Sulfobacillus disulfidooxidans sp. nov., a New Acidophilic, Disulfide-Oxidizing, Gram-Positive, Spore-Forming Bacterium

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An acidophilic, disulfide-oxidizing, mesophilic, aerobic bacterium was isolated from wastewater sludge. The new organism is a gram-positive sporulated rod. It can use elemental sulfur and pyrite as sole energy sources and grows on organic substrates such as glutamate and glucose. It also grows on the following organic sulfur substrates: oxidized and reduced glutathione, cysteine, cystine, and dithio(bis)benzothiazole and clearly shows a preference for disulfide bond-containing substrates. The optimal pH of growth is between 1.5 and 2.5, depending on the substrate used, and the growth temperature range varies from 4 to 40°C, with an optimal value at 35°C. The G+C chromosomal DNA content was measured at 53 ± 1 mol%. Phylogenetic analysis of 16S genes coding for rRNA sequences places the new isolate in the genus *Sulfobacillus*. In addition, unique phenotypic and physiologic characteristics and DNA homology values assign the isolate to a new species in the genus. Therefore, this new isolate has been named *Sulfobacillus disulfidooxidans* and has been assigned ATCC number 51911.

Many facultatively heterotrophic bacteria are known for their ability to grow under highly acidic conditions. Some of these within the genera *Sulfolobus* (5) and *Acidianus* (31) are extremely thermophilic archaeobacteria. They obtain their energy by the oxidation or reduction of elemental sulfur or from organic substrates. Others are mesophilic gram-negative bacteria belonging to the genus *Acidiphilium* (2, 18) and are able to use organic substrates at pH 3.0 or belong to the genus *Thiobacillus*, such as *Thiobacillus acidophilus* (17), which is also known for its ability to grow with elemental sulfur or glucose at pH 2.5.

However, the only acidophilic, facultatively autotrophic, gram-positive, spore-forming bacillus so far characterized is *Sulfobacillus thermosulfidooxidans* (16), together with two subspecies ("thermotolerans" and "asporogenes") that do not produce spores (19). Golovacheva and Karavaiko (16) first described the genus *Sulfobacillus* in 1978 as gram-positive, spore-forming bacteria growing on elemental sulfur, ferrous iron, and other metal sulfides. The rod-shaped cells were described as fairly pleomorphic. The spores were spherical or slightly oval, terminal, subterminal, or paracentral. *S. thermosulfidooxidans* is a typical acidophilic, thermotolerant, and facultatively autotrophic bacterium. Since then, four isolates which are gram-variable, thermotolerant acidophiles have been recovered from mines: ALV (27), BC (27), C-MT1 (15), and TH3 (4), and have been described as belonging presumably to this genus. Some other gram-positive bacteria from the genus *Alicyclobacillus* (7–9) can also grow under acidic conditions, but they are heterotrophic bacteria.

This work reports on the isolation and characterization of a new facultatively autotrophic, mesophilic, acidophilic, gram-positive, spore-forming bacterium (SD-11) that could belong to the genus *Sulfobacillus*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The acidophilic strain SD-11 was isolated from a mixed culture obtained after enrichment of wastewater sludge of Blake Lake City, Québec, Canada, with elemental sulfur and further plating on thiosulfate agar adjusted to pH 4.0 (11). *Escherichia coli* K-12 ATCC 10798 was

used for reference purposes with regard to molecular DNA analysis. Modified 9K medium (9K-EL) (32) was used as the basalt growth medium for strain SD-11, and the ferrous sulfate used as the energy source in 9K medium was replaced by organic and inorganic substrates at a final concentration of 2.5 g/liter. Yeast extract was added (0.1 g/liter) as a growth factor. The pH of the liquid medium was adjusted to 2.25 with 2 N H₂SO₄. Four different substrates were tested: sodium glutamate, oxidized and reduced glutathione (Aldrich Chemical Co.) sterilized by filtration, and tyndallized elemental sulfur. They were added to autoclaved 250-ml flasks containing 100 ml of basalt salt medium 9K-EL. The isolate was cultured at 30°C for 7 days in a gyratory incubator shaker at 200 rpm. *E. coli* was cultured in Trypticase soy broth (BBL) overnight at 37°C. All chemical reagents used in this study were analytical grade.

Phenotypic characterization. The pH and temperature range for growth were determined for three different substrates (sulfur, sodium glutamate, and oxidized glutathione). The growth was monitored by measurement of the protein content determined according to the method of Lowry et al. (23) as modified by Fieg (12) for culturing on sulfur. Growth was also estimated in 9K-EL with oxidized glutathione as the substrate in an anaerobic chamber at 30°C after 10 and 20 days of incubation. No electron acceptor besides oxidized glutathione, sulfates, or traces of nitrate was added during anaerobic incubation.

