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Geovibrio thiophilus sp. nov., a novel sulfur-reducing bacterium belonging to the phylum *Deferribacteres*

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Strain AAFu3^T (= DSM 11263^T = ATCC BAA-311^T), a Gram-negative, non-sporulating bacterium, was isolated from a methanogenic mixed culture growing with acetone, in which acetate was the major intermediate. The cells of strain AAFu3^T were slender spirilla, usually of less than one turn, and were motile by means of a single polar flagellum. The cells contained *c*-type cytochromes and the G+C content of the genomic DNA was 50·2 mol%. Sulfur, nitrate, fumarate, DMSO and oxygen (microaerophilically) were used as electron acceptors, but sulfate, sulfite, thiosulfate and ferric iron were not. Sulfide, hydrogen, formate and acetate acted as electron donors for respiratory growth, while fumarate, maleate and L-malate supported fermentative growth. Neither fermentative nor respiratory growth was supported by carbohydrates, fatty acids more than two carbons long, alcohols or amino acids. The strain was a mesophile. Comparative sequence analysis of the 16S rRNA gene and comparison of phenotypic characteristics showed that strain AAFu3^T is closely related to *Geovibrio ferrireducens*, within the phylum *Deferribacteres*. Strain AAFu3^T was designated as the type strain of a new species, for which the name *Geovibrio thiophilus* is proposed.

Keywords: *Geovibrio thiophilus* sp. nov., sulfur reduction, microaerophile, anaerobe

We have been studying a mixed culture of anaerobic micro-organisms that degrades acetone to methane and carbon dioxide (Platen & Schink, 1987; Platen *et al.*, 1994). The mixed culture consists of (1) a rod-shaped bacterium that is postulated to convert acetone to acetate, (2) a methanogen, *Methanosaeta concilii*, that metabolizes the acetate produced by the rod-shaped bacterium to methane and carbon dioxide, and (3) a slender, curved bacterium with no known role in the mixed culture. We isolated this third bacterium, which we have named strain AAFu3^T, in pure culture and found that it could not metabolize acetone (Platen *et al.*, 1994). To understand its possible role in the mixed culture, we characterized this strain in detail.

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The GenBank accession number for the 16S rRNA sequence of strain AAFu3^T is AJ299402.

Growth

Strain AAFu3^T was routinely grown in the sulfide-reduced, bicarbonate-buffered, vitamin-supplemented medium FM (Janssen *et al.*, 1997), at 30 °C. Screw-capped bottles were filled, leaving a small gas bubble, or serum bottles were partly filled [the headspace being gassed with N_2 plus CO_2 (80:20, v/v)] and closed with butyl-rubber stoppers. Unless noted otherwise, the growth substrate was 10 mM acetate plus 20 mM fumarate. L-isomers of chiral organic and amino acids and D-isomers of sugars were used, unless noted otherwise. 2,3-Butanediol (Fluka) was a mix of racemic and meso forms. Substrates and other supplements were prepared as neutralized (with NaOH or HCl as required) 200 mM to 2 M stock solutions and sterilized by autoclaving or, in the case of heat-labile compounds and sugars, by sterile filtration (0·2 μm pore size). Substrates were added to sterile media just before inoculation. Elemental sulfur was prepared as an aqueous slurry (Janssen *et al.*, 1997) and added at approximately 100 mmol l^{-1}. Hydrogen was added to the N_2/CO_2 headspace of partially filled serum bottles

to an overpressure of 0·6 bar. Oxygen was added to the N_2/CO_2 headspace of similar cultures to the desired final partial pressure, using FM without Na_2S and with 2 mM $Na_2S_2O_3$ as a sulfur source. Nitrate, sulfate and thiosulfate were added as sodium salts. Amorphous ferric iron was prepared as described by Lovley & Phillips (1986) and added from an autoclaved stock suspension at 30 mmol Fe(III) per litre FM with 2 mM L-cysteine as the reducing agent (instead of Na_2S) and with 2 mM Na_2SO_4 or 1 mM $Na_2S_2O_3$ as an additional sulfur source.

