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 thiooxidans 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 hydrolyase 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 (1). 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 comes 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) 1% [vol/vol] is 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.

Received 1 July 2013 Accepted 25 August 2013
Published ahead of print 30 August 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02167-13.
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equivalent to 1.8% [wt/vol] and 0.18 M sulfuric acid). Strains were also
grown in minichemostat 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 sys-
tem (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 enrich-
ments to MM with higher H2SO4 concentrations. Pure cultures were ob-
tained from enrichment cultures grown in 4 to 6% H2SO4 on Gelrite
plates. Single colonies were serially transferred three times to fresh plates
and checked microscopically for purity. Fungal contamination (present
mainly in the enrichments from industrial samples) was eliminated by
adding 150 μg ml−1 chlorothalonil either to the plates or to liquid cul-
tures. The H2SO4 tolerance of each isolated strain was confirmed by re-
peated subculturing at least three times in liquid MM containing 4 to 5% H2SO4.

**Screening of known Acidithiobacillus strains for CS2 conversion ca-

cacity.** 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. ferroxi-
dans* DSM14882 (882), *A. caldus* DSM8584 (150a), *A. thioxidans*
DSM14887 (71), *A. thiioxidans* 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 (Fermenta-
tas) 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 supple-
mental material), and 1 μl Taq polymerase (Fermentas). The PCR proto-
ocol 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 trans-
formed into *Escherichia coli* strain TOP10 (Invitrogen) according to the
manufacturers’ instructions. Plasmids with a correct insert were Sanger
sequenced by the following facility at the Department of Human Genet-
ics of the University Medical Center St. Radboud, Nijmegen, the Neth-
erlands. 16S ISR contigs were assembled by using Contig Express (Vector
NTI; Invitrogen), and phylogenetic relationships were inferred by the
neighbor-joining method (19) in MEGA 4.0 (20) with the following set-
tings: maximum composite likelihood nucleotide substitution model,
gaps and missing data eliminated, transitions and transversions included,

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 float-
ing-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
placed 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 contin-
uous flow of 45 ml min−1 air containing 10 nmol CS2 ml−1. Growth at
different pHs was determined by measuring colony diameters and count-
ing 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 deter-
mined by a modified form of the method of Rosenberg et al. (23). The pH
of culture samples from the minichemostats 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 − A/A600) × 100. The use of
OD600 to measure suspension turbidity yielded similar results.

**Preparation of cell extracts.** Cell extracts from steady-state reac-
tor-grown bacterial cells were prepared as follows. Thirty to 50 ml was re-
moved 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 resus-

cipated by measuring the H2S production in the media suggested (DSMZ medium number in
parentheses after the strain name) or as otherwise stated, i.e., *A. ferroxi-
dans* DSM14882 (882), *A. caldus* DSM8584 (150a), *A. thiioxidans*
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neighbor-joining method (19) in MEGA 4.0 (20) with the following set-
ings: maximum composite likelihood nucleotide substitution model,
gaps and missing data eliminated, transitions and transversions included,

uniform rates among sites, and homogeneous pattern among lineages.

**Enzyme kinetics based on H2S measurements.** The Michaelis-Men-
ten 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 hydrolyase 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 nonlin-
er regression with the Michaelis-Menten equation

\[ V = \frac{V_{\text{max}} \times [CS2]}{K_{\text{m}} + [CS2]} \]

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

November 2013 Volume 79 Number 21 aem.asm.org 6785

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minichemostats ($D = 0.02$) diluted 200 × in reactor medium (MM containing 1% $\text{H}_2\text{SO}_4$). Rates were normalized to the reactor OD$_{600}$.

**Sulfur ($S_\text{O}$)** 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 Cycloparen track etched membrane 7060-2501 or Millipore Isopore VCTP filters) were rinsed with Milli-Q 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 $S_\text{O}$ before processing. Up to 5 ml culture was carefully loaded directly onto the membrane to prevent $S_\text{O}$ from sticking to the glass of the vacuum unit and filtered under vacuum. Controls consisted of 3 ml ML containing 1% $\text{H}_2\text{SO}_4$. The filters were rinsed with 1 ml Milli-Q 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 $S_\text{O}$. The samples were incubated at 90°C for 10 min and cooled to RT, and 200 μl 0.75 M Fe(NO$_3$)$_3$ 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 $S_\text{O}$ in acetone (259 mg in 100 ml) diluted in MilliQ water to a final amount of up to 75 μg $S_\text{O}$ per filter.