The commercially available API 50 CH system (BioMérieux), described by Deinhard et al. (8) for the biochemical profile characterization of *Alicyclobacillus acidoterrestris*, was used with minor modifications to evaluate carbohydrate utilization by strain SD-11. The 9K-EL at pH 2.25, to which bromophenol blue was added as a pH indicator, replaced the *Bacillus acidocaldarius* medium. The inoculated medium was incubated at 30°C, and growth was monitored by examining turbidity and/or acidification every 3 days for 15 days.

Cellular morphology was recorded with a Nikon Optiphot phase-contrast microscope. Polyphosphate granules were stained with Loeffler's methylene blue solution (28). Fine morphological features were revealed by transmission and scanning electron microscopy. A washed cell suspension of strain SD-11 was fixed in a 5.0% glutaraldehyde solution in 0.2 M phosphate buffer (pH 7.2), followed by post-fixation in a 3% OsO₄ solution. Dehydration was carried out in a graded ethanol series. Cells were embedded in LR white resin, and cell sections were obtained with a Reichert OM V2 ultramicrotome. They were stained with lead hydroxide (5%) and uranyl acetate (5%) solutions and examined on a Philips EM 300 transmission electron microscope at 80 kV. Negative staining was carried out as follows: 5 µl of a culture suspension was allowed to air dry on a formvar-coated copper grid (size 200 HH), 5 µl of sodium phosphotungstate solution (2% [pH 7.2]) was added, and the grid was dried on filter paper. The specimens were examined by transmission electron microscopy at 60 kV. Scanning electron microscopy was performed on a deposit obtained from a sample of culture (100 µl) passed through a 13-mm polycarbonate (0.1 µm pore size) Nucleopore filter. The filters were treated according to the procedure of Blais et al. (3), and the fixed specimen was examined with a JEOL JMS-T330A electron scanning microscope.

DNA base composition. Genomic DNA was extracted and purified according to the method of Marmur (25), and G+C content was determined from the thermal melting profile (midpoint temperature) (26).

16S rDNA phylogenetic analyses. *E. coli* and SD-11 cells were harvested and washed and nucleic acids were extracted by a previously published procedure (1).

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TABLE 1. Positions of the primers used for PCR amplification of 16S rDNA

Primer	Position ^a	Sequence	Reference
SSU 27	8–27	5'-AGAGTTTGATCMTGGCTCAG-3'	10
SSU 785'	785–805	5'-GGACTACCAAGGGTATCTAATC-3'	This study
SSU 536	515–536	5'-GTGCCAGCMGCCGCGGTAATAC-3'	This study
SSU 1175'	1175–1195	5'-GACGTCATCCCCCTCCTTCCTC-3'	This study
SSU 926	908–926	5'-AAACTYAAKGAATTGACGG-3'	This study
SSU 1492'	1492–1508	5'-TACGGYTACCTTGTTACGACTT-3'	10

^a Relative to *E. coli* 16S rDNA sequence.

Genes coding for 16S rRNA (rDNA) fragments corresponding to the positions 8 to 1510 of *E. coli* 16S rDNA (6) were amplified by PCR. The later sequence was amplified in overlapping fragments with the three sets of primers described in Table 1. DNA amplification was conducted in a Perkin-Elmer Cetus GenAmp PCR System 9600 under the following conditions: a preheating cycle at 98°C for 1 min, followed by 30 amplification cycles with profiles for DNA denaturation, primer annealing, and primer extension steps of 98, 62, and 72°C for 10, 5, and 15 s, respectively; for the SSU 27-SSU 785' primer pair and 98, 55, and 72°C for 10, 5, and 15 s for the other two primer pairs. After 30 cycles, the reactions were stopped, and PCR products were purified by QIA quick-spin column chromatography (QIAGEN, Chatworth, Calif.). Both strands were sequenced with an ABI 372 automated sequencer with *Taq* dyedexy cycle sequencing (PE-ABI, Foster City, Calif.).

The 16S rDNA sequence of strain SD-11 was first aligned with one of its most similar sequences, the 16S rDNA of *S. thermosulfidooxidans* by the ALIGNMENT procedure of the Ribosomal Database Project (24). The SD-11 sequence was aligned manually on the already available alignment of 16S rDNA sequences from 15 species, including *S. thermosulfidooxidans*, obtained from the Ribosomal Database Project (SUBALIGN procedure). Ambiguous regions (positions 1 to 225, 452 to 481, 998 to 1044, and 1510 to 1546) were removed, and the resulting alignment of 1,208 nucleotides was submitted to phylogenetic analyses. Standard parsimony analysis was conducted with the heuristic algorithm of PAUP, version 3.1.1 (34), in which minimal gaps were considered as missing values. Sequences were submitted in 100 different random orders. Pairwise sequence identities were also estimated and transformed in rates of substitution by the two-parameter method of Kimura (20), which takes into account transitions and transversions. The resulting matrix of substitution rates was submitted to neighbor-joining analysis (30) with MEGA, version 1.0 (21). Confidence levels for the tree topologies obtained were estimated by a bootstrap procedure (14) with 100 replicates for both parsimony (PAUP) and neighbor-joining analysis (MEGA). In all cases, the gram-negative *E. coli*, *T. acidophilus*, and *T. thiooxidans* were used as outgroups for rooting the gram-positive tree.

