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Sporanaerobacter acetigenes gen. nov., sp. nov., a novel acetogenic, facultatively sulfur-reducing bacterium

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A strictly anaerobic, moderately thermophilic, sporulating rod, designated strain Lup 33^T, was isolated from an upflow anaerobic sludge blanket (UASB) reactor in Mexico. Strain Lup 33^T possessed a few laterally inserted flagella, had a DNA G+C content of 32·2 mol% and grew optimally at pH 7·4 and 40 °C. Growth was observed at temperatures of up to 50 °C and was inhibited in the presence of 5% NaCl. Strain Lup 33^T is heterotrophic and utilized some sugars, peptides and various single amino acids. Gelatin and casein were not used as energy sources. It performed the Stickland reaction and reduced elemental sulfur to sulfide. Acetate was the only fatty acid detected from glucose fermentation, whereas acetate together with isobutyrate and isovalerate were found as end products from peptone fermentation. Phylogenetically, strain Lup 33^T branched with members of cluster XII of the order *Clostridiales*, with *Clostridium hastiforme* as the closest relative (similarity of 93%). On the basis of the phenotypic, genotypic and phylogenetic characteristics of the isolate, it is proposed as a novel species of a new genus, *Sporanaerobacter acetigenes* gen. nov., sp. nov. The type strain is strain Lup 33^T (= DSM 13106^T = CIP 106730^T).

Keywords: *Sporanaerobacter acetigenes*, acetogenic, sulfur reduction, taxonomy, phylogeny

INTRODUCTION

During the last two decades, 16S rRNA gene sequence analysis has provided new and valuable insights into the phylogenetic interrelationships between micro-organisms (Woese, 1987) and is now considered essential for bacterial taxonomy. It has been very helpful in designating novel micro-organisms at the species level and in reorganizing bacterial genera taxonomically. This is especially true for the genus *Clostridium*, order *Clostridiales*, first defined as containing Gram-positive, anaerobic, rod-shaped and spore-forming bacteria unable to carry out dissimilatory sulfate reduction (Cato *et al*., 1986; Hippe *et al*., 1992). Most members of this genus are ubiquitous chemo-organotrophic micro-organisms that may use carbohydrates and/or proteinaceous compounds as energy sources (Cato *et al*., 1986; Hippe *et al*., 1992). Although these bacteria have long been recognized as phenotypically heterogeneous, it was only with the availability of both oligonucleotide cataloguing and 16S rRNA sequencing data that it became possible to appreciate their phylogenetic diversity (Cato & Stackebrandt, 1989; Collins *et al*., 1994; Lawson *et al*., 1993; Rainey & Stackebrandt, 1993; Rainey *et al*., 1993; Stackebrandt & Rainey, 1997). After studying the phylogeny of the genus *Clostridium* on the basis of numerous clostridial 16S rRNA gene sequences, Collins *et al*. (1994) proposed five new genera and 11 new species combinations. Clostridia and their relatives were finally distributed within 19 clusters, some of them (e.g. cluster XII) containing a phylogenetically

Abbreviations: COD, chemical oxygen demand; UASB, upflow anaerobic sludge blanket.

The GenBank accession number for the 16S rRNA gene sequence of strain Lup 33^T is AF358114.

and phenotypically diverse assemblage of micro-organisms. One of the important results reported by these authors was a phylogenetic intermixing of spore-forming and non-spore-forming micro-organisms. These observations led to the reconsideration of the value of spore formation as a phenotypic character in bacterial taxonomy.

In this study, we report the isolation of a novel glycolytic, spore-forming bacterium that reduces elemental sulfur to sulfide and belongs to cluster XII of the order *Clostridiales*. Because of its peculiar phenotypic and phylogenetic characteristics, we propose to designate it as a novel species of a new genus of the order *Clostridiales*, *Sporanaerobacter acetigenes* gen. nov., sp. nov.