$S_\text{O}$, $H_2S$, and $SO_3^{2-}$ 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 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) O$_2$ respiration with an O$_2$ sensor (Unisense), (ii) $H_2S$ production with an $H_2S$ sensor (Unisense), and (iii) $S_\text{O}$ production by following the change in OD$_{480}$ with an Agilent 8453 spectrophotometer. In addition and simultaneously, a 100-ml glass syringe containing a stir bar was filled with diluted culture and incubated in a 22°C water bath for 35 min with continuous stirring. CS$_2$ was also added to the syringe, and at intervals during the respiration and $H_2S$ production curves, samples were pushed out of the syringe into an Eppendorf tube and frozen immediately for subsequent $SO_3^{2-}$ determination.

In order to study $H_2S$ and $S_\text{O}$ formation kinetics during CS$_2$ respiration in more detail, these parameters were also simultaneously measured in one cylindrical cuvette with three entry ports, which was positioned in a single-beam spectrophotometer. Two ports were used for $O_2$ and $H_2S$ sensors, and the third served for the addition of CS$_2$.

The percentage of $S_\text{O}$ accumulated was calculated as follows. The total O$_2$ consumption is represented by the formula $H_2S + 2O_2 \rightarrow H_2SO_4$. In a respiration curve, the total O$_2$ consumed (A) will depend on the total amount of $H_2S$ (produced from CS$_2$), and so $A/2$ represents the amount of sulfate oxidized to sulfate. Sulfur $S_\text{O}$ is an intermediate and is oxidized according to the formula $S_\text{O} + 1.5SO_3^{2-} + H_2O \rightarrow H_2SO_4$. At the sulfur peak ($A_{SO_3}$), only $S_\text{O}$ is present and the amount of oxygen consumed (a) until then is the end represents sulfur oxidation, and so $a/1.5$ represents the amount of $S_\text{O}$ present at the peak. This gives a $S_\text{O}$/total sulfide ratio of $[a/1.5]/[A/2] = 1.33 \times a/A$, where A is the fraction of the oxygen consumption after the sulfur peak compared to the total consumption, determined from the respiration curve.

**Sulfite ($SO_3^{2-}$)** determination. Sulfite ($SO_3^{2-}$) was measured by the method described by Truemp and Schlegel (25) but with reduction of the amount of $H_2SO_4$ in the assay to take into account the amount of $H_2SO_4$ that is present in the samples (in these experiments, 1% [vol/vol]). Dilutions of an anaerobic 1 M stock of Na$_2$SO$_3$ were used as standards.

**Starvation experiments.** Starvation experiments were performed in steady-state chemostats by stopping the influent and effluent pumps and removing the CS$_2$ supply. One-milliliter samples were removed at regular intervals from the reactors during starvation and recovery, diluted 10 × in MM containing 1% [vol/vol] $H_2SO_4$ and incubated in a 7.63-ml double-port cuvette at 22.0 to 22.2°C. Ten minutes after the sample was removed from the chemostat, a 10 μM CS$_2$ pulse from a 6 mM stock bottle (see above) was injected into the cuvette. $H_2S$ production and removal, as well as respiration, were measured simultaneously with an $H_2S$ 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 KC902829 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% $H_2SO_4$. Ultimately, some enrichment cultures showed growth at 6% $H_2SO_4$ (Table 1). This concentration is equal to a theoretical pH of ~0.05. Enrichments containing 4 to 6% $H_2SO_4$ (Table 1) were used to isolate 16 pure cultures on Gelrite plates (0.1% $H_2SO_4$). To reconfirm their acid tolerance, all isolates were successfully transferred to 4% $H_2SO_4$ 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 rRNA 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 (see 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 Solfatara...
(Rome, Italy), strain BC6-1, and strain BDW2 from the Oy Visko plant.

**Screening of Acidithiobacillus reference strains for CS₂ conversion.** To check whether CS₂ conversion is a general trait of acidithiobacilli under our growth conditions, we tested four publicly available Acidithiobacillus species. 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 DSM504 was found to be capable of CS₂ conversion as soon as CS₂ was added to a growing culture.

