination of pH optima.** pH optima were determined by a floating-filter method. Samples taken from the steady-state chemostats (1% H2SO4 measured pH, 0.72) were diluted 106-fold in MM at pH 2, and 1 ml of this dilution was filtered through a sterile, 0.2-μm, 25- or 47-mm-diameter Cyclopore polycarbonate filter (Whatman). Filters were then floated on 20 ml MM acidified with sulfuric acid to pHs of 0.5 to 6. They were placed in airtight jars and incubated at RT for 16 days with a continuous flow of 45 ml min−1 air containing 10 nmol CS2 ml−1. Growth at different pHs was determined by measuring colony diameters and counting colonies. The growth of strain BBW1 was quantified visually, as cells had spread over the filter and over the surface of the medium.

**Cell surface hydrophobicity.** Cell surface hydrophobicity was determined by a modified form of the method of Rosenberg et al. (23). The pH of culture samples from the minichromosats was adjusted to 3 or 7 with PUM buffer (23). The suspensions were diluted to an OD600 of 0.45 (A600) in PUM buffer with the appropriate pH. In a test tube, a 1-ml suspension was mixed with 200 μl n-octane or n-hexane. The mixture was incubated at RT for 10 min, mixed vigorously for 1 min, and left to stand at RT for at least 25 min. OD600 was measured (A600), and the percent adherence to the solvent was calculated with the equation (1 − A600/A600) × 100. The use of OD600 to measure suspension turbidity yielded similar results.

**Preparation of cell extracts.** Cell extracts from steady-state reactor-grown bacterial cells were prepared as follows. Thirty 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 H2O and resuspended in 0.5 ml 20 mM KP at pH 7. Approximately 350 μM glass beads (80- to 110-μm size) were added, and the cells were broken by bead beating for 2 × 2 min at 30 Hz (Retsch) with intermittent cooling on ice. The broken cell mixtures were centrifuged for 5 min at 16,000 × g, and the supernatants were stored at −20°C with a final concentration of 10% glycerol.

**Enzyme kinetics based on H2S measurements.** The Michaelis-Menten constants Km and Vmax were determined for the CS2 conversion rates of cell extracts of the different strains by measuring the H2S production rate with an H2S microsensor (Unisense) in 20 mM HEPES (pH 7) as described previously (15). Experiments were performed at pH 7, as the CS2 hydrolase is predicted to reside in the cytoplasm of the cell because of the absence of a signal sequence at the N terminus of the enzyme. The Km and Vmax values were calculated from Michaelis-Menten plots by nonlinear regression with the Michaelis-Menten equation V = Vmax × [CS2]/(Km + [CS2]). Experiments were repeated at least three times, and average values of three independent experiments ± the standard errors of the means were calculated. The same method was used to determine Vmax values (with 600 μM CS2 as the substrate) for steady-state cells from the
minichemostats \( (D = 0.02) \) diluted 200× in reactor medium (MM containing 1% \( \text{H}_2\text{SO}_4 \)). Rates were normalized to the reactor \( \text{OD}_{600} \).

**Sulfur (S\(^0\)) determination.** Sulfur was determined by a modified form of the method of Sorbo (24) to reduce interference from medium components and loss of sulfur during processing. Poly carbonate filters (0.1-μm Whatman Cyclopore track etched membrane 7060-2501 or Milli-pore Isopore VCTP filters) were rinsed with MilliQ water and placed on a vacuum filter unit. Liquid samples from batch and continuous cultures, as well as bacteria removed from Gelrite plates and resuspended in 0.5 ml sterile demineralized water, were immediately filtered to prevent the bacteria from metabolizing the S\(^0\) before processing. Up to 5 ml culture was carefully loaded directly onto the membrane to prevent S\(^0\) from sticking to the glass of the vacuum unit and filtered under vacuum. Controls consisted of 3 ml MM containing 1% \( \text{H}_2\text{SO}_4 \). The filters were rinsed with 1 ml MilliQ water and inserted into a 2-ml Eppendorf tube, and 1.5 ml 0.1 M KCN was added immediately to stop the cells from metabolizing the S\(^0\). The samples were incubated at 90°C for 10 min and cooled to RT, and 200 μl 0.75 M FeNO\(_3\) in 20% HNO\(_3\) was added. Samples were centrifuged for 5 min at 16,000 × g to pellet precipitate cell debris and filters, and the supernatant absorbance was measured immediately at a wavelength of 460 nm. Standard curves were prepared in the same manner, with a solution of S\(^0\) in acetone (259 mg in 100 ml) diluted in MilliQ water to a final amount of up to 75 μg S\(^0\) per filter.