Strain AAFu3^T grew most rapidly at 37 and 40 °C. Growth was possible at 4 °C, but not at 45 °C. All further experiments were incubated at 30 °C. Growth was not inhibited when the NaCl and $MgCl_2.6H_2O$ concentrations in the medium were increased from 1 and 0·4 g l^{-1} to 20 and 3 g l^{-1}, respectively.

General characteristics

Cells of strain AAFu3^T were slender spirilla (Fig. 1a). The cells were 0·35 μm in diameter, 2·5–6 μm long and each had a single polar flagellum (Fig. 1b). The cells were motile, speeds of up to 50–60 μm s^{-1} being commonly observed under phase-contrast microscopy of wet mounts. Sporulation was tested by adding 1 ml sterile soil extract (Cote & Gherna, 1994) to 50 ml medium supplemented with 0·5 g thiamin l^{-1}, 0·5 g $CaCl_2.2H_2O$ l^{-1} and 0·3 g $MnCl_2.4H_2O$ l^{-1}. No spores were observed by phase-contrast microscopy of cultures grown in this medium. The Gram stain (Süßmuth *et al.*, 1987) reaction was negative. Colonies in agar deeps (Widdel & Bak, 1992) were lens-shaped and red in colour. Difference spectra of crude cell-free extracts (Janssen *et al.*, 1996b) of acetate + fumarate-grown cells revealed the presence of *c*-type cytochromes, with absorption maxima of 424, 523·5 and 553·5 nm. The G + C content of the genomic DNA was 50·2 mol % (SD = 0·3, $n = 3$), determined by reversed-phase HPLC (Janssen *et al.*, 1996a). Tests for aesculin hydrolysis, indole production from L-tryptophan and urease activity (Janssen *et al.*, 1997) were all negative.

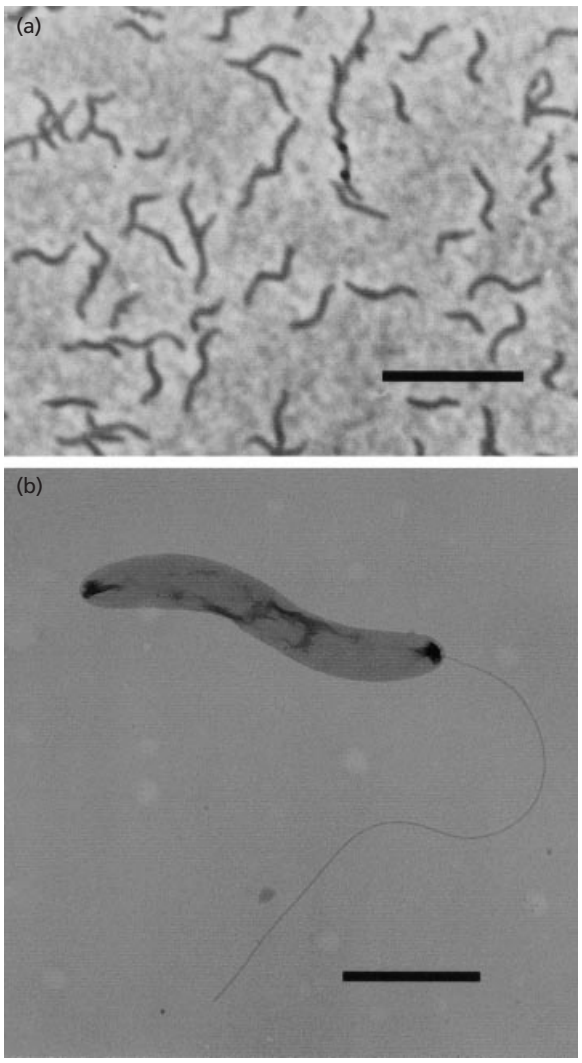

Fig. 1. Morphology of cells of strain AAFu3^T. (a) Living cells on an agar-coated slide (Pfennig & Wagener, 1986) photographed using phase-contrast microscopy. Bar, 10 μm. (b) Electron micrograph (Chin *et al.*, 2001) of a negatively stained cell, showing the polar flagellum. Bar, 1 μm.