**Maximum acid tolerance.** The maximum acid tolerance of all 16 isolates in batch cultures was determined by serially transferring the strains to medium containing gradually higher concentrations of H₂SO₄. The maximum H₂SO₄ concentration in which growth could be observed within 2 months was taken as the growth limit of each organism. Table 1 shows that all of the isolated strains were able to grow at 4% H₂SO₄, which corresponds to a pH of 0.12. All of the strains but G8 grew at 5% H₂SO₄ (pH 0.03), and several of the strains isolated from industrial biofilters still showed growth at 6% H₂SO₄ (pH ~0.05). Growth at concentrations higher than 4% H₂SO₄ was slow; it took up to 2 to 3 months to reach an OD₆₀₀ of about 0.1 at 6% H₂SO₄.

**Chemostat growth of CS₂-converting A. thiooxidans strains 2Bp, Sts 4-3, S1p, G8, and BBW1.** To identify the strains that have the highest affinity for CS₂ and produce little sulfur under highly acidic conditions, 5 of the 16 isolated CS₂-converting A. thiooxidans strains were selected for further comparison. Selection was based on sample location, different genetic groups as determined by 16S ISR analysis (Fig. 1), and different colony morphologies on plates. The five strains were grown in continuous culture under identical conditions in MM with 1% H₂SO₄. The strains had comparable growth yields, as determined by comparing OD, total protein, and total carbon measurements (see Table S3 in the supplemental material). The OD of strain Sts 4-3 was consistently lower than that of the other four strains, probably because of the observed smaller amount of elemental sulfur in the culture.

The μ_{\text{max}} values of the strains growing under these conditions were determined by increasing the medium flow and simultaneously increasing the [CS₂] supplied to the reactors with the same factor (resulting in a constant CS₂ load per milliliter of medium input), up to and beyond the point where S⁰ formation started becoming visible in the reactors. Strains S1p and G8 had slightly higher μ_{\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

### TABLE 1 Comparison of isolated strains of CS₂-converting microorganisms

<table>
<thead>
<tr>
<th>Sample type and origin</th>
<th>Sample pH</th>
<th>Strain</th>
<th>% H₂SO₄ (vol/vol)</th>
<th>Enrichment</th>
<th>Max</th>
<th>Colony morphology</th>
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<td></td>
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<td>Various hot springs</td>
<td>3.1</td>
<td>2Ap</td>
<td>2</td>
<td>5</td>
<td></td>
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<tr>
<td></td>
<td>2.5</td>
<td>2Bp</td>
<td>4</td>
<td>5</td>
<td></td>
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<tr>
<td></td>
<td>1.9</td>
<td>1Bp</td>
<td>3</td>
<td>5</td>
<td></td>
<td>Cream colored, spread out, large</td>
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<tr>
<td>Solfatara, Italy</td>
<td>1.9</td>
<td>S1p</td>
<td>4</td>
<td>5</td>
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<td>0.3</td>
<td>Sts 4-3</td>
<td>4</td>
<td>5</td>
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<td>Fabelta</td>
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<td>G8</td>
<td>ND</td>
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<td>5</td>
<td>6</td>
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<td>Cream-brown, small</td>
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<td>BAW3</td>
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<td>6</td>
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<td>3.2</td>
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<td>6</td>
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<td></td>
<td>3.2</td>
<td>BBW1</td>
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<td>6</td>
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<td>3.9</td>
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<td>5</td>
<td></td>
<td>Shiny, cream colored</td>
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<td>Od Visko</td>
<td>1.2</td>
<td>BDW2</td>
<td>5</td>
<td>6</td>
<td></td>
<td>White, spread out, large</td>
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<tr>
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<td>BEF1</td>
<td>5</td>
<td>5</td>
<td></td>
<td>Cream to brown, small</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>BEF3</td>
<td>5</td>
<td>5</td>
<td></td>
<td>Cream to brown, small</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>BED2</td>
<td>5</td>
<td>5</td>
<td></td>
<td>Cream to brown, small</td>
</tr>
</tbody>
</table>

---

(Rome, Italy), strain BC6-1, and strain BDW2 from the Oy Visko plant.

