INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, Oct. 1986, p. 559–564 Vol. 36, No. 4
0020-7713/86/040559-06$02.00/0

Acidianus infernus gen. nov., sp. nov., and *Acidianus brierleyi* comb. nov.: Facultatively Aerobic, Extremely Acidophilic Thermophilic Sulfur-Metabolizing Archaebacteria

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A new genus, *Acidianus*, is characterized from studies of 26 isolates of thermoacidophilic archaebacteria from different solfatara fields and marine hydrothermal systems; these isolates grow as facultative aerobes by lithotrophic oxidation and reduction of S^0, respectively, and are therefore different from the strictly aerobic *Sulfolobus* species. The *Acidianus* isolates have a deoxyribonucleic acid guanine-plus-cytosine content of 31 mol%. In contrast, two of three *Sulfolobus* species, including the type species, have a guanine-plus-cytosine content of 37 mol%; *Sulfolobus brierleyi* is the exception, with a guanine-plus-cytosine content of 31 mol%. In contrast to its earlier descriptions, *S. brierleyi* is able to grow strictly anaerobically by hydrogen-sulfur autotrophy. Therefore, it is described here as a member of the genus *Acidianus*. The following species are assigned to the genus *Acidianus*: *Acidianus infernus* sp. nov. (type strain, strain DSM 3191) and *Acidianus brierleyi* comb. nov. (type strain, strain DSM 1651).

The following two major groups of extremely thermophilic S^0-metabolizing archaebacteria (12) that thrive within acidic solfatara fields have been described previously: (i) the genus *Sulfolobus*, which due to its isolated phylogenetic position may represent a still undescribed order (8, 32, 39) and (ii) the *Thermoproteales* (16, 44). Thus far, *Sulfolobus* is represented by three species, which are characterized by the irregular coccoid shape of the cells, the low pH and high temperature optima for growth, the lipid composition (25), and the common mode of chemolithotrophic energy conservation by oxidation of elemental sulfur (8, 31, 45). Alternatively, these organisms are able to grow by oxidizing organic materials without S^0 as an electron acceptor (8, 45). *Sulfolobus acidocaldarius* (8, 32) and *Sulfolobus solfataricus* (15, 45) are extreme thermophiles with guanine-plus-cytosine (G+C) contents of about 37 mol% and maximum growth temperatures of 85 to 87°C, while *Sulfolobus brierleyi*, which has a G+C content of only 31 mol%, is less thermophilic, growing at temperatures up to 75°C (5, 15, 45). In contrast to the aerobic *Sulfolobus* species, all members of the order *Thermoproteales*, which is comprised of the genera *Thermoproteus* (16, 44), *Desulfurococcus* (17, 43), and *Thermofilum* (18, 42), are strict anaerobes, exhibiting G+C contents between 51 and 56 mol%. These bacteria grow optimally at slightly acidic to neutral pH values on organic compounds by means of sulfur respiration (28). The rod-shaped organism *Thermoproteus tenax* (16, 44) and some still undescribed morphologically similar isolates are able to grow lithoautotrophically by anaerobic reduction of S^0 by H_2 (11). Recently, we isolated some strains of new extremely thermoacidophilic archaebacteria that could grow facultatively aerobically by either oxidation or reduction of elemental sulfur (30). These new isolates, together with *Sulfolobus brierleyi*, constitute a new genus, which we have named *Acidianus*.

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MATERIALS AND METHODS

Bacterial strains. *S. acidocaldarius* DSM 639^T (T = type strain), *S. solfataricus* DSM 1616^T, and *S. brierleyi* DSM 1651^T were obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany. All other strains were isolates obtained from our laboratory.

Culture conditions. Unless stated otherwise, the isolates from our laboratory, *S. acidocaldarius*, and *S. solfataricus* were grown at 85°C in Allen medium (1) in the presence of 2 g of S^0 per liter (pH 2.5). *S. brierleyi* was grown at 70°C. For optimal growth, aerobic media were supplemented with 0.5 g of beef extract (E. Merck AG, Darmstadt, Federal Republic of Germany) per liter. Anaerobic media were prepared by using the technique of Balch et al. (2). Media were reduced by the addition of sodium sulfide (0.75 g/liter), which was visualized by reduction of resazurin (1 mg/liter). Samples (20 ml) of media were distributed into 100-ml serum bottles (type III glass; Bormioli) and then pressurized with H_2-CO_2 (80:20, vol/vol; 300 kPa) and sterilized by tyndallization. For large-scale preparations organisms were grown in enamel-protected fermentors (Bioengineering, Wald, Switzerland) with 100- and 300-liter working volumes.