S\(^0\), \( \text{H}_2\text{S} \), and \( \text{SO}_3\)\(^2-\) accumulation in bacterial cells. The accumulation of intermediates during CS\(_2\) respiration was measured simultaneously but in separate reaction chambers as follows. Samples from the minichemostats were diluted 10-fold with \( \text{O}_2\)-saturated MM containing 1% \( \text{H}_2\text{SO}_4 \). The diluted cultures were used to fill three glass cuvettes containing stir bars, after which the cuvettes were closed and placed in a 22°C water bath for 35 min with vigorous stirring. CS\(_2\) (17 μM) was added to each cuvette, and simultaneous measurements were then made of (i) \( \text{O}_2 \) respiration with an \( \text{O}_2 \) sensor (Unisense), (ii) \( \text{H}_2\text{S} \) production with an \( \text{H}_2\text{S} \) sensor (Unisense), and (iii) \( \text{S}^0 \) production by following the change in OD\(_{480}\) with an Agilent 8453 spectrophotometer. In addition and simultaneously but in separate reaction chambers as follows. Samples from the chemostat, a 100-ml glass syringe containing a stir bar was filled with diluted MM containing 1% \( \text{H}_2\text{SO}_4 \). At the sulfur peak (above) was injected into the cuvette. \( \text{H}_2\text{S} \) production and removal, as well as respiration, were measured simultaneously with an \( \text{H}_2\text{S} \) microsensor (Unisense) and an oxygen sensor (Strathkelvin Instruments), respectively.

**Nucleotide sequence accession numbers.** The 16S ISR sequences determined in this research have been deposited in the GenBank database under accession numbers KC902816 to KC902839 and KC902831.

**RESULTS**

Enrichment and isolation of CS\(_2\)-utilizing bacteria. To obtain extremely acidophilic CS\(_2\)-converting microorganisms, samples from naturally acidic, sulfur-rich environments and from industrial biotrickling filters were incubated in acidified MM and with CS\(_2\) as the sole energy source. Significant CS\(_2\) conversion was observed within a few days, and dense cultures were obtained within 2 to 4 weeks of incubation with 1 to 4% \( \text{H}_2\text{SO}_4 \). Ultimately, some enrichment cultures showed growth at 6% \( \text{H}_2\text{SO}_4 \) (Table 1). This concentration is equal to a theoretical pH of ~0.05. Enrichments containing 4 to 6% \( \text{H}_2\text{SO}_4 \) (Table 1) were used to isolate 16 pure cultures on Gelrite plates (0.1% \( \text{H}_2\text{SO}_4 \)). To reconfirm their acid tolerance, all isolates were successfully transferred to 4% \( \text{H}_2\text{SO}_4 \) medium.

Descriptions of growth characteristics on plate cultures and in liquid cultures are given in the supplemental material and shown in Fig. S1 and S2 in the supplemental material. The 16 isolated strains showed distinctly different colony morphologies, ranging from large, dry, white colonies to smooth, shiny, compact colonies. Stationary-phase liquid cultures showed white or yellow aggregates or little aggregation. From the cryo-SEM analysis of surfaces and cross sections of colonies, we conclude that on solid medium, the compactly growing strains produce more extracellular polymeric substance (EPS) than the spreading strains do.

**Phylogenetic analysis.** All of the new CS\(_2\)-converting isolates were identified as *Acidithiobacillus thiooxidans* strains by conventional 16S rRNA gene analysis (data not shown). Improved discrimination was achieved by analysis of the 16S-23S ISR sequences, containing two tRNA genes and three intergenic transcribed spacers (ITS). The total length of the ISR sequences of the CS\(_2\)-converting isolates varied between 456 and 460 bp, with one exception; isolate BDW2 from the Oy Visko reactor had an ISR of 439 bp (Table S2 in the supplemental material). Within the ISR, most of the variation in nucleotide composition was observed in the third of the three ITS. Strains BAW3 and BBW1, which originated from two parallel reactors operating at the Loudon factory, were distinctly different with respect to their nucleotide composition, differing by 57 bp and harboring three insertions and three deletions compared with reference strain G8. In a neighbor-joining phylogenetic tree constructed from the 16S ISR sequences of the CS\(_2\)-converting strains and other *A. thiooxidans* strains, strains BAW3 and BBW1 form a distinct cluster within the *A. thiooxidans* strains (Fig. 1). Three more clusters could be distinguished, i.e., cluster 2, which did not contain any of the CS\(_2\)-converting strains; cluster 3, comprising most of the CS\(_2\)-converting strains and also *A. albertensis*, described as a distinct species but phylogenetically indistinguishable from *A. thiooxidans* (26); and cluster 4, containing strain S1p from the Solfatarea
Acidithiobacillus strains were able to grow at 4% H2SO4, which corresponds to the highest affinity for CS2 and produce little sulfur under highly acidic conditions. Of these, the three mesophilic Acidithiobacillus species (A. albertensis DSM14366, A. ferrooxidans DSM14882, and A. thiooxidans DSM504 and DSM14887) were not able to convert CS2 during the 2 to 5 days the cultures were monitored. Since a hierarchical utilization of energy sources may still be possible, CS2 utilization cannot be completely ruled out. However, the moderate thermophile A. caldus 8584 was found to be capable of CS2 conversion as soon as CS2 was added to the reactors with the same input), up to and beyond the point where S0 formation started becoming visible in the reactors. Strains S1p and G8 had slightly higher \( \mu_{\text{max}} \) values than the other three strains (Table 2).