Metabolism

Strain AAFu3^T grew fermentatively with fumarate, L-malate and maleate (Table 1). The concentrations of growth substrates and organic end-products of metabolism were measured by ion-exclusion HPLC (Krumböck & Conrad, 1991). Succinate was the sole organic end-product of catabolism of these substrates, 6 mol being produced from 7 mol substrate (Table 2). No acetate was formed. This indicated a complete oxidation of 1 mol substrate to CO_2, the electrons arising from the oxidation being used to reduce fumarate to succinate. In the presence of sulfur, sulfide was formed with formate, acetate, hydrogen and D-malate, but not with L-malate, fumarate or maleate as electron donors. Sulfide production was measured colorimetrically (Cline, 1969). Formate and acetate were no longer detectable after growth and no organic catabolic products were detected, so these substrates were presumably oxidized to CO_2. Succinate was formed from D-malate. Other organic acids, alcohols, sugars and amino acids did not support fermentative growth or sulfur reduction (Table 1). Sulfate (20 mM), sulfite (10 mM), thiosulfate (20 mM), nitrite (2 or 10 mM) and amorphous ferric iron (30 mM) were not used as electron acceptors with acetate as the electron donor. Fumarate, L-malate and formate were also tested as potential electron donors, with ferric iron as the potential electron acceptor, but iron was not reduced, although growth occurred in the cultures containing fumarate and L-malate. This indicated that ferric iron was not inhibitory and that growth could occur in the medium used for these experiments. The addition of

Table 1. Substrates supporting growth of, and sulfur reduction by, strain AAFu3^T

Initial concentrations, in mM, are given in parentheses after each compound. Unless noted otherwise, L-isomers of organic and amino acids and D-isomers of sugars were tested. +, Growth or sulfide production observed; −, no growth or sulfide production observed. Compounds not used: glucose (4), cellobiose (2), fructose (4), L-rhamnose (4), xylose (4), galactose (4), lactose (2), mannose (4), maltose (2), ribose (4), sucrose (2), arabinose (4), melibiose (2), succinate (20), lactate (20), citrate (20), DL-3-hydroxybutyrate (20), crotonate (20), tartrate (20), pyruvate (20), acetone (10), methanol (20), ethanol (20), propanol (10), butanol (10), pentanol (5), ethylene glycol (20), glycerol (20), acetoin (20), mannitol (20), 2,3-butanediol (20), alanine (20), glutamate (20), glycine (20), threonine (20), lysine (20), aspartate (20), valine (20) and leucine (20). Propionate (10), butyrate (5), valerate (5), caprate (2), palmitate (1) and stearate (1) were tested only in the presence of sulfur.

Compound used	Fermentative growth	Growth in the presence of sulfur	Sulfur reduction
Fumarate (20)	+	+	−
Maleate (20)	+	+	−
L-Malate (20)	+	+	−
D-Malate (20)	−	−	+
Hydrogen*	−	+	+
Formate (20)	−	+	+
Acetate (10)	−	+	+

* Added at 0·6 bar in the headspace (70 ml) over a liquid culture (50 ml).

Table 2. Comparison of the utilization of growth substrates and electron acceptors with the products formed by strain AAFu3^T

NQ, Not quantified.

Substrate	Amount utilized (mmol l^{-1})	Acceptor	Amount utilized (mmol l^{-1})	Product	Amount formed (mmol l^{-1})
Fumarate	13·70	None		Succinate	10·66
Acetate	5·61	Fumarate	19·08	Succinate	19·13
Acetate	2·32	Sulfur	NQ	Sulfide	10·23
Acetate	3·68	DMSO	NQ	Dimethylsulfide	13·06
Acetate	12·94	Nitrate	NQ	Ammonium	7·22
Hydrogen	10·91	Sulfur	NQ	Sulfide	10·14
Formate	2·21	Sulfur	NQ	Sulfide	2·54
Sulfide	2·66	Nitrate	NQ	Sulfur	2·02
				Nitrite	2·13
				Ammonium	0·36