Electron microscopy. Thin sections were prepared and electron microscopy was performed as previously described (14).

Bacterial growth. Cell concentrations were determined with a light microscope by using a Thoma counting chamber (depth, 0.02 mm).

Organic substrates. Each of the following organic substances (obtained from Difco Laboratories, Detroit, Mich., except where noted otherwise) was added to Allen mineral medium in concentrations of 0.2, 0.5, and 2 g/liter in the presence (2 g/liter) and absence of S^0 under either aerobic or anaerobic culture conditions: D-(−)-ribose, L-(+)-arabinose, D-(+)-xylose, D-(+)-glucose, L-(−)-glucose, D-(+)-galactose, maltose, lactose, sucrose, melibiose, raffinose, cellulose, starch, sorbitol, mannitol, glycerol, acetate, butyrate, i-butyrate, i-valerate, citrate, alanine plus glycine, 20

TABLE 1. Distribution and origin of the new isolates

Source		No. of samples	Original temp (°C)[a]	Original pH[a]	No. of positive samples[a]	Strain designations
Country	Locality					
Italy	Solfatara Crater and Pisciarelli Solfatara, Naples	11	53–96	1.5–6.5	11	So1d, So3d, So4a^T, So5c, PS3d, PS6c, PS7c, PS8c, PS11c, PS14d, PS17c
	Vulcano, Porto di Levante[b]	17	40–100	2.5–5	2	Vc11, Vc16
Azores	Caldeira da Velha	1	96	3.5	1	HW1
Iceland	Krafla	5	100	3	1	Kra1
	Krisuvik	14	85–100	1.5–3.5	5	K4, K5, K6, K7, K8
	Kerlingarfjöll	6	100	4	1	KF2
	Hveravellir	2	85	4	1	HV7
United States	Yellowstone National Park	15	75–94	3–4.5	4	YFP1, YFP2, YMV1, YWTh4

[a] Samples containing the new isolates.
[b] Marine environment.

different L-amino acids (Sigma Chemical Co., St. Louis, Mo.), Casamino Acids, yeast extract, tryptone, peptone, and beef extract (Merck).

Metabolic products. H_2S was analyzed as described elsewhere (35, 38). SO_4^{2-} was determined gravimetrically after precipitation of $BaSO_4$ with $BaCl_2$ by the method of Williams (38). Organic products, such as volatile acids and C_1 through C_5 monoalcohols, were analyzed by gas chromatography (41).

Cell walls. Muramic acid and *meso*-diaminopimelic acid were analyzed as previously described (24, 29).

DNA analyses. Deoxyribonucleic acid (DNA) was prepared by the method of Lauerer et al. (26a). DNA base composition was analyzed by the melting point method of Marmur and Doty in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (27). Calf thymus DNA (42 mol% G+C) (27) and DNA from *Lactobacillus casei* phage PL-1 (47 mol% G+C) (36) were used as references. The level of DNA homology between organisms was determined by challenging genomic DNA bound to nitrocellulose filters with nick-translated, ^{32}P-labeled, genomic DNAs from other organisms (3, 20) in 3× SSC containing 10% formamide at 25°C below the melting point, as described by König (23) and Segerer et al. (30).

RESULTS AND DISCUSSION

Isolation. The new bacteria were enriched anaerobically (2, 34) in culture medium which was inoculated with samples (5% inocula) and incubated in the presence of S^0 under an H_2-CO_2 (80:20, vol/vol) atmosphere at 85°C. Organisms were obtained from the enrichment cultures by four sequential serial dilutions, which yielded a total of 26 isolates from samples from all areas with original temperatures ranging from 40 to 100°C and with pH values between 1.5 and 6.5 (Table 1). Colonies of type strain So4a, which is described in detail below, were obtained by plating the organism onto 9% starch containing about 12 g of colloidal sulfur per liter and 0.2 g of yeast extract per liter at 85°C with an H_2-CO_2 gas phase.