Nucleotide sequence accession number. The 16S rDNA sequence of strain SD-11 obtained in this study has been deposited in the GenBank nucleotide sequence database under accession number U34974. The accession numbers used as reference sequences are as follows: *Alicyclobacillus acidocaldarius*, X60742; *Alicyclobacillus acidoterrestris*, X60743; *Alicyclobacillus cycloheptanicus*, X51928; *Bacillus subtilis*, K00637, M10606, and X00007; *Clostridium innocuum*, M23732; *E. coli*, J01695; *Mycobacterium bovis*, M20940; *Sporolactobacillus inulinis*, M58838; *Streptomyces lividans*, Y00484; strain ALV, M79375, M79376, and M80290; strain BC, M79380 and M79382; strain TH3, M79433 and M79434; *Thiobacillus thiooxidans*, X75269; and *Thiobacillus acidophilus*, M79399 and M79400.

RESULTS

Isolation and growth. The new species was isolated from a mixed culture obtained after enrichment from a Blake Lake City sludge sample with elemental sulfur and further plating on thiosulfate agar adjusted to pH 4.0 (11). A pure culture of SD-11 was obtained through micromanipulation of tiny colonies of the isolated mixed culture and further grown in enrichment broth 9K-EL supplemented with oxidized glutathione at pH 2.25. Since strain SD-11 showed active sporulation after prolonged growth on the latter liquid medium, the mixed population was subjected to heat treatment. Mixed suspensions of cells were therefore boiled for 30 min, after which spore preparations were left to cool at room temperature. Successful germination could only be reached after storage at 2 to 4°C for 18 to 24 h. Culture purity was verified by plating of a 10-μl inoculum on a thiosulfate agar surface and on other solidified media. It should be noted that under the indicated conditions,

strain SD-11 could not be grown in pure culture on solidified media. The growth on solid medium was obtained only when a thiosulfate-oxidizing, acidophilic bacterium was cocultivated with strain SD-11. Absence of growth when a pure culture inoculum was used indicated the absence of contaminating bacteria. Pure culture flocculated when grown in liquid media.

Morphology and ultrastructure. Isolate SD-11 is a pleomorphic, rod-shaped, spore-forming, nonmotile bacterium; it has been impossible to obtain a satisfactory Gram staining of a fixed or unfixed bacterial cell suspension, even if the preparation originated from a young and active culture. Under electron microscopy, it exhibits the typical smooth texture of the membrane of gram-positive bacteria (Fig. 1A and B). The cells occur singly and often in pairs when grown on sulfur, or they tend to form filaments when grown on oxidized glutathione (Fig. 1A and B). Cellular divisions seem to be atypical, as shown on Fig. 1A; asymmetric divisions and mini-cell-like structures were regularly observed under various growth conditions. Polyphosphate granules and spores were also observed at opposite ends of the cells; polyphosphate granules seem to disappear slowly when the sporulation process is initiated (Fig. 1B and C).

Physiological and biochemical characteristics. Isolate SD-11 appears to be an aerobic microorganism; even if strain SD-11 grows poorly under anaerobic conditions, only one fifth of the cellular yield obtained in aerobiosis was achieved in the absence of oxygen with oxidized glutathione as the energy source. Strain SD-11 is a mixotroph and uses a wide variety of organic and inorganic substrates as described in Table 2. Growth yields were measured with inorganic substrates, such as elemental sulfur and pyrite, and with organic substrates such as glucose, glutamic acid, and oxidized glutathione. Among organic sulfur compounds, those containing disulfide bonds were preferred over the thiol groups (—SH). On the basis of total protein content, generation time varied from 6 to 49 h. Culture of the bacteria on yeast extract as sole substrate was not possible. However, when yeast extract was added to the culture media at a final concentration of 0.1 g/liter, it enhanced growth by providing essential growth factors. Table 3 presents results on growth and utilization of various carbohydrates by isolate SD-11 in the API 50 CH galleries: only a few substrates, including amygdalin, glucose, maltose, and melezitose, could support fast growth. However, glucose was the sole substrate on which the organism was able to achieve a noticeable growth. No decrease in pH was observed for any of the 49 other substrates.