2 mM nitrilotriacetic acid or 20 mM citric acid as a chelating agent (each from a stock solution that had been adjusted to pH 8·0 with NaOH) did not facilitate the utilization of ferric iron as an electron acceptor, even after 3 months incubation. Sulfate and thiosulfate were added individually as sulfur sources in the presence of L-cysteine in the experiments testing the use of ferric iron as the potential electron acceptor, in case the inability to grow with ferric iron was really due to the lack of a suitable sulfur source in the absence of sulfide, but these additions did not result in growth with ferric iron. Sulfide was not formed from L-cysteine. Acetate was oxidized with fumarate (20 mM), with nitrate (20 mM) or with DMSO (20 mM) as the electron acceptor (Table 2). Fumarate was reduced to succinate, nitrate to nitrite and ammonium, and DMSO to dimethylsulfide (Table 2). Colorimetric assays were used to measure the concentrations of ammonia (Chaney & Marbach, 1962) and nitrite (Drews, 1983). Dimethylsulfide production was measured by GC (Kappler *et al.*, 1997). Cultures growing with hydrogen and sulfur with no added organic compounds other than the vitamins in the medium could be repeatedly transferred, although growth was

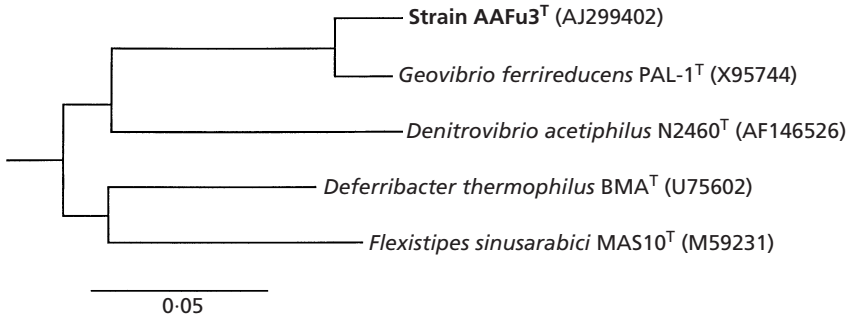

Fig. 2. 16S rRNA gene-based distance dendrogram showing the phylogenetic position of strain AAFu3^T in relation to currently recognized members of the phylum *Deferribacteres*. Evolutionary distances were calculated using the Jukes–Cantor correction (Jukes & Cantor, 1969) and 1324 alignment positions which contained identical nucleotides in at least 50% of the 16S rRNA gene sequences were compared (Friedrich *et al.*, 1996; Finster *et al.*, 1998). The dendrogram was constructed from the resulting distance matrix using the neighbour-joining technique (Saitou & Nei, 1987). The membership of all five organisms in this lineage was confirmed in all neighbour-joining treeing analyses (1000 data resamplings) with bootstrap values $>95\%$ (data not shown). The root was determined in relation to a set of outgroup bacterial reference sequences (not shown). The GenBank accession numbers are shown in parentheses. Scale bar, 0·05 changes per nucleotide position.

slower than when 2 mM acetate was added. This suggested that strain AAFu3^T may be able to grow autotrophically, but the capacity for CO_2 fixation was not investigated.