Metabolism. All 26 isolates, including strain So4a^T, were able to grow facultatively aerobically by means of two contrary modes of chemolithotrophy (30). At extremely anaerobic conditions (2), the organisms grew autotrophically, forming H_2S (11) (Table 2). Growth depended strictly on H_2 and S^0; H_2 could not be replaced by the organic components listed in Materials and Methods, and S^0 could not be replaced by sulfite, thiosulfate, or sulfate. *S. brierleyi* DSM 1651^T also grows on H_2 and S^0 (30), but *S. acidocaldarius* DSM 639^T and *S. solfataricus* DSM 1616^T are unable to grow by this metabolic pathway (30) (Table 2).

Under aerobic conditions in the presence of S^0, all of the new isolates were able to grow by oxidation of S^0, forming sulfuric acid (30), like the three *Sulfolobus* type strains. A switch in growth from aerobic to anaerobic conditions was demonstrated with a culture of isolate So4a^T; after a lag time of about 2 h, growth began again (Fig. 1), accompanied by formation of H_2S instead of H_2SO_4 (data not shown). In contrast to *S. acidocaldarius* DSM 639^T, *S. solfataricus* DSM 1616^T, and *S. brierleyi* DSM 1651^T, the new isolates, including So4a^T, were unable to grow without S^0 by oxidizing organic compounds and were, therefore, strictly chemolithotrophic. However, the final cell yields of the new isolates could be increased by adding low concentrations (e.g., 0.2 g/liter) of yeast extract, tryptone, peptone, beef extract, or (slightly) Casamino Acids, indicating the presence of unknown stimulating factors within these heterogenous compounds; concentrations of 2 g/liter did not further stimulate growth. No positive influence on growth could be detected after the addition of amino acids, sugars, organic acids, and alcohols. As reported previously for *S. brierleyi* DSM 1651^T and *S. acidocaldarius* DSM 639^T (6), the new isolates, including So4a^T, were able to grow anaerobically by means of S^0 oxidation, forming sulfuric acid, in the presence of high amounts of molybdate as an electron acceptor. However, since molybdenum is only a minor metal within

TABLE 2. Formation of H_2S by aerobically grown cultures of isolate So4a^T and the *Sulfolobus* type strains after a change to anaerobic conditions

Strain	Optical density at 578 nm[a]	Presence of H_2[b]	H_2S concn (μmol/ml)[c]
So4a^T	0.12	+	24
A. brierleyi DSM 1651^T	0.18	+	22
		−	0
S. acidocaldarius DSM 639^T	0.20	+	0
		−	0
S. solfataricus DSM 1616^T	0.19	+	0
		−	0

[a] At the beginning of the experiment.
[b] +, H_2-CO_2 atmosphere (80:20, vol/vol; 300 kPa); −, N_2-CO_2 atmosphere (80:20, vol/vol; 300 kPa).
[c] Determined after 24 h of incubation under anaerobic conditions.

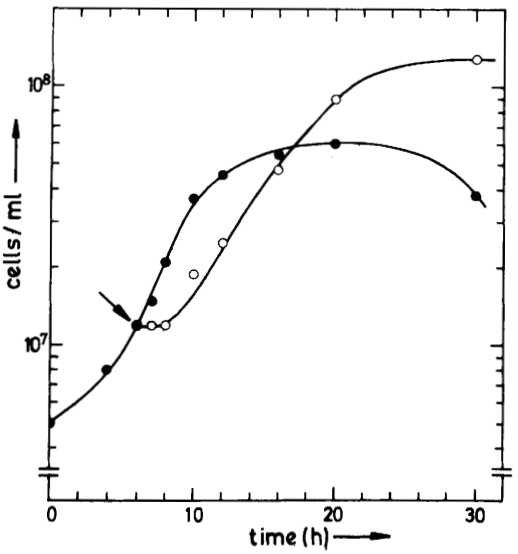

FIG. 1. Growth curves of *A. infernus* So4a^T under aerobic (●) and anaerobic (○) (2) conditions. A sample of the aerobically growing culture was reduced (arrow) and then incubated separately under anaerobic conditions.