Temperature and pH conditions allowing growth were tested in 9K-EL supplemented with elemental sulfur, sodium glutamate, or oxidized glutathione. Growth was possible from 4 to 40°C, with an optimum at 35°C; no growth was detected above 40°C. Isolate SD-11 grew between pH 0.5 and 6.0, and the optimum value varied between 1.5 and 2.5, depending on

FIG. 1. Electron micrographs of SD-11 isolates. (A) Scanning electron micrograph showing a short chain of abnormally dividing (arrows) and normal-length (*) cells. (B) Transmission electron micrograph of negatively stained preparation showing the polyphosphate granules (PG) both in abnormally dividing (white arrow) and normal-length (*) SD-11 cells. (C) Transmission electron micrographs of ultrathin longitudinal and transverse sections of SD-11 cells at different stages of growth: 1, dividing vegetative cells; 2, mature vegetative cell (normal length [*]); 3, sporulating cell with polyphosphate granule and highly deforming prespore at opposite end. (Insert) Transverse section of sporulating SD-11 cell showing prespore multilamellar membrane (MM) and cortex (CO). Bar, 1 μ m.

the substrate used. The initial pH of the culture media did not change during growth.

DNA analyses and phylogenetic relationships. The genomic DNA G+C content of strain SD-11 was 53 ± 1 mol%, and the G+C nucleotide ratio of the 16S rDNA was 59.3 mol%. From 16S rDNA nucleotide sequence data, isolate SD-11 shares 95.5% homology with *A. cycloheptanicus* and 95.2% homology with *S. thermosulfidooxidans* (Table 4). The transition/transversion ratios were 1.3 for strain SD-11 versus *S. thermosulfidooxidans* and 1.1 for strain SD-11 versus *A. cycloheptanicus*.

The tree topologies estimated from both standard parsimony and neighbor-joining analyses of two-parameter substitution rates showed close relatedness between the genera *Sulfobacillus* and *Alicyclobacillus*. This group was supported by a high bootstrap value, which indicates the degree of statistical confidence (Fig. 2). In both analyses, the genus *Alicyclobacillus* did not appear monophyletic, because its taxa were intermingled with the genus *Sulfobacillus*. This arrangement was supported by high bootstrap values. The only topological difference between parsimony and neighbor-joining trees was the placement of the *Bacillus-Sporolactobacillus-Clostridium* group, which shows low percentage G+C content together with the *Sulfobacillus-Alicyclobacillus* group. Parsimony placed both groups together, and this was supported by a bootstrap value of 85% (Fig. 2A); this topology is in accordance with previous studies (22). Neighbor-joining analysis placed the *Sulfobacillus-Alicyclobacillus* group next to the intermediate-percentage-G+C-content group formed by the strains BC and ALV (Fig. 2B). However, this alternative arrangement was supported by a lower bootstrap value (58%). To further investigate this lack of congruence, the phylogeny was estimated with Gary Olsen's fastDNAm1 program (Ribosomal Database Project) (24), which is based on the maximum-likelihood approach (13). The 16S rDNA sequence of the strain C-MT1 (GenBank entry X75270), isolated by Goebel and Stackebrandt (15) and presumably identified as a member of the genus *Sulfobacillus* on preliminary physiological analysis, was also included. The max-

imum-likelihood tree obtained was very similar to the parsimony tree, and the strain C-MT1 clustered with the strains BC and ALV. The newly isolated strain SD-11 was found with the low-percentage-G+C strains of the genera, which were re-grouped by the analysis.

DISCUSSION

This study has provided phenotypic and genotypic information about the newly isolated strain SD-11. This strain, isolated from a wastewater sludge treatment plant, is a gram-positive, mesophilic, acidophilic, and aerobic bacterium. However, the organism may grow as a facultative anaerobe, since oxidized glutathione seems to be used as a substrate under anaerobiosis. Under the anaerobic growth conditions used during the experiments, we favor the anaerobic respiration hypothesis, although no significant data support it except for the very low cellular yield during incubation. We estimated that only 20% of the anaerobiosis cellular yield was reached after 20 days of culturing under anaerobiosis. Its morphological features and mixotrophic metabolism suggest that strain SD-11 most likely belongs to the genus *Sulfobacillus*. The morphological characteristics of isolate SD-11 are similar to those described for *S. thermosulfidooxidans* (Table 5). Both are gram-positive, non-motile, rod-shaped bacteria which form chains or straight or interwoven filaments (*S. thermosulfidooxidans* subsp. "asporogenous"). SD-11 exhibits subterminal, deforming spores. The analysis of the fatty acid composition in bacteria belonging to the genera *Alicyclobacillus* and *Sulfobacillus* revealed the pres-