### Cell surface hydrophobicity

The differences in growth characteristics on plates and in liquid culture suggested that there may be differences in the surface properties of the strains. To test this, cell surface hydrophobicity was measured by using adherence to n-octane and n-hexane (23). Strains 2Bp and Sts 4-3 had less hydrophobic cell surfaces than strains S1p, G8, and BBW1 (see Fig. S3 in the supplemental material). On plates, strains 2Bp and Sts 4-3 produced compact, smooth, and opaque colonies versus the dry and spread-out colonies of strains S1p, G8, and BBW1. Similar results were obtained when experiments were performed at pH 7 (see Fig. S3A and B) or 3 (see Fig. S3C and D); therefore, a different response to high-pH shock is not the cause of the differences in surface hydrophobicity observed.

### pH optimum and pH tolerance

The pH optima of the five selected strains were determined with a floating-filter assay on media with pHs ranging from 0.5 to 6. The pH optima varied among 1 (2Bp), 1.5 (Sts 4-3 and G8), and 2 (S1p) (Table 2). Strain BBW1 did not form colonies but showed spreading surface growth on the filter and the medium as well. On the basis of visual observations, the pH optimum of this strain was between 1 and 1.5 (Table 2).

Tolerance of different pH levels was estimated by counting the colonies that appeared on the filters after the transfer of cells growing at 1% H2SO4 (measured pH, 0.72) to media with lower or higher pHs. Survival rapidly decreased when cells were exposed to...
more acid (pH 0.5) and more gradually decreased when cells were exposed to higher-than-optimum pHs. Strain BBW1 was the most sensitive to high-pH stress; no growth was observed in medium with a pH higher than 3.5.

Enzyme kinetic analysis.

To assess the CS2 removal efficiencies of the five new CS2-converting A. thiooxidans isolates, the CS2 affinity constants ($K_m$) and maximum CS2 conversion rate ($V_{max}$) of crude protein extracts from continuous cultures were determined by adding a pulse of CS2 to diluted protein extracts and measuring the rate of H2S production. Table 2 shows that the $K_m$ values of the five strains are of the same order of magnitude, ranging between 81 and 130 μM CS2 for strains G8 and 2Bp, respectively. Comparison of the $V_{max}$ values revealed crude extracts from strain S1p to have a consistently higher $V_{max}$ under these conditions than crude extracts from the other four strains tested, resulting in a 2-fold higher $k_{cat}/K_m$ value for S1p. In accordance, the $V_{max}$ for CS2 of steady-state, minichemostat-grown, intact S1p cells was consistently slightly higher than the $V_{max}$ of the other strains (Table 2).

Sulfur production and consumption. (i) Sulfur production.

Production of S0 by CS2-converting acidithiobacilli as an intermediate in the oxidation of H2S to SO4$^{2-}$ can block biotrickling filters and subsequently decrease performance. Therefore, we compared the H2S and S0 production and consumption of the five selected A. thiooxidans strains upon CS2 pulses. When concentrations as low as 1.8 μM CS2 (equivalent to 36 ppm CS2 in the gas phase) were added to a cuvette containing diluted samples from the minichemostats, there was an almost immediate increase in S0, just above the detection level. Peak concentrations of H2S ranged from 0.5 to 1.0 μM under these conditions, and so, S0 formation already started at H2S concentrations below this value. In subsequent experiments, H2S and S0 production was measured simultaneously in one cuvette. Addition of 17 μM CS2 resulted in an immediate and large accumulation of H2S for all strains. Also, S0 formation started almost without delay and continued until virtually all of the H2S had been consumed (Fig. 2). The respiration curves show an initial period of fast respiration when H2S is still present, followed by a sudden decrease in the respiration rate when the cells start respiring solely on S0. This decrease in the respiration rate indicates that the processing of the S intermediates in the cell, and not respiration, is the rate-limiting step. This was observed in all of the strains, but it was most prominent in strain Sts 4-3 (Fig. 2). The chemical reaction of H2S with O2 was less than 2 μM H2S h$^{-1}$ and therefore did not significantly contribute to the observed oxygen consumption rates.