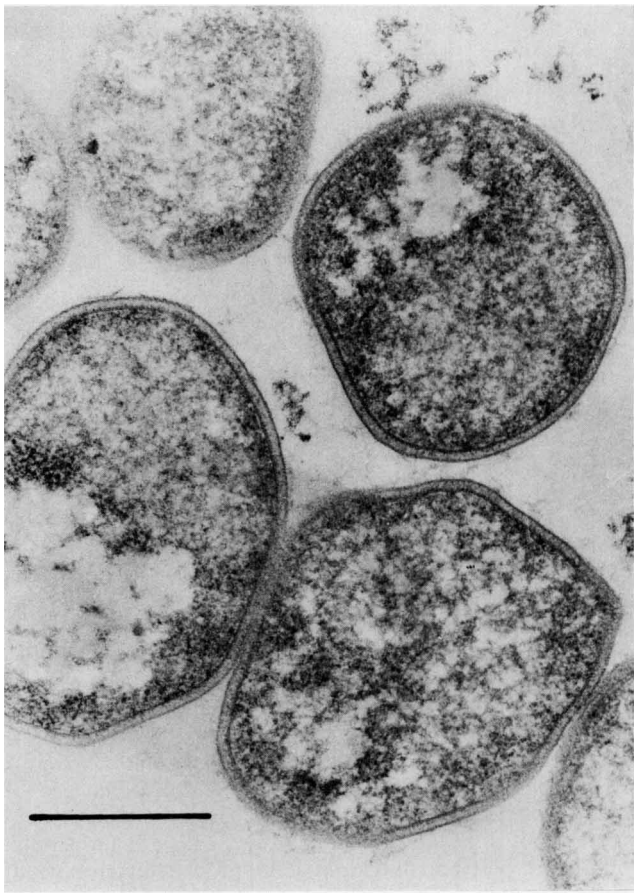

FIG. 2. Electron micrograph of a thin section of *A. infernus* So4a^T. Bar = 0.5 μm.

solfatara fields (37), the ecological significance of this reaction is unclear.

Criteria for a pure culture. Strain So4a^T was cloned by plating. Further evidence for the purity of this strain came from the genetic identity of aerobically and anaerobically grown cells (33) (Table 3) and the very short lag time in growth after a culture was switched from aerobic to anaerobic conditions (Fig. 1).

On the basis of the unique metabolic properties and the distinct common G+C contents (31 mol%) of our isolates and *S. brierleyi* DSM 1651^T, we describe below the new genus *Acidianus*, with *Acidianus infernus* as the type species. This genus comprises the species *Acidianus infernus* sp. nov. (type strain, DSM 3191) and *Acidianus brierleyi* comb. nov. (type strain, DSM 1651); previously, the latter organism has been placed in the genus *Sulfolobus* (8, 15, 32, 45).

Description of *Acidianus* gen. nov. *Acidianus* (A.ci.d.ia'nus. L. masc. adj. *acidus* acid; L. masc. n. *Ianus* a mythical Roman figure with two faces looking in opposite directions; M. L. masc. n. *Acidianus* acidic bifaced [bacterium], reflecting the growth conditions and the metabolism of the organisms) cells are gram negative and are found almost exclusively singly; the cells have an irregular coccoid morphology (sometimes lobed or showing sharp bends and looking like tetrahedrons, pyramids, disks, or dishes). They are nonmotile. The cell width is between 0.5 and 2 μm,

TABLE 3. Levels of DNA homology for three facultatively aerobic isolates and *Sulfolobus* type strains

Source of filter-bound DNA	% Homology with the following sources of ^{32}P-labeled DNA:						
	Isolate So4a^T grown aerobically	Isolate So4a^T grown anaerobically	Isolate Vc11[a]	Isolate Vc16[a]	*A. brierleyi* DSM 1651^{T}[a]	*S. acidocaldarius* DSM 639^T	*S. solfataricus* DSM 1616^T
Isolate So4a^T grown aerobically	100	100	ND[b]	ND	24	23	ND
Isolate So4a^T grown anaerobically	94	100	86	94	16	7	11
Isolate Vc11[a]	ND	88	100	96	5	1	4
Isolate Vc16[a]	ND	85	92	100	4	5	8
A. brierleyi DSM 1651^{T}[a]	19	7	10	9	100	6	10
S. acidocaldarius DSM 639^T	12	14	6	8	ND	100	13
S. solfataricus DSM 1616^T	ND	6	7	10	ND	11	100

[a] Grown anaerobically.
[b] ND, Not determined.