TABLE 2. Generation time of strain SD-11 grown on different substrates in 9K-EL liquid medium at pH 2.25 at 30°C

Substrate	Mean generation time (h)	Growth (%) ^a
Inorganic		
Elemental sulfur	13 (6 h at 35°C)	66
Pyrite	16	56
Ferrous sulfate	25	30
Organic		
Glutamate	12	68
Glycine	26	12
Glucose	16	88
Oxidized glutathione (—S—S—)	12 (7 h at 35°C)	100
Reduced glutathione (—SH)	28	72
Thiourea (—S—S—, =S)	24	61
Cystamine (—S—S—)	28	54
Cystine (—S—S—)	42	53
Cysteine (—SH)	49	17

^a Recorded as percentage of growth relative to oxidized glutathione at pH 2.25 and 30°C.

TABLE 3. Relative growth of strain SD-11 on the different substrates of the API 50 CH system

Substrate on which relative growth is ^a :			
—	+	++	+++
Control	Adonitol	Starch	Amygdalin
Arbutin	D- and L-Arabinose	L-Arabitol	Glucose
Dulcitol	D-Arabitol	N-Acetyl-glucos-amine	Maltose
Erythritol	Cellobiose		Melezitose
Fructose	Esculin	Glycogen	
L-Fucose	D-Fucose	Inulin	
Galactose	Gentiobiose	Sucrose	
Inositol	Glycerol		
Lactose	Gluconate		
D-Lyxose	2-Keto-gluconate		
Mannitol	5-Keto-gluconate		
Mannose	α -Methyl-D-glucoside		
Rhamnose	Melibiose		
Salicin	α -Methyl-D-mannoside		
Trehalose	Ribose		
D-Turanose	Raffinose		
Xylitol	Sorbitol		
β -Methyl-D-xyloside	Sorbose		
	D-Tagatose		
	D and L-Xylose		

^a Growth was evaluated visually under phase-contrast microscopy in the absence of turbidity. —, presence of sporulated cells only or no growth; +, few isolated cells; ++, short chains of long cells; +++, chains of short cells (fast dividing cells).

TABLE 4. 16S rDNA sequence identities between isolate SD-11 and various gram-positive bacteria and proteobacteria^a

Species or strain	% Sequence identity with:														
	<i>S. thermosulfidooxidans</i>	<i>A. cycloheptanicus</i>	<i>A. acidocaldarius</i>	<i>A. acidoterrestris</i>	<i>B. subtilis</i>	<i>S. inulinis</i>	<i>C. innocuum</i>	Strain BC	Strain ALV	Strain TH3	<i>M. bovis</i>	<i>S. lividans</i>	<i>T. thiooxidans</i>	<i>T. acidophilus</i>	<i>E. coli</i>
SD-11	95.2	95.5	93.7	93.6	87.7	87.1	83.3	84.3	83.3	82.2	81.9	83.3	81.2	79.7	79.9
<i>Sulfobacillus thermosulfidooxidans</i>															
<i>Alicyclobacillus cycloheptanicus</i>		94.9	94.2	94.1	87.4	86.4	83.3	84.9	82.9	82.3	82.1	83.4	82.6	80.5	80.4
<i>Alicyclobacillus acidocaldarius</i>			95.0	94.9	88.3	87.3	83.7	84.8	84.5	83.1	83.3	84.2	82.3	80.2	81.0
<i>Alicyclobacillus acidoterrestris</i>				99.6	88.3	87.5	84.3	85.0	83.6	83.0	82.9	83.8	82.6	80.0	80.5
<i>Bacillus subtilis</i>					88.2	87.3	84.0	85.1	83.6	82.9	83.0	83.8	82.4	79.9	80.1
<i>Sporolactobacillus inulinis</i>						93.1	86.4	83.7	82.4	84.2	83.0	84.3	82.3	80.9	81.7
<i>Clostridium innocuum</i>							84.5	80.0	79.8	82.7	81.1	82.6	81.1	80.8	81.8
Strain BC								80.8	80.7	79.7	80.7	82.1	80.7	79.6	79.6
Strain ALV									94.5	81.4	83.2	83.3	82.3	80.0	79.6
Strain TH3										81.3	81.4	82.0	79.6	79.8	78.5
<i>Mycobacterium bovis</i>											87.3	88.6	82.6	82.0	82.4
<i>Streptomyces lividans</i>												91.7	80.9	80.1	80.2
<i>Thiobacillus thiooxidans</i>													80.8	80.2	81.9
<i>Thiobacillus acidophilus</i>														85.1	85.6
<i>Escherichia coli</i>															82.2

^a Based on an alignment of 1,208 nucleotides.

ence of ω -cyclohexane acids (or ω -cycloheptane in *A. cycloheptanicus*) (8, 9, 36), which are rarely seen in other bacteria. The fatty acid composition for strain SD-11 closely resembles that of *S. thermosulfidooxidans*. The presence of ω -cyclohexane C₁₇ and C₁₉ was revealed in this new organism (29). The G+C contents were found to be 53 ± 1 mol% and 54 ± 1 mol%, respectively, for SD-11 and *S. thermosulfidooxidans*, and were identical at 59 ± 1 mol% for their 16S rDNA sequences (35).