METHODS

Sample collection and sample source. The sludges used as inocula for the enrichments originated from the anaerobic digesters of three private Mexican companies: la Caperucita, Imexa and Unipak. Reactors were of the upflow anaerobic sludge blanket (UASB) type and had been operating for several years at the time of sampling. They were initially inoculated with sludge from different origins: (i) sludge from the anaerobic lagoon of another local cheese factory for Caperucita, (ii) sludge from a UASB reactor treating wastewater from citrus processing in California (USA) for Unipak and (iii) a mixture of granular sludges from two UASB systems treating paper wastewater in Mexico and brewery wastewater in the USA, for Imexa. The reactors were operated with different hydraulic retention times and chemical oxygen demand (COD) loading rates. Wastewaters treated contained a very broad range of organics, COD concentrations and COD/SO_4^{2-} ratios. The pH of the liquid phase in all reactors was around 7 and the temperature was between 32 and 35 °C, except for the cheese factory, where it was around 23 °C. The sludges of the three reactors were collected by completely filling 500 ml sterile plastic bottles, which were maintained at room temperature until processing.

Enrichment, isolation and growth conditions. The Hungate technique (Hungate, 1969) was used throughout this study. The basal medium (BM) contained (l^{-1} distilled water): 1 g NH_4Cl, 3 g K_2HPO_4, 3 g KH_2PO_4, 0·2 g $MgCl_2.6H_2O$, 0·1 g $CaCl_2.2H_2O$, 0·1 g KCl, 0·6 g NaCl, 0·5 g cysteine hydrochloride, 1 mg resazurin and 10 ml trace mineral element solution (Balch *et al.*, 1979). The pH was adjusted to 7·4 with 10 M KOH. The medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Five ml aliquots were dispensed into Hungate tubes and 20 ml in serum bottles under a stream of N_2/CO_2 (80:20, v/v) gas and the sealed vessels were autoclaved for 45 min at 110 °C. Prior to inoculation, $Na_2S.9H_2O$ and $NaHCO_3$ were injected from sterile stock solutions to final concentrations of 0·04 and 0·2 % (w/v), respectively. Enrichment was performed in 120 ml serum bottles. Peptone (5 g l^{-1}) and thiosulfate (20 mM) were added to BM as the electron donor and acceptor, respectively. The serum bottle was inoculated with 4 ml sludge, corresponding to 10 % (v/v) of the final liquid volume (40 ml). After inoculation, the serum bottle atmosphere was changed to H_2 at a final pressure of $2{\cdot}026 \times 10^5$ Pa. The bottles were incubated at 35 °C in a controlled temperature room for 2–3 weeks. Isolation was performed in the same medium with N_2 instead of H_2 in the gas phase. Four enrichment series were performed before isolation.

pH, temperature and NaCl concentration ranges for growth. Growth experiments were performed in duplicate using Hungate tubes containing BM. For pH growth experiments, medium without NaCl was adjusted to different pH values by injecting $NaHCO_3$ or Na_2CO_3 from 10 % (w/v) sterile anaerobic stock solutions and incubated at 40 °C. The temperature range for growth was determined using the culture medium without NaCl, adjusted to the optimum growth pH. For studies on NaCl requirements, NaCl was weighed directly into Hungate tubes and the culture medium, adjusted to pH 7·4, was dispensed into tubes as described above. The tubes were incubated at 40 °C. The strain was subcultured at least once under the same experimental conditions prior to inoculation for growth experiments.

Tests for sporulation. The ability to produce endospores was checked by growth in an appropriate medium (Mouné *et al.*, 2000). The presence of spores was determined by microscope examination of the culture at different phases of growth.

Substrate utilization tests. For substrate utilization tests, BM supplemented with 1 g yeast extract l^{-1} (Difco Laboratories) was used. The substrates were injected into Hungate tubes to a final concentration of 10 mM for amino acids, 5 g l^{-1} for peptides and proteins and 20 mM for sugars, fatty acids and alcohols. All substrates were tested in the presence of elemental sulfur (2 %, w/v, final concentration). To test for sulfur-containing electron acceptors, sodium thiosulfate (20 mM), sodium sulfate (20 mM), elemental sulfur (2 %) and sodium sulfite (2 mM) were added to the growth medium. Nitrate (10 mM) and nitrite (10 mM) were also tested as potential electron acceptors.