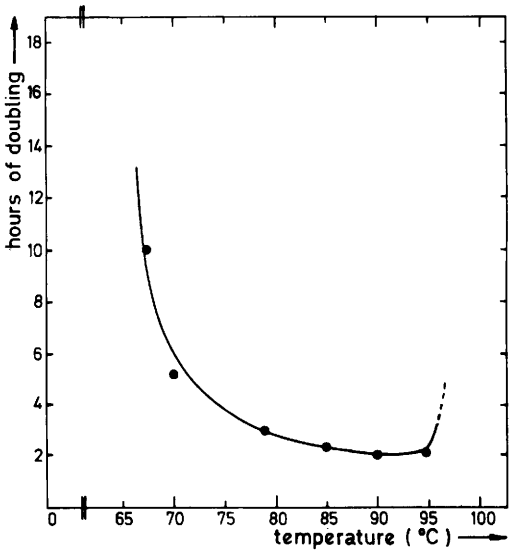

FIG. 3. Optimal growth temperature for *A. infernus* So4a^T grown under anaerobic conditions.

depending on the culture conditions. Thin sections reveal a surrounding envelope about 25 nm wide covering the cell membrane (Fig. 2). The envelope is composed of subunits in a hexagonal array (45; U. Sleytr, personal communication).

The cells are facultative aerobes. Lithotrophic growth occurs aerobically by means of S^0 oxidation or anaerobically by means of S^0 reduction with H_2. The cells are autotrophic and mixotrophic and may or may not grow heterotrophically on yeast extract without S^0 in the presence of O_2.

The organisms are thermoacidophiles, thriving between pH 1 and 6 and between 45 and 96°C. They grow in the presence of 0.1 to 4% NaCl. Neither muramic acid nor *meso*-diaminopimelic acid is present, indicating the absence of murein (19).

Elongation factor 2 is sensitive to adenosine diphosphate ribosylation by diphtheria toxin (21, 22; F. Klink, personal communication).

Cells contain isopranyl ether lipids (26; T. Langworthy, personal communication) calditol (9, 25; Langworthy, personal communication), and caldariellaquinone (10, 25; M. D. Collins, personal communication).

Acidianus spp. are resistant to vancomycin, ampicillin, and kanamycin (150 μg/ml each).

DNA-dependent ribonucleic acid polymerase shows incomplete immunological cross-reactions with antibody against DNA-dependent ribonucleic acid polymerase from *S. acidocaldarius* DSM 639^T (45).

The purified DNA has a G+C content of about 31 mol%.

Members of the genus *Acidianus* occur in acidic solfataras and in marine hydrothermal systems.

The type species is *Acidianus infernus*.

Description of *Acidianus infernus* sp. nov. *Acidianus infernus* (in.fer'nus. L. masc. adj. *infernus* emerged from Hades, referring to the place of isolation at the Solfatara Crater, where Dante suggested the gate to hell was in his *Divina Commedia*) exponentially growing cells are about 0.5 to 1.5 μm wide in an aerobic cultures and 0.8 to 2 μm wide in aerobic cultures, becoming smaller in the stationary growth phase. Packed cells from aerobic cultures are ochre colored, and packed cells from extremely anaerobic cultures are greenish black. Growth is obligately chemolithotrophic and S^0 dependent by oxidation or reduction of S^0. Cells grow at temperatures ranging from 65 to 96°C, with optimum growth at around 90°C, as shown for strain So4a^T (Fig. 3). No growth is detected at 60 and 98°C. The pH suitable for growth ranges from 5.5 to 1.0, with the optimum pH around 2 (isolate So4a^T) (data not shown). No growth is obtained at pH 0.5 or 6.0. Strains So4a^T, Vc11, and Vc16 exhibit a broad salt tolerance, with an optimum around 0.2% NaCl (data not shown); this tolerance is independent of the salinity of their place of origin (7) (Table 1). Anaerobically grown cultures of isolate So4a^T at pH 2 can be used for at least 2 years as inocula, when they are stored at 4°C. However, we were unable to obtain growth from aerobically grown cultures after storage at pH 2 and 4°C for 2 months. Viability of aerobic cultures can be increased to about 4 months by raising the pH to 5.5 by adding $CaCO_3$. Cells lyse at pH values greater than 8.5.

Isolates were obtained from hot water, mud, and marine sediments at geothermal springs in Italy, the Azores, Iceland, and the United States (Table 1).

The type strain is strain So4a (= DSM 3191).

Emended description of *Acidianus brierleyi* comb. nov. *Acidianus brierleyi* (brier'ley.i. M. L. gen. n. *brierleyi* of Brierley, named for J. Brierley, the American bacteriologist who isolated this organism) cells are about 1 to 1.5 μm wide. Packed cells from aerobic cultures are ochre colored and packed cells from extremely anaerobic cultures are grayish black. Growth is chemolithotrophic by means of oxidation or reduction of S^0 or by means of oxidation of ferrous iron. Organotrophic growth occurs in the presence of O_2 and yeast extract, peptone, tryptone, beef extract, or Casamino Acids. The temperature range for growth is 45 to 75°C; the optimum temperature is 70°C. The pH range for growth is 1 to 6; the optimum pH is 1.5 to 2. Anaerobically grown cultures at pH 2 cannot be used as inocula when they are stored for more than 4 months at 4°C. The viability of such cultures can be increased up to 6 to 8 months by raising the pH to 5.5 by adding $CaCO_3$.