Strain AAFu3^T could not grow with acetate under a gas headspace containing 2 or 20% oxygen. No colonies developed in the upper 20 mm of agar deep cultures growing with acetate plus fumarate under a headspace of $N_2/CO_2/O_2$ (3:1:1, by vol.). Cultures were also grown in opposing gradients of oxygen and sulfide prepared by using a modification of the method of Nelson & Jannasch (1983). These 'gradient cultures' were prepared in test tubes (150 × 18 mm), capped with Kim-Kap polypropylene closures (Kimble-Kontes). Three millilitres of anoxic 3% (w/v) agar containing a potential growth substrate were overlaid with 10 ml anoxic FM without sulfide containing 0·3% agar and inoculated with 0·1 ml well-grown culture. When sulfide was the growth substrate being tested, 10 mM Na_2S neutralized with 13 mM HCl was added to the lower agar layer. A bacterial plate less than 0·3 mm thick formed about 3 mm under the surface of the upper agar layer in these cultures. The plate consisted of highly motile spiral-shaped cells and refractile globules that were presumably sulfur. No such plate formed in uninoculated tubes or in tubes incubated under a headspace of N_2/CO_2 (4:1, v/v). This suggested that strain AAFu3^T can oxidize sulfide under micro-oxic conditions, gaining energy from the chemolithotrophic oxidation of the reduced sulfur species. Growth in sulfide and oxygen gradients occurred in the absence of added acetate, providing additional evidence that autotrophic growth may be possible. No growth occurred in opposing gradients of oxygen and other substrates [10 mM acetate, 20 mM formate ($\pm$1 mM acetate in the upper agar layer), 20 mM fumarate, 20 mM L-malate or 20 mM maleate], with either 2 mM Na_2SO_4 or 1 mM $Na_2S_2O_3$ as the potential sulfur source. It is possible that strain AAFu3^T could not grow with organic substrates in these experiments because sulfide was required as a sulfur source or to reduce the oxygen partial pressure. The oxygen partial pressures allowing growth have not been determined, nor have all of the potential end-products of sulfide oxidation been assayed. Cultures were able to oxidize sulfide to sulfur, which was measured spectrophotometrically in benzene extracts of cell suspensions (Javor *et al.*, 1990), when nitrate was added to the growth medium (Table 2).

Strain AAFu3^T has a very versatile metabolic repertoire, even though the range of energy sources it can use is relatively small. In the presence of sulfur or other electron acceptors, a range of products of fermentation can be oxidized in growth-supporting reactions. Growth is also possible under micro-oxic conditions. This suggests that the natural habitat of strain AAFu3^T is near the oxic/anoxic interface of freshwater sediments. The rapid motility displayed by the strain could enable the bacterium to position itself within gradients of oxygen and sulfide, or to move towards areas with favourable substrates. The persistence of strain AAFu3^T within the enrichment culture from which it was isolated is explained by its flexible metabolism: it can use acetate, the major intermediate in the mixed culture (Platen *et al.*, 1994), and either oxygen, which diffuses even through butyl-rubber stoppers during the long incubation times required for cultivation of the mixed culture, or sulfur, which may be formed by the chemical oxidation of the sulfide reductant used in the growth medium. The strain may not have been involved in a direct metabolic or nutritional association with the other organisms present in the acetone-degrading consortium, comprising *M. concilii* and an unidentified bacterium (Platen & Schink, 1987; Platen

Table 3. Characteristics of strain AAFu3^T and *G. ferrireducens*

Data on *G. ferrireducens* are from Caccavo *et al.* (1996). +, Characteristic present; −, characteristic absent; NR, not reported.

Characteristic	Strain AAFu3^T	*G. ferrireducens*
Cell shape	Spiral	Spiral
Flagella	Single polar	Single polar
G+C content of genomic DNA (mol%)	50	43
Cytochromes	*c*-type	*c*-type
Electron acceptors used		
Ferric iron	−	+
Sulfur	+	+
Nitrate	+	−
Sulfate, sulfite, thiosulfate	−	−
Oxygen (microaerophilic)	+	NR
Oxygen (20% in air)	−	−
Electron donors used		
Acetate	+	+
Propionate	−	+
Hydrogen	+	+
Formate	+	−
Lactate	−	+
Pyruvate	−	+
Succinate	−	+
Proline	−	+
Sulfide	+	NR
Fermentative growth	+	−

et al., 1994), in the original sediment system from which the culture was derived. The degree of spatial heterogeneity over small distances in freshwater sediments (Brune *et al.*, 2000) means that a sample of a surface sediment will encompass many different habitats. We conclude that strain AAFu3^T is probably not directly involved in acetone metabolism by the consortium. Nevertheless, it may still contribute to the stability of the mixed culture, perhaps by providing essential growth factors for the other members, or by scavenging oxygen that diffuses through the butyl-rubber stoppers, thus protecting the strictly anaerobic *M. concilii* and perhaps also the unidentified acetone-catabolizing bacterium.