However many physiological differences were noted between isolate SD-11 and *Sulfobacillus* sp. and *Alicyclobacillus* sp. The species of the genera *Sulfobacillus* and *Alicyclobacillus* grow better at temperatures above 45°C, whereas isolate SD-11 is more typical of a mesophilic species (35°C). The new isolate does not grow on solid media, even with the use of phytagel, agarose, silicate gel, or starch as solidifying agents. It grows autotrophically on elemental sulfur and on pyrite with the production of sulfur oxyanions, including sulfate, in contrast with *S. thermosulfidooxidans*, which oxidizes sulfur to sulfuric acid. It can also grow heterotrophically on carbohydrates like glucose, without any production of acidic end products, and also on amino acids like glutamate. The new organism shows a net preference for organic sulfur substrates (Table 6). The API 50 CH system was also used to compare the utilization of carbohydrates by isolate SD-11 and by strains from the genus *Alicyclobacillus*, the major difference again being the lack of acidification observed after growth of isolate SD-11 (8, 9). As a consequence of the differences in phenotypic and physiological characteristics, the isolate SD-11 may be classified as a new species of the genus *Sulfobacillus*.

The analysis of the 16S rDNA sequences showed that strain SD-11 is phylogenetically closely related to the genera *Sulfobacillus* and *Alicyclobacillus*. Even if the genera *Sulfobacillus* and *Alicyclobacillus* possess a number of distinctive phenotypic features that could justify their separation into two genera (Table 7), phylogenetic analyses confirm their close proximity (35). Since *A. cycloheptanicus* is found to be more related to *Sulfobacillus* strains than to other *Alicyclobacillus* strains, and because both groups share growth factor requirements (Table 7), a revision of the taxonomic nomenclature of the group at the genus level may be necessary, but this will require the description of additional species.

Signature sequences (513-A, 1207-C, 168-G, 906-G, 955-G, and 1167-A according to the *E. coli* numbering convention) support the assignment of strain SD-11 as a gram-positive organism of the low-percentage-G+C subdivision (22). The 14 conserved nucleotide positions reported by Tourova et al. (35), which link the genera *Alicyclobacillus* and *Sulfobacillus* and differentiate them from the other low-percentage-G+C gram-positive groups were also found in isolate SD-11 (154-G, 452-G, 480-A, 672-G, 734-C, 748-G, 862-G, 867-C, 1122-G, 1123-T, 1150-A, 1151-C, 1297-G, and 1298-T). However, the clade formed by strains ALV, BC, and C-MT1 was shown by parsimony and maximum-likelihood methods to be quite remote from the genera *Sulfobacillus* and *Alicyclobacillus* (22, 35), even if the isolate BC has been described as a gram-negative rod-shaped bacterium (15, 27).

For every type of phylogenetic analysis (neighbor joining, parsimony, and maximum likelihood), the closest strain to the isolate SD-11 was *S. thermosulfidooxidans*, and this is supported by the high bootstrap values (Fig. 2). Two signature sequences, 5'-ATGCTACGGCATCGC-3' and 5'-AACCCCTGAAAACCGC-3' at positions 200 to 222 and 1279 to 1296, were found only in both *S. thermosulfidooxidans* and strain SD-11. This further reinforces the very high phylogenetic affinity observed between the Golovacheva's *Sulfobacillus* isolate

FIG. 2. Phylogenetic relationships among gram-positive bacteria and position of strain SD-11 based on analysis of 16S rDNA gene sequences. Gram-negative strains were used as outgroup to root the gram-positive tree. (A) Tree obtained from neighbor-joining analysis of two-parameter substitution (subst.) rates. (B) Tree obtained from standard parsimony analysis. Numbers on branches indicate bootstrap estimates from 100 replicates. For full names of strains, refer to Table 4. str., strain.

and the bacterium SD-11 isolated from a completely different environment.

Given that the highest 16S rDNA sequence homology of strain SD-11 compared with the other bacteria is less than 97.0% (Table 4), total DNA will not reassociate to more than 60% with any other piece of DNA from different bacterial strains, no matter which hybridization method is used (33).