Cells lyse at pH 7.

The DNA-dependent ribonucleic acid polymerase consists of nine subunits with different molecular weights; it requires Mg^{2+} and is resistant to rifampin and streptolydigine.

The organism was isolated from an acidic solfataric spring.

The type strain is strain DSM 1651.

The new isolates described here are extreme thermoacidophiles which were obtained from distant acidic geo- and hydrothermally heated areas; therefore, these organisms may be distributed worldwide in similar biotopes. Although these organisms grow in laboratories only at temperatures ranging from 65 to 96°C at pH 1 to 5.5, they were also isolated from places with much lower temperatures (e.g., 40°C) and higher pH values (e.g., pH 6.5). These bacteria may thrive at such places only within distinct temperatures and pH gradients formed by small hot fumaroles which heat the surface water or sediments. Because of their broad salt tolerance, the new isolates are found within solfatara fields with low ionic strength (7), as well as in hydrothermal seawater in high-ionic-strength environments. Because of their unique ability to employ two contrary modes of chemolithotrophy, in which S^0 is oxidized or reduced depending on the oxygen supply (30), these organisms are capable of growing in the oxygen-rich surfaces of solfataric biotopes and at reduced depths that are well supplied with H_2 and CO_2 by escaping volcanic gasses (37).

The new organisms, represented by strain So4a^T, belong to the archaebacteria on the basis of their lack of murein

(19), their resistance to the antibiotics vancomycin, ampicillin, and kanamycin (13), the occurrence of isopranyl ether lipids in their cells (26; Langworthy, personal communication), and the presence of elongation factor 2 which is sensitive to diphtheria toxin (21, 22; Klink, personal communication).

Isolate So4a^T, which is described above, resembles the type species of *Thermoproteus*, *T. tenax*, in its anaerobic H_2-S^0 lithotrophy (11, 44), but strongly differs in its shape, its ability to grow aerobically, its extremely acidophilic mode of life, and its much lower G+C content (16, 44). On the other hand, it shows a distinct similarity to the type species of *Sulfolobus*, *S. acidocaldarius* (8, 32), in its morphology, its extreme thermoacidophily, its lithotrophy based on S^0 oxidation, the presence of calditol and caldariellaquinone (25), the same strongly acidic biotope, and the low, although different, G+C content of its DNA (30, 45). However, isolate So4a^T diverges from the *Sulfolobus* type species and from *S. solfataricus* by its lower G+C content and its ability to grow anaerobically by means of S^0 reduction. *A. brierleyi* has the same G+C content as isolate So4a^T and is also able to grow anaerobically by means of S^0 reduction; the latter feature was not recognized in its earlier description (15, 45). It differs from So4a^T by its ability to grow organotrophically without S^0 in the presence of O_2 (4, 45), by its much lower growth temperature, and by the lack of significant DNA homology, indicating that it is a separate species (33) (Table 3). Marine isolates Vc11 and Vc16 exhibit a high degree of DNA homology with So4a^T and each other (Table 3), indicating that they belong to the same species. There is also no significant DNA homology between *A. brierleyi* DSM 1651^T and *S. acidocaldarius* DSM 639^T and *S. solfataricus* DSM 1616^T. As determined by 16S ribosomal ribonucleic acid cataloging, *S. acidocaldarius* DSM 639^T and *S. solfataricus* DSM 1616^T show a similarity coefficient of only 0.41 (40), indicating a large phylogenetic difference between these two species of the same genus. However, no further distinguishing taxonomic markers are present now to justify their placement in different genera.

ACKNOWLEDGMENTS

We thank Roswitha Semmler and Helmut König for preparation of thin sections and electron micrographs, Matthew D. Collins, Friedrich Klink, Thomas A. Langworthy, and Uwe B. Sleytr for providing unpublished data, Christine Stadler for preparation of the manuscript, and the Icelandic government and the United States Department of the Interior National Park Service for research permits.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 43) and the Fonds der Chemischen Industrie to K.O.S.

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