**Screening of Acidithiobacillus reference strains for CS₂ conversion.** To check whether CS₂ conversion is a general trait of acidithiobacilli under our growth conditions, we tested four publicly available Acidithiobacillus species. 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 DSM504 was found to be capable of CS₂ conversion as soon as CS₂ was added to a growing culture.

**Maximum acid tolerance.** The maximum acid tolerance of all 16 isolates in batch cultures was determined by serially transferring the strains to medium containing gradually higher concentrations of H₂SO₄. The maximum H₂SO₄ concentration in which growth could be observed within 2 months was taken as the growth limit of each organism. Table 1 shows that all of the isolated strains were able to grow at 4% H₂SO₄, which corresponds to a pH of 0.12. All of the strains but G8 grew at 5% H₂SO₄ (pH 0.03), and several of the strains isolated from industrial biofilters still showed growth at 6% H₂SO₄ (pH ~0.05). Growth at concentrations higher than 4% H₂SO₄ was slow; it took up to 2 to 3 months to reach an OD₆₀₀ of about 0.1 at 6% H₂SO₄.

**Chemostat growth of CS₂-converting A. thiooxidans strains 2Bp, Sts 4-3, S1p, G8, and BBW1.** To identify the strains that have the highest affinity for CS₂ and produce little sulfur under highly acidic conditions, 5 of the 16 isolated CS₂-converting A. thiooxidans strains were selected for further comparison. Selection was based on sample location, different genetic groups as determined by 16S ISR analysis (Fig. 1), and different colony morphologies on plates. The five strains were grown in continuous culture under identical conditions in MM with 1% H₂SO₄. The strains had comparable growth yields, as determined by comparing OD, total protein, and total carbon measurements (see Table S3 in the supplemental material). The OD of strain Sts 4-3 was consistently lower than that of the other four strains, probably because of the observed smaller amount of elemental sulfur in the culture.

The μ_{\text{max}} values of the strains growing under these conditions were determined by increasing the medium flow and simultaneously increasing the [CS₂] supplied to the reactors with the same factor (resulting in a constant CS₂ load per milliliter of medium input), up to and beyond the point where S⁰ formation started becoming visible in the reactors. Strains S1p and G8 had slightly higher μ_{\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 (*Kₘ*ₗ) and maximum CS₂ conversion rate (*Vₘₚₓ*) 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 *Kₘ* 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 *Vₘₚₓ* values revealed crude extracts from strain S1p to have a consistently higher *Vₘₚₓ* under these conditions than crude extracts from the other four strains tested, resulting in a 2-fold higher *kₗₚₓ/Kₘ* value for S1p. In accordance, the *Vₘₚₓ* of steady-state, minichemostat-grown, intact S1p cells was consistently slightly higher than the *Vₘₚₓ* 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 SO₄²⁻ 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-
TABLE 2 Comparison of CS₂-converting *A. thiooxidans* strains 2Bp, Sts 4-3, S1p, G8, and BBW1a

<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><em>A. thiooxidans</em> 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</td>
<td>1.5</td>
<td>2</td>
<td>1.5</td>
<td>1–1.5</td>
</tr>
<tr>
<td>pH tolerance</td>
<td>0.5–6</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 S₀ formation</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 $K_m$ ($\mu M$ S₀) ± 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 $V_{max}$ ($\mu M$ H₂S min⁻¹ mg protein⁻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>$k_{cat}/K_m$ ($\mu M$⁻¹ s⁻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 $V_{max}$ ($\mu M$ H₂S min⁻¹ ml culture⁻¹ for OD₆₀₀ 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 S₀ 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>NDb</td>
</tr>
<tr>
<td>Mean measured S₀ 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 H₂S and S₀ combined after a 10 µM CS₂ pulse (nmol O₂ min⁻¹ ml culture⁻¹ for OD₆₀₀ 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 S₀ only after 10 µM CS₂ pulse (nmol O₂ min⁻¹ ml culture⁻¹ for OD₆₀₀ 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 S₀ globules attached to cells after 10 17 µM CS₂ 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 S₀ globules after 10 17 µM CS₂ 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 CS₂ stress</td>
<td>Low</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>Speed of recovery after CS₂ starvation</td>
<td>Slow</td>
<td>Slow</td>
<td>Slow</td>
<td>Fast</td>
<td>Fast</td>
</tr>
</tbody>
</table>

a Strains were grown in continuous culture (D = 0.02) with CS₂ as the sole energy source. Samples were taken from these reactors for the comparison experiments. The $K_m$ and $V_{max}$ of each strain were calculated from Michaelis-Menten plots by nonlinear regression with the Michaelis-Menten equation $V = V_{max} \times S/(K_m + S)$. Mean values from at least three independent experiments are shown.