During the period of S0 respiration, we often observed a temporary reduction in the respiration rate. Although it is possible that an intermediate temporarily accumulated that inhibited res-
pitation on S\textsuperscript{0}, at this point, the S\textsuperscript{0} consumption rate did not decrease. Also, although detectable levels of the potentially inhibiting intermediate SO\textsubscript{3}\textsuperscript{2-} were present throughout the experiment, there was no increase at the reduced respiration “bump.” Therefore, the apparent temporary reduction in respiration is more likely due to the sensor being affected by an intermediate at that point in the experiment.

At the point where S\textsuperscript{0} peaks, the H\textsubscript{2}S and CS\textsubscript{2} had been completely consumed and only 30 to 40% of the total oxygen was consumed. Therefore, the remaining O\textsubscript{2} consumption resulted entirely from S\textsuperscript{0} oxidation, if the accumulation of any other intermediates is excluded. With this O\textsubscript{2} consumption, and taking into account that this is an underestimate of the actual O\textsubscript{2} consumption because of a small baseline drift upward of the O\textsubscript{2} sensor during the experiment, we calculated that at least 80% of the S added as CS\textsubscript{2} was converted to S\textsuperscript{0}. This was similar for all of the strains tested (Table 2), and the percentage also remained identical when CS\textsubscript{2} concentrations as low as 1.8 \mu M were added. Below this value, H\textsubscript{2}S formation could not be observed any more, making such calculations impossible.

The rates of S\textsuperscript{0} formation and consumption were calculated from the slopes before and after the apex of the OD\textsubscript{480} trace (Fig. 2). Consistently, S\textsuperscript{0} production (corrected for consumption) was between four times (strains 2Bp, S1p, G8, and BBW1) and nine times (strain Sts 4-3) faster than S\textsuperscript{0} consumption. This matches with a buildup of S\textsuperscript{0} of at least 80% of the total S added, as calculated above from the oxygen respiration. In separate experiments, chemical analysis for S\textsuperscript{0} at the moment OD\textsubscript{480} reached its maximum value consistently showed S\textsuperscript{0} recovery between 60 and 67% (Table 2). Therefore, chemical analysis appeared to underestimate the amount of S\textsuperscript{0} produced during CS\textsubscript{2} respiration. However, both methods of determining S\textsuperscript{0} accumulation upon the application of CS\textsubscript{2} pulses indicated that all of the strains tested produced about equal amounts of extracellular S\textsuperscript{0} under conditions of excess CS\textsubscript{2}.

(ii) Sulfur consumption. Although all of the strains tested behaved similarly in terms of S\textsuperscript{0} production, there were some obvious differences in subsequent S\textsuperscript{0} respiration (Fig. 2); strain Sts 4-3 respired on S\textsuperscript{0} five to eight times more slowly than the other four strains. The H\textsubscript{2}S combined with S\textsuperscript{0} respiration rates (the first part of the respiration curve) showed a maximum 2-fold difference between the strains (Fig. 2 and Table 2). Although strain Sts 4-3 respired more slowly on S\textsuperscript{0}, the respiration rate did not decline until all of the S\textsuperscript{0} had been depleted.