Indeed, the highest 16S rDNA sequence homology values observed with *A. cycloheptanicus* and *S. thermosulfidooxidans* were less than 96%. Therefore, the isolate SD-11 should be considered formally as a distinct species both from the 16S rDNA composition and homology perspective, even if formal DNA-DNA reassociation studies were not conducted.

On the basis of all phenotypic, genotypic, and phylogenetic

TABLE 5. Morphological and chemotaxonomical characteristics of the genera *Sulfobacillus* and *Alicyclobacillus*

Characteristic	SD-11	Result for:					
		<i>S. thermosulfidooxidans</i> VKM B-1269 ^a	<i>S. thermosulfidooxidans</i> subsp. thermotolerans ^b	<i>S. thermosulfidooxidans</i> subsp. asporogenes ^b	<i>A. cycloheptanicus</i> DMS 4006 ^c	<i>A. acidocaldarius</i> ^d	<i>A. acidoterrestri</i> s ^e
Origin	Wastewater sludge from Blake Lake, Québec, Canada	Spontaneous thermo- genesis zones of the leached dump of the Nikolaev cop- per-zinc-pyrite de- posit, Eastern Ka- zakhstan	Dumps of oxidizing lead-zinc ores, Uz- beck SSR	Drainage waters of the polymetal sul- fide deposit of Ar- manis, Armenian SSR	Soil from car service station, Losheim, Saarland, Germany	Thermal acid water and soil	Forest and garden soils
% G+C	53 ± 1	54 ± 1	49 ± 1	46 ± 1	55 ± 1	61 ± 1	52 ± 1
Cell morphology (gram positive)	Straight rods, 0.3–0.5 by 0.9–3.6 µm; single, paired, short chain; filaments which could be interwoven	Straight rods, 0.6–0.8 by 1–3 µm; single, paired, in short chain; palisade, Y- shaped cells and other forms	Straight rods, 0.9–1.0 by 3–6 µm; single, paired, short chain, polymorphism	Straight rods, 0.5–0.9 by 2–4 µm; single, paired, short chain, filamentous cells which are interwo- ven	Rods, 0.3–0.6 by 2.5–4.5 µm; single, pair or short chain	Rods, 0.7–0.8 by 2–3 µm; single, pair or short chain	Rods, 0.6–0.8 by 2.9–4.3 µm; single, pair or short chain
Wall lipids	ω-Cyclohexane fatty acids	ω-Cyclohexane fatty acids	ND ^f	ND	ω-Cycloheptane fatty acids	ω-Cyclohexane fatty acids	ω-Cyclohexane fatty acids
Menaquinones	ND	ND	ND	ND	MK-6, MK-7, MK-9	MK-7, MK-8	MK-6, MK-7
Spore: Presence or absence	+	+	+	–	+	+	+
Morphology	Oval, 0.7–0.9 by 0.9–1.8 µm; subterminal or terminal dilated spo- rangia	Spherical, 0.7 by 0.7– 0.8 µm; subterminal or terminal dilated sporangia	Oval, terminal slightly dilated sporangia	–	Oval, 0.75 by 1.0 µm; subterminal slightly bulging sporangia	Ellipsoidal, subtermi- nal or terminal, not swollen spo- rangia	Oval, 0.9–1.0 by 1.5– 1.8 µm; subtermi- nal or terminal slightly bulging sporangia
Motility	–	–	–	–	–	ND	ND

^a Golovacheva and Karavaiko (16).
^b Karavaiko et al. (19).
^c Deinhard et al. (9).
^d Group of *A. acidocaldarius* isolates; Darland and Brock (7).
^e Group of *A. acidoterrestri* isolates; Deinhard et al. (8).
^f ND, not determined.

TABLE 6. Physiological characteristics of the genera *Sulfobacillus* and *Alicyclobacillus*