b ND, not determined.

piration on S₀, at this point, the S₀ consumption rate did not decrease. Also, although detectable levels of the potentially inhibiting intermediate SO₃²⁻ 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 S₀ peaks, the H₂S and CS₂ had been completely consumed and only 30 to 40% of the total oxygen was consumed. Therefore, the remaining O₂ consumption resulted entirely from S₀ oxidation, if the accumulation of any other intermediates is excluded. With this O₂ consumption, and taking into account that this is an underestimate of the actual O₂ consumption because of a small baseline drift upward of the O₂ sensor during the experiment, we calculated that at least 80% of the S added as CS₂ accumulated as S₀. This was similar for all of the strains tested (Table 2), and the percentage also remained identical when CS₂ concentrations as low as 1.8 µM were added. Below this value, H₂S formation could not be observed any more, making such calculations impossible.

The rates of S₀ formation and consumption were calculated from the slopes before and after the apex of the OD₄₈₀ trace (Fig. 2). Consistently, S₀ production (corrected for consumption) was between four times (strains 2Bp, S1p, G8, and BBW1) and nine times (strain Sts 4-3) faster than S₀ consumption. This matches with a buildup of S₀ of at least 80% of the total S added, as calculated above from the oxygen respiration. In separate experiments, chemical analysis for S₀ at the moment OD₄₈₀ reached its maximum value consistently showed S₀ recovery between 60 and 67% (Table 2). Therefore, chemical analysis appeared to underestimate the amount of S₀ produced during CS₂ respiration. However, both methods of determining S₀ accumulation upon the application of CS₂ pulses indicated that all of the strains tested produced about equal amounts of extracellular S₀ under conditions of excess CS₂.

(ii) Sulfur consumption. Although all of the strains tested behaved similarly in terms of S₀ production, there were some obvious differences in subsequent S₀ respiration (Fig. 2); strain Sts 4-3 respired on S₀ five to eight times more slowly than the other four strains. The H₂S combined with S₀ 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 S₀, the respiration rate did not decline until all of the S₀ had been depleted.
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, Sts 4-3, 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 $\mu$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% H$_2$SO$_4$. Therefore, chemostat samples of the newly isolated A. thiooxidans strains were incubated and subjected to repeated 17 $\mu$M CS$_2$ pulses. Each new pulse was given when the H$_2$S produced from the previous pulse had been
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 BBW1) and 3.4 µmol H$_2$S liter$^{-1}$ min$^{-1}$ unit of OD$_{600}^{-1}$ (strain G8) and decreased for all of the strains after repeated CS$_2$ pulses, with strain 2Bp appearing to be the most strongly affected (1.0 µmol H$_2$S liter$^{-1}$ min$^{-1}$ unit of OD$_{600}^{-1}$, 41% of the initial rate) and strain BBW1 appearing to be the least strongly affected (1.5 µmol 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.

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 reconnected 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 µM CS$_2$. Of the four strains tested at this time point, G8 appeared to be the most strongly affected, with a 40% reduction of its initial respiration activity, when both H$_2$S and S$^0$ were present and a 22% reduction in S$^0$ respiration activity (Fig. 4). After 24 h of CS$_2$ starvation, all of the strains showed reduced respiratory activity. Although not tested extensively, the reduction was largest for 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 rates. Strain 2Bp also seemed to recover slowly from starvation, whereas the other four strains showed a doubling of initial respiratory activity back to steady-state levels after only 4 h of resumed CS$_2$ supply, the activity of strain 2Bp increased only 1.4-fold to 29% of the steady-state level. This slow recovery corresponds to the observation described above that strain 2Bp suffers more from CS$_2$ stress than the other four strains do. At 24 h of recovery, the culture density of all five strains was higher than during steady state. This indicates that the cultures had started growing and/or had S$^0$ present.