### Table 2: Comparison of CS\textsubscript{2}-converting A. thiooxidans strains 2Bp, Sts 4-3, S1p, G8, and BBW1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>2Bp</th>
<th>Sts 4-3</th>
<th>S1p</th>
<th>G8</th>
<th>BBW1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony morphology</td>
<td>Smooth, domed</td>
<td>Small, smooth, domed</td>
<td>White, dry, compact</td>
<td>White, dry, spreading</td>
<td>White, dry, spreading</td>
</tr>
<tr>
<td>A. thiooxidans 16S ISR cluster (Fig. 1)</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Maximum acid tolerance (% vol/vol sulfuric acid)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>pH optimum</td>
<td>1–5–6</td>
<td>0.5–5</td>
<td>1–6</td>
<td>0.5–6</td>
<td>0.5–3.5</td>
</tr>
<tr>
<td>Cell surface hydrophobicity</td>
<td>Low</td>
<td>Low-intermediate</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Reactor dilution rate at point of S\textsuperscript{0} formation (approximating (\mu_{\text{max}}))</td>
<td>0.09</td>
<td>0.08</td>
<td>0.1</td>
<td>0.1</td>
<td>0.09</td>
</tr>
<tr>
<td>Mean cell extract (K_{\text{m}}) (\muM CS\textsubscript{2}) ± SEM</td>
<td>130 ± 22</td>
<td>97 ± 10</td>
<td>100 ± 5</td>
<td>81 ± 7</td>
<td>116 ± 4</td>
</tr>
<tr>
<td>Mean cell extract (V_{\text{max}}) (\mumol H\textsubscript{2}S min\textsuperscript{-1} mg protein\textsuperscript{-1}) ± SEM</td>
<td>38 ± 3</td>
<td>17 ± 0</td>
<td>48 ± 7</td>
<td>23 ± 1</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>(k_{\text{cat}}/K_{\text{m}}) (\muM\textsuperscript{-1} s\textsuperscript{-1})</td>
<td>3.5</td>
<td>2.1</td>
<td>6.5</td>
<td>3.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Mean whole-cell (V_{\text{max}}) (\mumol H\textsubscript{2}S min\textsuperscript{-1} ml culture\textsuperscript{-1} for OD\textsubscript{600} of 1) ± SEM</td>
<td>11 ± 0</td>
<td>10 ± 0</td>
<td>12 ± 0</td>
<td>8 ± 0</td>
<td>11 ± 0</td>
</tr>
<tr>
<td>Mean calculated S\textsuperscript{0} accumulated (% of total S pulse added) ± SEM</td>
<td>76 ± 4</td>
<td>83 ± 1</td>
<td>82 ± 1</td>
<td>83 ± 1</td>
<td>ND\textsuperscript{a}</td>
</tr>
<tr>
<td>Mean measured S\textsuperscript{0} accumulated (% of total S pulse added) ± SEM</td>
<td>67 ± 2</td>
<td>63 ± 2</td>
<td>65 ± 1</td>
<td>61 ± 1</td>
<td>ND</td>
</tr>
<tr>
<td>Mean respiration rate on H\textsubscript{2}S and S\textsuperscript{0} combined after a 10 \muM CS\textsubscript{2} pulse (nmol O\textsubscript{2} min\textsuperscript{-1} ml culture\textsuperscript{-1} for OD\textsubscript{600} of 1) ± SEM</td>
<td>45 ± 9</td>
<td>43</td>
<td>87 ± 10</td>
<td>101 ± 1</td>
<td>66 ± 7</td>
</tr>
<tr>
<td>Mean respiration rate on S\textsuperscript{0} only after 10 \muM CS\textsubscript{2} pulse (nmol O\textsubscript{2} min\textsuperscript{-1} ml culture\textsuperscript{-1} for OD\textsubscript{600} of 1) ± SEM</td>
<td>41 ± 3</td>
<td>7</td>
<td>63 ± 3</td>
<td>44 ± 1</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>Mean % of S\textsuperscript{0} globules attached to cells after 10 17 \muM CS\textsubscript{2} pulses ± SEM</td>
<td>87 ± 7</td>
<td>17 ± 4</td>
<td>9 ± 2</td>
<td>52 ± 2</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Mean % of cells attached to S\textsuperscript{0} globules after 10 17 \muM CS\textsubscript{2} pulses ± SEM</td>
<td>35 ± 10</td>
<td>13 ± 3</td>
<td>7 ± 2</td>
<td>30 ± 6</td>
<td>13 ± 0</td>
</tr>
<tr>
<td>Resistance to CS\textsubscript{2} stress</td>
<td>Low</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>Speed of recovery after CS\textsubscript{2} starvation</td>
<td>Slow</td>
<td>Slow</td>
<td>Slow</td>
<td>Fast</td>
<td>Fast</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Strains were grown in continuous culture (\(D = 0.02\)) with CS\textsubscript{2} as the sole energy source. Samples were taken from these reactors for the comparison experiments. The \(K_{\text{m}}\) and \(V_{\text{max}}\) of each strain were calculated from Michaelis-Menten plots by nonlinear regression with the Michaelis-Menten equation \(V = V_{\text{max}} \times S/(K_{\text{m}} + S)\). Mean values from at least three independent experiments are shown.

\textsuperscript{b} ND, not determined.
This was in contrast to the other strains, especially strain S1p, which showed a continuous decline in the rate of respiration and concurrent $S^0$ depletion.

We hypothesized that different strains produce differently bioavailable $S^0$; strains 2Bp, G8, and BBW1 produce $S^0$ that can be efficiently metabolized again, whereas strains Sts 4-3 and S1p produce $S^0$ that is difficult to remove. To test this hypothesis, we examined sulfur accumulation by different strains upon the application of repeated CS$_2$ pulses to undiluted chemostat samples by counting $S^0$ globules under a light microscope (Table 2). We counted 160 to 240 cells of each strain, and a representative picture of strains 2Bp, S1p, and G8 is shown in Fig. S4 in the supplemental material. All five of the strains tested showed the accumulation of $S^0$ globules of similar sizes, either attached to the bacteria or loose in the medium (see Fig.S4). There were large differences in the observed cell attachment to $S^0$ globules. Although a correlation between the attachment of cells to $S^0$ globules and cell surface hydrophobicity might be expected, we did not observe this despite the fact that all of the cells had identical physiological backgrounds. Strain S1p clearly had the fewest $S^0$ globules that were attached to cells (9% ± 2%), while in cultures of strain 2Bp, most of the $S^0$ globules were attached to cells (87% ± 7%, Table 2). This supports our hypothesis that S1p produces $S^0$ that appears less bioavailable; attachment of $S^0$ globules to bacteria is required for rapid $S^0$ consumption upon H$_2$S depletion. Strain Sts 4-3, which consumes $S^0$ very slowly, also had a low percentage (13%) of cells attached to $S^0$ globules, and 17% of the $S^0$ globules were attached to cells (Table 2). This percentage was not as low as that of strain S1p, and it therefore cannot entirely explain the very low observed $S^0$ consumption rate of this strain. However, strain Sts 4-3 produced more H$_2$S than the other strains when pulsed with CS$_2$ (25 versus 20 M, Fig. 2), which could have had an inhibitory effect on overall respiration.