Light and electron microscopy. Light and electron microscopy were performed as described previously (Cayol *et al.*, 1994).

Analytical techniques. Growth was measured by inserting Hungate tubes directly into a model UV-160A spectrophotometer (Shimadzu) and measuring the optical density at 580 nm. Sulfide was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). Hydrogen and fermentation products (alcohols and volatile and non-volatile fatty acids) were quantified as described previously (Fardeau *et al.*, 1993). Amino acid concentrations were determined by HPLC (Moore *et al.*, 1958).

Determination of G+C content. The G+C content of DNA was determined at the DSMZ. The DNA was isolated and purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977) and the G+C content was determined by HPLC as described by Mesbah *et al.* (1989). Non-methylated lambda DNA (Sigma) was used as the standard.

16S rRNA sequence studies. The methods for the purification and extraction of DNA and the amplification and sequencing of the 16S rRNA gene have been described previously (Andrews & Patel, 1996; Love *et al.*, 1993; Redburn & Patel, 1993). The 16S rRNA gene sequence determined was aligned manually with reference sequences of various members of the domain *Bacteria* by using the editor ae2 (Maidak *et al.*, 1996). Reference sequences were obtained from the Ribosomal Database Project (Maidak *et al.*, 1996), EMBL and GenBank databases. Positions of sequence and alignment uncertainty were omitted from the analysis. A phylogenetic analysis was performed by using the various programs implemented as part of the PHYLIP