Characteristic	Result for:						
	SD-11	<i>S. thermosulfidooxidans</i> VKM B-1269	<i>S. thermosulfidooxidans</i> subsp. <i>thermotolerans</i>	<i>S. thermosulfidooxidans</i> subsp. <i>asporogenes</i>	<i>A. cycloheptanicus</i> DMS 4006	<i>A. acidocaldarius</i>	<i>A. acidoterrestris</i>
Anaerobic growth	+	—	—	—	+	—	—
pH							
Optimum	1.5–2.5	1.9–2.4	2.5–2.7	1.6–2.5	3.5–5.5		
Growth range	0.5–6.0	1.5–5.5	1.2–4.2	1.5–4.5	3.0–5.5	2.0–6.0	2.2–5.8
Temp							
Optimum (°C)	35	50	37–42	50	48		42–53
Growth range (°C)	4–40 ^a	20–60	20–50	20–60	40–53	45–70	35–55
Growth factor	Yeast extract	Yeast extract	Yeast extract	Yeast extract	Ile Met or B ₁₂ pantothenate	—	—
Growth in ^b :							
S ⁰	+	+	+	+	ND ^c	ND	ND
Fe ²⁺	(+)	+	+	+	ND	ND	ND
S ₂ O ₃ ²⁻	—	+	ND	ND	ND	ND	ND
Fe ₂ S	+	+	+	+	ND	ND	ND
Yeast extract	—	+	+	+	+	+	+
Acetate	—	+	ND	ND	—	ND	ND
Casamino Acids	ND	+	ND	+	+	ND	ND
Cystamine	+	ND	ND	ND	ND	ND	ND
Cysteine	+	+	ND	+	ND	ND	ND
Cystine	+	ND	ND	ND	ND	ND	ND
Dithio(bis)benzo- thiazole	+	ND	ND	ND	ND	ND	ND
Galactose	—	—	ND	ND	+	+	+
Glucose	+	+	+	+	+ acid ^d	+ acid ^d	+ acid ^d
Glutamate	+	+	ND	ND	ND	ND	ND
Glutathione	+	ND	ND	+	ND	ND	ND
Glycerol	+	ND	ND	ND	+	+	+
Meat peptone	—	—	ND	ND	ND	ND	ND
Growth on:							
FeTSB	—	+	ND	ND	ND	ND	ND
<i>B. acidocaldarius</i> medium agar	ND	ND	ND	ND	+	+	+

^a Spore germination at 4°C.^b *Sulfobacillus* sp. strain SD-11 was grown in liquid medium at pH 2.25. *Alicyclobacillus* sp. was grown in liquid medium at pH 4.0.^c ND, not determined.^d Acid formation when growth occurs with the substrate.

evidence, the present isolate is formally proposed as a new species within the genus *Sulfobacillus*.

Description of *S. disulfidooxidans* sp. nov. *S. disulfidooxidans* (di.sul.fi.do.ox'i.dans. L. adj. *duplus*, double; L. n. *sulfur*, sulfur; M.L. v. *oxido*, oxidize; M.L. adj. *disulfidooxidans*, disulfide-oxidizing bacteria). The new species appears as a aerobic, endospore-forming rod shaped bacterium (length, 0.9 to 3.6 µm; width, 0.3 to 0.5 µm). The strain is nonmotile; it is a typical gram-positive bacterium, even if Gram staining is variable. *S. disulfidooxidans* produces oval spores, located subterminally in a swollen sporangium. Cells sometime appear pleomorphic and increase in width with aging. Long chains of rods appear only on selected substrates. *S. disulfidooxidans* does not grow alone on solid medium, but it forms colonies only when an accompanying acidifying bacterium is present and growing.

S. disulfidooxidans grows in liquid medium where it literally flocculates. Growth is maximal between pH 1.5 and 2.5, and culture is possible within a wide range of pH, from 0.5 to 6.0. The *Sulfobacillus* strain grows best at 35°C and within an interval of 4 to 40°C. *S. disulfidooxidans* utilizes glucose, glutamate, oxidized glutathione, or dithio(bis)benzothiazole during its heterotrophic growth. When the bacterium uses elemental

sulfur as its sole energy source, it does not oxidize it to sulfuric acid, but yeast extract is needed as a growth factor. No acid formation has been observed during culture on carbohydrates (API 50 CH).

The G+C content of the extracted DNA is 53 ± 1 mol% as

TABLE 7. Features differentiating the genera *Sulfobacillus* and *Alicyclobacillus*

Characteristic	Result for genus:	
	<i>Sulfobacillus</i> ^a	<i>Alicyclobacillus</i> ^b
Growth at 25°C	+	—
Growth at pH 2	+	—
Growth factors required	+	— ^c
Autotrophic growth	+	DU ^d
Growth on galactose	—	+
Acid produced from carbohydrates	—	+

^a This work and that of Golovacheva and Karavaiko (16).^b Darland and Brock (7) and Deinhard et al. (8, 9).^c Except *A. cycloheptanicus* (see Table 6).^d DU, data unavailable.

determined by the thermal denaturation profiles. Its highest 16S rDNA sequence identities were 95.5 and 95.2% with *A. cycloheptanicus* and *S. thermosulfidooxidans*, respectively. As determined by neighbor-joining, parsimony, and maximum-likelihood approaches, the closest phylogenetically to *S. disulfidooxidans* is *S. thermosulfidooxidans*, followed by strains from the genus *Alicyclobacillus*, both genera forming a highly supported clade of low-percentage-G+C gram-positive strains.

S. disulfidooxidans SD-11 has been deposited at the American Type Culture Collection, Rockville, Md., and has been designated ATCC 51911.

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