**CS$_2$ stress and starvation.** The effect of fluctuations in CS$_2$ concentrations in biofilters can be simulated by applying CS$_2$ pulses to undiluted culture samples from CS$_2$-limited steady-state chemostats in MM with 1% M$_2$SO$_4$. Therefore, chemostat samples of the newly isolated $A$. thiooxidans strains were incubated and subjected to repeated 17 M CS$_2$ pulses. Each new pulse was given when the H$_2$S produced from the previous pulse had been

**FIG 2 Conversion of CS$_2$ by five $A$. thiooxidans strains.** The arrows indicate the times when 17 M CS$_2$ was injected into a cuvette containing cells from a continuous culture growing at $D = 0.02$, diluted $6 \times$ in MM containing 1% H$_2$SO$_4$. Respiration (O$_2$ consumption, black dashed line), H$_2$S production (black solid line), and $S^0$ production (gray line) were monitored. The production and consumption rates in these graphs were used to determine the percentages of $S^0$ accumulation in Table 2. Note the different scale on the $x$ axis of the graph for strain Sts 4-3.
consumed by the cells (after about 1.5 to 3 min). The H$_2$S consumption rates after the first CS$_2$ pulse varied between 1.9 (strain indicated that the strains cope reasonably well with short periods consumption rates and respiratory activity were measured. Results strain 2Bp, where H$_2$S/S$_0$ respiration activity was reduced by 80% and S$_0$ respiration was reduced by 85% below the steady-state stressed when subjected to CS$_2$ loads above the concentration the cells were adapted to in the chemostat. This is probably due to the toxic effect of the repeated buildup of H$_2$S in the cells.

To test the effect of intermittent interruption of the factory CS$_2$ supply, the five strains growing in chemostats as described above were subjected to CS$_2$ starvation by temporarily shutting down the medium and CS$_2$ supply for 24 h. During this period of starvation and the subsequent 24-h recovery period when the CS$_2$ supply was reconnecting again, samples were taken from the chemostats and subjected to a CS$_2$ pulse. Subsequent H$_2$S production and consumption rates and respiratory activity were measured. Results indicated that the strains cope reasonably well with short periods of starvation. After 4 h of CS$_2$ starvation, there was only modest to no reduction in respiration activity when the cells were pulsed with 10 $\mu$M CS$_2$. Of the four strains tested at this time point, G8 appeared to be the most strongly affected (1.0 $\mu$mol H$_2$S liter$^{-1}$ min$^{-1}$ unit of OD$_{600}^{-1}$, 41% of the initial rate) and strain BBW1 appeared to be the least strongly affected (1.5 $\mu$mol H$_2$S liter$^{-1}$ min$^{-1}$ unit of OD$_{600}^{-1}$, 76% of the initial rate, Fig. 3). This indicates that the cells were becoming increasingly stressed when subjected to CS$_2$ loads above the concentration the cells were adapted to in the chemostat. This is probably due to the toxic effect of the repeated buildup of H$_2$S in the cells.

The new Acidithiobacillus isolates even showed slow growth in 6% H$_2$SO$_4$. The constant supply of CS$_2$ to the cultures was the key.
factor in the maintenance of cultures at these very high \( \text{H}_2\text{SO}_4 \) concentrations. Culture activity decreased dramatically upon storage without a substrate at or above 4% \( \text{H}_2\text{SO}_4 \). Acidophiles require a continuous and high supply of maintenance energy to be able to actively pump out protons that leak from the acidic environment into the nearly neutral \( \text{pH} \) cell cytoplasm (29). Operation of biofilters under highly acidic conditions therefore puts additional stress on the microorganisms during periods of fluctuating \( \text{CS}_2 \) concentration and factory shutdown.