**DISCUSSION**

Our approach to the enrichment of extremely acidophilic CS$_2$-converting microorganisms from volcanic regions and CS$_2$-converting trickling filters proved successful. Conversion of CS$_2$ was observed within days after inoculation. Furthermore, the enrichments and isolated *Acidithiobacillus* strains could grow at very high H$_2$SO$_4$ concentrations. The only acidophilic CS$_2$-converting *Acidithiobacillus* strain described to date, TJ330, was isolated from an acidic (pH 6.1 to 1.4) CS$_2$- and H$_2$S-treating peat biofilter (9). The neutrophilic CS$_2$-converting species *Paracoccus denitrificans* was isolated from oak leaves and soil beneath the leaf canopy (7, 27). Our attempt to enrich acidophilic microorganisms from soil samples taken under an oak tree and from compost was unsuccessful. Of the *Acidithiobacillus* strains from culture collections screened for CS$_2$ conversion (at micromolar concentrations), only *A. caldus* DSM 8584 was positive. This trait was not previously reported for this species. Our screening confirmed the observation of Smith and Kelly (28), although they used a potentially toxic liquid CS$_2$ concentration (2 mM).

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 component for the successful enrichment under these conditions.

**FIG 3** Effect of repeated addition of CS$_2$ on the H$_2$S consumption capacity of minichemostat-grown cells of *A. thiooxidans* strains 2Bp, Sts 4-3, S1p, G8, and BBW1. Reactor samples were starved for 5 min in a cuvette, and 10 pulses of 17 µM CS$_2$ were sequentially injected. Strain Sts 4-3 was starved for 15 min before the first injection. The experiment was repeated three times independently, and data from a representative experiment are shown as percentages of the H$_2$S consumption rate after the first CS$_2$ pulse. Measurements were done with an H$_2$S sensor, and rates (in nanomoles of H$_2$S per minute per milliliter of culture) were normalized to a culture OD$_{600}$ of 1.

**FIG 4** Effects of CS$_2$ starvation and recovery on respiration rates $\pm$ the standard error of the mean ($n = 3$) on internally produced H$_2$S and S$^0$ (A) and on only S$^0$ (B) after a CS$_2$ pulse. The experiment was performed with five CS$_2$-converting *A. thiooxidans* strains (2Bp, Sts 4-3, S1p, G8, and BBW1), all growing simultaneously in five identically run chemostats. Rates are given in nanomoles of O$_2$ per minute per milliliter of culture. Note that the respiration rates of strain 2Bp were monitored only at steady state, after 24 h of starvation, and after 4 h of recovery.
factor in the maintenance of cultures at these very high H2SO4 concentrations. Culture activity decreased dramatically upon storage without a substrate at or above 4% H2SO4. 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 pH cell cytoplasm (29). Operation of biofilters under highly acidic conditions therefore puts additional stress on the microorganisms during periods of fluctuating CS2 concentration and factory shutdown.

Although growth was very slow at 6% H2SO4, dense growth was already observed at 4% for all new Acidithiobacillus strains. Similar H2SO4 tolerance was reported for Acidithiobacillus sp. strain AZ11, which is able to respire on elemental sulfur in the presence of 4.2% H2SO4 (30). The pH optima determined for 5 of the 16 new CS2-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 Acidithiobacillus strains (29, 31).

Growth at around pH 0, as observed for 15 of our A. thiooxidans isolates, is among the lowest pHs reported in the literature for any organism. Ferroplasma acidarmanus (32), Picrophilus oshimae, and Picrophilus torridus could grow at pH 0 (optimum pH, 0.7) (33). Picrophilus torridus adapted to growth at pH 0.1 even showed significant growth at 1.2 M H2SO4 (pH −0.06) (34). The eukaryotic red alga Cyanidium caldarium was cultured at 0.5 M H2SO4 (35), and the green alga Dunaliella acidophila is able to survive pH 0.2 (36). Also, some fungal species were reported to grow at pH 0, i.e., Acontium cylatium (37), Cephalosporium sp., and Trichosporon cerebræ (38). We also observed fungal growth at 6% H2SO4 (pH −0.05) in our enrichment cultures but did not further investigate the species present in these cultures. With growth at pH as low as −0.05 (6% H2SO4), the new Acidithiobacillus isolates obtained in this research exceed the previously reported pH limit of 0.5 for microbial CS2 conversion for Acidithiobacillus TJ330 (9).