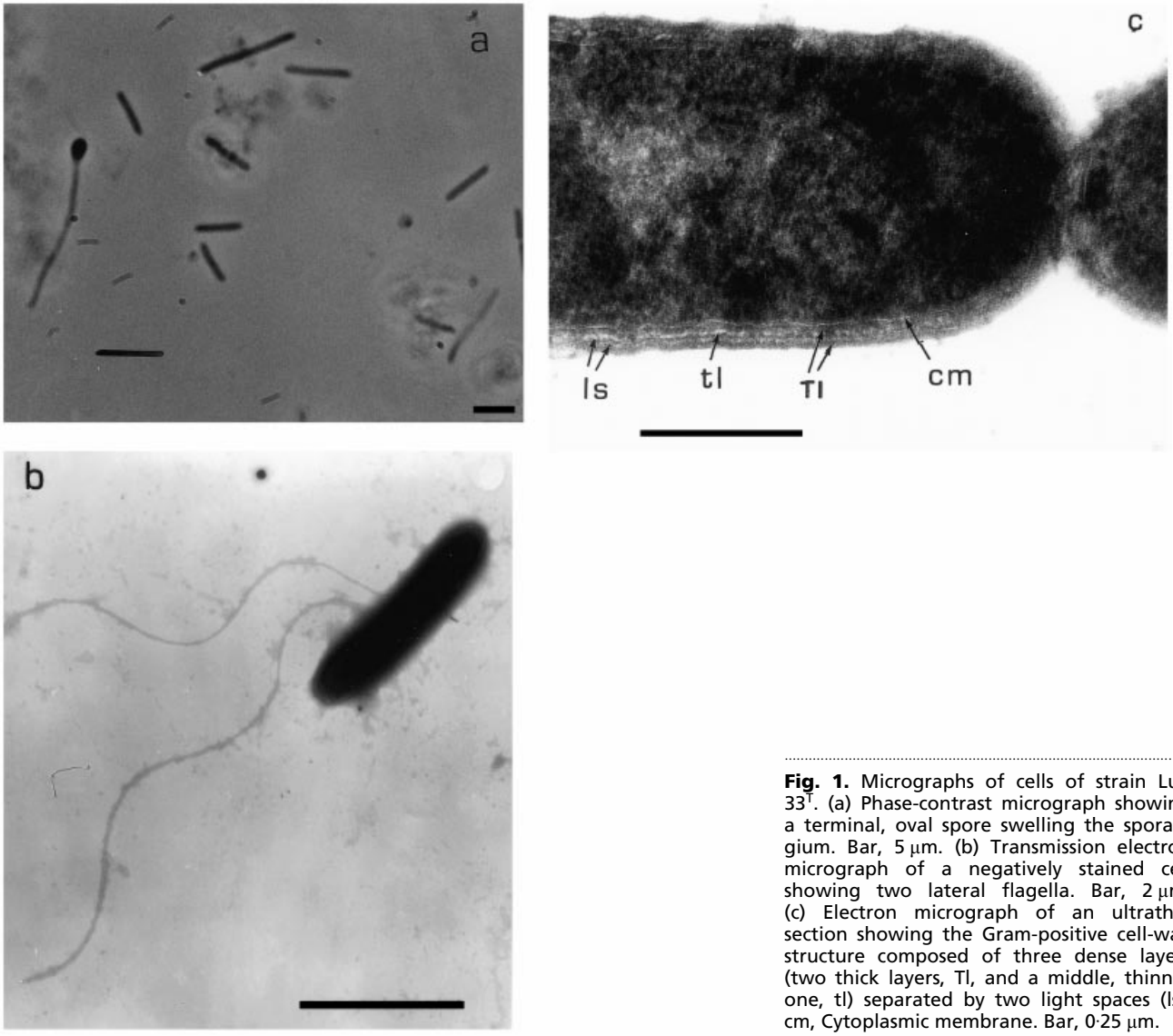

Fig. 1. Micrographs of cells of strain Lup 33^T. (a) Phase-contrast micrograph showing a terminal, oval spore swelling the sporangium. Bar, 5 μm. (b) Transmission electron micrograph of a negatively stained cell showing two lateral flagella. Bar, 2 μm. (c) Electron micrograph of an ultrathin section showing the Gram-positive cell-wall structure composed of three dense layers (two thick layers, Tl, and a middle, thinner one, tl) separated by two light spaces (ls). cm, Cytoplasmic membrane. Bar, 0·25 μm.

package (Felsenstein, 1993). Pairwise evolutionary distances based on 1304 unambiguous nucleotides were computed by using the method of Jukes & Cantor (1969) and dendrograms were constructed from these distances by using the neighbour-joining method. The accession numbers for rDNA sequences of reference organisms are included in Fig. 3.

RESULTS

Enrichment and isolation

Enrichment cultures were incubated at 35 °C for 2–3 weeks. Growth was regarded as positive on the basis of H_2S production. Microscope examination of the enrichment culture revealed the presence of various bacterial morphotypes, including rods and vibrios. Colonies, 2–3 mm in diameter, appeared after 2 days of incubation at 37 °C in roll tubes containing peptone-rich agar medium. Single colonies were picked using the techniques developed by Hungate (1969) and the process of serial dilution in roll tubes was repeated at least twice in order to purify the cultures. Three rod-shaped bacteria designated strains Lup 33^T, Lup 7a and Lup 27 were isolated. Strain Lup 33^T originated from the sludge of the cheese factory (la Caperucita), strain Lup 7a from the sludge of the paper factory (Unipak) and strain Lup 27 from the sludge of the yeast factory (Imexa). Analysis of the 16S rRNA gene sequences of the three strains showed that they were closely related phylogenetically, with a degree of similarity close to 99 % (data not shown). Strain Lup 33^T was characterized further.