Phylogenetic analysis

The almost complete sequence of the 16S rRNA gene of strain AAFu3^T was determined, as described by Finster *et al.* (1998). The gene sequence was added to a database of about 7200 bacterial 16S rRNA gene sequences with at least 1300 unambiguously determined nucleotide sequence positions, using the automatic alignment tool of the ARB program package and manual editing of the resulting alignment. This database is part of the ARB program package [version 2.5b, developed by O. Strunk and W. Ludwig, Technische Universität München (http://www.arb-home.de)].

The initial analysis clearly showed that strain AAFu3^T was phylogenetically affiliated with members of the phylum *Deferribacteres* (Garrity & Holt, 2001). Phylogenetic placement of strain AAFu3^T was carried out in relation to the currently recognized members of the phylum *Deferribacteres* (Garrity & Holt, 2001) and a set of reference sequences representative of other main lines of bacterial descent. The phylum *Deferribacteres*, equivalent to the division *Flexistipes* of Hugenholtz *et al.* (1998), is characterized by only four described genera and species (Fig. 2). These are *Geovibrio ferrireducens* (Caccavo *et al.*, 1996), *Flexistipes sinusarabici* (Fiala *et al.*, 1990), *Deferribacter thermophilus* (Greene *et al.*, 1997) and *Denitrovibrio acetiphilus* (Myhr & Torsvik, 2000). Strain AAFu3^T is most closely related to *G. ferrireducens* strain PAL-1^T (Fig. 2). The two strains share an overall similarity in their 16S rRNA gene sequences of 96·5%, while the corresponding values to *F. sinusarabici* strain MAS10^T, *Deferribacter thermophilus* strain BMA^T and *Denitrovibrio acetiphilus* strain N2460^T are clearly lower, i.e. 84·4, 85·6 and 85·0%, respectively.

The metabolic capabilities of strain AAFu3^T increase the already wide range of metabolisms found in the few members of the phylum *Deferribacteres* that have been studied to date. Strain AAFu3^T displays phenotypic characteristics that differentiate it from *G. ferrireducens* (Table 3). The most significant of these is the

apparent inability to reduce ferric iron, which is a key characteristic of *G. ferrireducens* (Caccavo *et al.*, 1996). However, the similarity of the 16S rRNA gene sequences and the similarities in morphology and general mode of metabolism suggest that AAFu3^T and *G. ferrireducens* are closely related. We propose that strain AAFu3^T be designated the type strain of a new species within the genus *Geovibrio*, for which the name *Geovibrio thiophilus* sp. nov. is proposed. The description is based on the type and only strain available at present.

Description of *Geovibrio thiophilus* sp. nov.

Geovibrio thiophilus (thi.o′phi.lus. Gr. n. *theion* sulfur; Gr. adj. *philos* loving; M.L. masc. adj. *thiophilus* sulfur-loving).

Gram-negative, spiral-shaped cells, 0·35 μm in diameter and 2·5–6 μm long, each motile by means of a single polar flagellum. Spores are not formed. Cells occur singly. Cells contain *c*-type cytochromes. The G+C content of the genomic DNA is 50·2 mol%. Uses sulfur, nitrate, fumarate, DMSO and oxygen (microaerophilically) as electron acceptors. Sulfide, hydrogen, formate and acetate act as electron donors for respiratory growth. Sulfate, sulfite, thiosulfate, nitrite and ferric iron are not used as electron acceptors. Fumarate, maleate and L-malate support fermentative growth. Neither fermentative nor respiratory growth is observed with carbohydrates, fatty acids more than two carbons long, acetone, alcohols or amino acids. Grows best in a growth medium with less than 20 g NaCl l^{-1}. Grows most rapidly at 37–40 °C. Growth is possible at 4 °C, but not at 45 °C. Sequence analysis of the 16S rRNA gene shows that the species is closely related to *Geovibrio ferrireducens*. The type strain, AAFu3^T, was isolated from the surface sediments of a ditch in Konstanz, Germany, and has been deposited in the DSMZ as DSM 11263^T and in the American Type Culture Collection as ATCC BAA-311^T.

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