The newly isolated CS2-converting A. thiooxidans strains differed in colony morphology. The genus 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 A. ferrooxidans strains has been described, resulting in the same large, dry, white spreading colonies, as opposed to compact colonies, as observed here for some of the newly isolated A. thiooxidans strains (43, 44). A. thiooxidans strains may be motile via a polar flagellum (40). A. ferrooxidans ATCC 19859 spreading variants displayed increased motility and chemotaxis toward thiosulfate, 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 A. ferrooxidans group of strains has recently been reclassified into four separate species (45). Of these, only A. ferrivorans and some A. ferridurans strains were shown to be motile (45). The type strain A. ferrooxidans ATCC 23270 lacks flagellum and chemotaxis genes (46). The draft genome of A. thiooxidans ATCC 19377 (47) and the draft genomes of both A. thiooxidans strains S1p and G9 (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 Mycobacterium smegmatis (22). In support, 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 hydrophobic compounds on the cell surface of the latter strains. 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 A. thiooxidans grown on S0 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 S0 available as an energy source. In addition, EPS is essential for successful attachment and bioleaching of A. ferrooxidans 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 CS2-contaminated air streams requires microorganisms with a higher affinity for CS2 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 S0 accumulation, and rapid S0 removal to prevent clogging of the biofilters. The affinities of the five strains tested for CS2 were similar (around 100 μM) and correspond to that reported for the purified CS2 hydrolase of Acidithiobacillus AJ-1 (10). Comparison of the Vmax values revealed strain S1p to have a consistently higher Vmax under these conditions than the other four strains tested, possibly because it has a relatively larger amount of CS2 hydrolase present in the cells than the other strains (15). The Vmax is only reached at substrate concentrations of 200 μM (nmol ml−1) or higher. Bioreactors treating CS2-contaminated air from the viscose industry are operational at much lower concentrations of around 4 to 20 nmol ml−1 (100 to 500 ppm) (2), indicating that although strain S1p converts CS2 with a higher Vmax than the other strains, it would not usually reach its full potential reaction rate in a bioreactor. Indeed, a higher Vmax may even be deleterious, as it might cause H2S to accumulate to toxic levels in the cell more rapidly upon CS2 peaks.

Comparison of the resistance to changes in operating conditions in the form of an interruption in the CS2 supply revealed that all five strains were affected by 24 h of CS2 starvation in terms of the ability the respire after a CS2 pulse during starvation, but they all recovered within 24 h after reconnection of the CS2 supply. However, during recovery, all five strains will also produce S0, as we found in all of the strains tested that CS2 pulses during starvation resulted in the transient accumulation of 80% of the total S added as S0, independently of the amount of CS2 added. This implies that it will be difficult to avoid S0 accumulation in biofiltration systems with uneven CS2 loading. However, differences in S0 removal were observed, with strain Sts 4-3 having a much lower but constant S0 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, $CS_2$-converting *A. 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 $CS_2$, cocultures of extremely acidophilic *A. thiooxidans* strains to combine the best acid tolerance, affinity for $CS_2$, $S^0$-removing potential, and stability during periods of fluctuating $CS_2$ loads are most promising for inoculation of industrial biofilters. This application is expected to result in reduced sulfur accumulation, increased $CS_2$ removal rates, reduced water consumption, a more stable operation, and recycling of the sulfuric acid produced.

ACKNOWLEDGMENTS

This work was funded by STW project 6353 and ERC 232937. Bart Kraakman is thanked for providing samples. Jorge Valdés and David Holmes are thanked for the *A. thiooxidans* ATCC 19377 and *A. caldus* ATCC 51756 sequences. Markus Schmid is thanked for advice on primers, and Wendel Brock is thanked for analyzing 16S IR sequences. Jelle Egenesteyn is acknowledged for total carbon measurements. We thank Nardy Kip for help with the pH optimum experiment and Geert-Jan Janssen for the cryo-SEM. Sacha van Hijum is thanked for genome assembly, and Daan Speth is thanked for providing an annotated protein list from the draft genome assembly.

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