Although growth was very slow at 6% \( \text{H}_2\text{SO}_4 \), dense growth was already observed at 4% for all new \( \text{Acidithiobacillus} \) strains. Similar \( \text{H}_2\text{SO}_4 \) tolerance was reported for \( \text{Acidithiobacillus} \) sp. strain AZ11, which is able to respire on elemental sulfur in the presence of 4.2% \( \text{H}_2\text{SO}_4 \) (30). The \( \text{pH} \) optima determined for 5 of the 16 new \( \text{CS}_2 \)-converting strains were between 1 and 2. This is slightly lower than the optimal-pH range of 1.8 to 2.5 described for other \( \text{Acidithiobacillus} \) strains (29, 31).

Growth at around \( \text{pH} 0 \), as observed for 15 of our \( \text{A. thiooxidans} \) isolates, is among the lowest \( \text{pHs} \) reported in the literature for any organism. \( \text{Ferroplasma acidarmanus} \) (32), \( \text{Picrophilus oshimae} \), and \( \text{Picrophilus torridus} \) could grow at \( \text{pH} 0 \) (optimum \( \text{pH}, 0.7 \)) (33). \( \text{Picrophilus torridus} \) adapted to growth at \( \text{pH} 0.1 \) even showed significant growth at 1.2 M \( \text{H}_2\text{SO}_4 \) (\( \text{pH} -0.06 \)) (34). The eukaryotic red alga \( \text{Cyanidium caldarium} \) was cultured at 0.5 M \( \text{H}_2\text{SO}_4 \) (35), and the green alga \( \text{Dunaliella acidophila} \) is able to survive \( \text{pH} 0.2 \) (36). Also, some fungal species were reported to grow at \( \text{pH} 0 \), i.e., \( \text{Acontium cylatium} \) (37), \( \text{Cephalosporium sp.} \), and \( \text{Trichosporon cerebriae} \) (38). We also observed fungal growth at 6% \( \text{H}_2\text{SO}_4 \) (\( \text{pH} -0.05 \)) in our enrichment cultures but did not further investigate the species present in these cultures. With growth at \( \text{pH} \) as low as \( -0.05 \) (6% \( \text{H}_2\text{SO}_4 \)), the new \( \text{Acidithiobacillus} \) isolates obtained in this research exceed the previously reported \( \text{pH} \) limit of 0.5 for microbial \( \text{CS}_2 \) conversion for \( \text{Acidithiobacillus} \) TJ330 (9).

The newly isolated \( \text{CS}_2 \)-converting \( \text{A. thiooxidans} \) strains differed in colony morphology. The genus \( \text{Acidithiobacillus} \) comprises a physiologically and genetically heterogeneous group of microorganisms (39, 40), despite the often low sequence diversity in the 16S rRNA gene. For that reason, the 16S-23S ISR is used to discriminate at the intra species level (41, 42).

Two main colony types on Gelrite plates were distinguished, compact, creamy, shiny colonies and dry, white, spreading colonies (40). Reversible variation in colony morphology of several \( \text{A. ferroxidans} \) strains has been described, resulting in the same large, white spreading colonies, as opposed to compact colonies, as observed here for some of the newly isolated \( \text{A. thiooxidans} \) strains (43, 44). \( \text{A. thiooxidans} \) strains might be motile via a polar flagellum (40). \( \text{A. ferroxidans} \) ATCC 19859 spreading variants displayed increased motility and chemotaxis toward thioulate, which may be a selective advantage over biofilm growth during periods of low substrate concentrations. These variants arose through the rearrangement of insertion sequences in the genome, potentially acting as a genetic switch (44). The \( \text{A. ferroxidans} \) group of strains has recently been reclassified into four separate species (45). Of these, only \( \text{A. ferrivorans} \) and some \( \text{A. ferriaridus} \) strains were shown to be motile (45). The type strain \( \text{A. ferroxidans} \) ATCC 23270 lacks flagellum and chemotaxis genes (46). The draft genome of \( \text{A. thiooxidans} \) ATCC 19377 (47) and the draft genomes of both \( \text{A. thiooxidans} \) strains S1p and G8 (Daan Speth, personal communication) do contain the operons for flagellum biosynthesis and chemotaxis. However, we did not observe swimming motility in liquid cultures of our strains when we examined them microscopically. Therefore, the mechanism and role of the spreading colony phenotype in these strains are not clear.

SEM studies of frozen colonies indicated that the difference in colony appearance may be caused by the absence of a clear EPS layer on the dry white colonies, as has been observed for colony morphology mutants of \( \text{Mycobacterium smegmatis} \) (22). In support, \( \text{A. thiooxidans} \) strains S1p, G8, and BBW1, which produced white dry colonies, had a considerably higher cell surface hydrophobicity than strains 2Bp and Sts 4-3, which produced compact shiny colonies, suggesting the presence of relatively more hydrophilic compounds on the cell surface of the latter strains. \( \text{Acidithiobacillus} \) strains produce EPS containing both neutral sugars and fatty acids (48, 49), the proportion varying depending on the substrate the cells are grown on (50, 51). The EPS of \( \text{A. thiooxidans} \) grown on \( \text{S}_0 \) consists of 40% sugars and 60% fatty acids (mainly eicosanoic acid) (50). The fatty acids are released from the outer membrane by blebbing (52) and cause the “wetting” of sulfur particles described in 1961 by Jones and Starkey (53), making \( \text{S}_0 \) available as an energy source. In addition, EPS is essential for successful attachment and bioleaching of \( \text{A. ferroxidans} \) to pyrite (50). Differences in EPS production and potential motility observed in our strains may have implications for the degree of colonization and clogging of biofilters.

Efficient biofiltration of \( \text{CS}_2 \)-contaminated air streams requires microorganisms with a higher affinity for \( \text{CS}_2 \) than the concentration present in the air stream, to ensure its removal to concentrations complying with increasingly stringent regulations, as well as resistance to changes in operating conditions, low biomass production, low \( \text{S}_0 \) accumulation, and rapid \( \text{S}_0 \) removal to prevent clogging of the biofilters. The affinities of the five strains tested for \( \text{CS}_2 \) were similar (around 100 \( \mu \text{M} \)) and correspond to that reported for the purified \( \text{CS}_2 \) hydrolase of \( \text{Acidamus} \) A1-3 (10). Comparison of the \( V_{\text{max}} \) values revealed strain S1p to have a consistently higher \( V_{\text{max}} \) under these conditions than the other four strains tested, possibly because it has a relatively larger amount of \( \text{CS}_2 \) hydrolase present in the cells than the other strains (15). The \( V_{\text{max}} \) is only reached at substrate concentrations of 200 \( \mu \text{M} \) (\( \text{nmol mL}^{-1} \)) or higher. Bioreactors treating \( \text{CS}_2 \)-contaminated air from the viscose industry are operational at much lower concentrations of around 4 to 20 \( \mu \text{mol mL}^{-1} \) (100 to 500 ppm) (2), indicating that although strain S1p converts \( \text{CS}_2 \) with a higher \( V_{\text{max}} \) than the other strains, it would not usually reach its full potential reaction rate in a bioreactor. Indeed, a higher \( V_{\text{max}} \) may even be deleterious, as it might cause \( \text{H}_2\text{S} \) to accumulate to toxic levels in the cell more rapidly upon \( \text{CS}_2 \) peaks.

Comparison of the resistance to changes in operating conditions in the form of an interruption in the \( \text{CS}_2 \) supply revealed that all five strains were affected by 24 h of \( \text{CS}_2 \) starvation in terms of the ability the reprise after a \( \text{CS}_2 \) pulse during starvation, but they all recovered within 24 h after reconnection of the \( \text{CS}_2 \) supply. However, during recovery, all five strains will also produce \( \text{S}_0 \), as we found in all of the strains tested that \( \text{CS}_2 \) pulses during starvation resulted in the transient accumulation of 80% of the total \( \text{S} \) added as \( \text{S}_0 \), independently of the amount of \( \text{CS}_2 \) added. This implies that it will be difficult to avoid \( \text{S}_0 \) accumulation in biofiltration systems with uneven \( \text{CS}_2 \) loading. However, differences in \( \text{S}_0 \) removal were observed, with strain Sts 4-3 having a much lower but constant \( \text{S}_0 \) removal rate than the other strains and strain S1p.
having a long period in which the \( S^0 \) respiration rate slowly declined. Whatever the cause, the strains that remove \( S^0 \) more slowly will cause more clogging problems in bioreactors because of \( S^0 \) accumulation.

In summary, we have successfully isolated extremely acidophilic, \( \text{CS}_2 \)-converting \( A. \text{thiooxidans} \) strains from both environmental and industrial ecosystems that grow optimally around pH 1 to 2 and can grow at sulfuric acid concentrations of up to 6% (vol/vol). Currently bioreactors are operated at pH 0.5 to 1 (2). Use of the new strains would reduce water use and improve the prospect for reuse of the produced sulfuric acid in the rayon/viscose industry. The isolated strains displayed different growth and colony morphology characteristics, which may be due to differences in motility and/or the presence or absence of an EPS layer surrounding the cells. To circumvent the bottlenecks in the biofiltration of \( \text{CS}_2 \), cocultures of extremely acidophilic \( A. \text{thiooxidans} \) strains to combine the best acid tolerance, affinity for \( \text{CS}_2 \), \( S^0 \)-removing potential, and stability during periods of fluctuating \( \text{CS}_2 \) loads are most promising for inoculation of industrial biofilters. This application is expected to result in reduced sulfur accumulation, increased \( \text{CS}_2 \) removal rates, reduced water consumption, a more stable operation, and recycling of the sulfuric acid produced.

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