Cell morphology

Cells of strain Lup 33^T were strictly anaerobic rods. The cells were 0·4–0·5 μm in width and 3–5 μm in length and occurred singly or in pairs (Fig. 1a). Cells were motile by a few laterally inserted flagella (Fig. 1b). In media appropriate for spore induction, spherical, terminal, oval spores which swelled the spor-

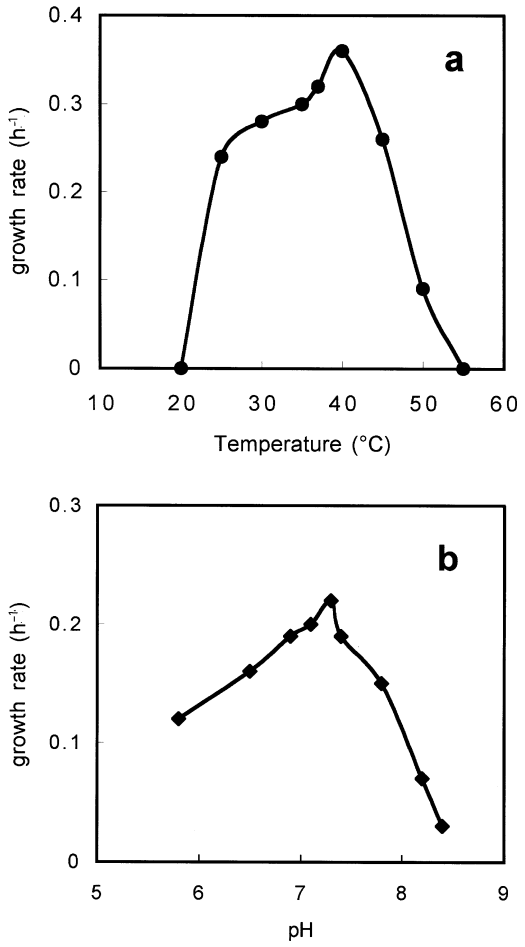

Fig. 2. Effects of temperature (0 g NaCl l^{-1}, pH 7·5) (a) and pH (0 g NaCl l^{-1}, 40 °C) (b) on growth of strain Lup 33^T cultivated in BM containing 1 g yeast extract l^{-1} and 5 g peptone l^{-1}.

angium appeared in the cells (Fig. 1a). Electron microscopy of sections of strain Lup 33^T exhibited a 33-nm-thick, stratified, Gram-positive-type cell wall, composed of three dense layers (two thick layers and a thinner middle layer) separated by two light spaces (Fig. 1c).

Optimum growth conditions

Strain Lup 33^T grew at temperatures ranging from 25 to 50 °C, with an optimum at 40 °C (Fig. 2a). The isolate grew in the presence of NaCl concentrations ranging from 0 to 40 g NaCl l^{-1}, with optimum growth in the absence of NaCl. The optimum pH for growth was around 7·5 and growth occurred between pH 5·5 and 8·5 (Fig. 2b).

Substrates and electron acceptors used for growth

Details of the substrates and electron acceptors used for growth are given in the genus and species descriptions below. Some further details are given here. Small amounts of the following amino acids (around 1 mM) were used as energy sources in the presence of yeast extract (2 g l^{-1}): DL-histidine, L-isoleucine, DL-leucine, L-methionine, L-phenylalanine, DL-tryptophan and DL-valine. In the presence of elemental sulfur as a terminal electron acceptor, the oxidation of the latter amino acids increased significantly (mean 2–3 mM). Lysine (1·7 mM) was only oxidized in the presence of elemental sulfur. The isolate fermented arginine (4·6 mM), and the use of arginine was not improved in the presence of elemental sulfur. No H_2 was detected from glucose metabolism in the presence of elemental sulfur as the electron acceptor.

G+C content of DNA and 16S rRNA sequence analysis

The G+C content of strain Lup 33^T was 32·2 mol% (HPLC). 16S rRNA sequence analysis revealed that strain Lup 33^T was a member of cluster XII of the *Clostridium* subphylum and its closest relatives were *Clostridium hastiforme* (92·7% similarity) and *Tissierella praeacuta* (92·5% similarity). Fig. 3 presents a dendrogram generated by the neighbour-joining method (Felsenstein, 1993) from the Jukes–Cantor evolutionary similarity matrix (Jukes & Cantor, 1969).

DISCUSSION

Strain Lup 33^T is an anaerobic, spore-forming micro-organism of the domain *Bacteria* that grows heterotrophically on carbohydrates, peptones and amino acids. A mixture of volatile fatty acids, including acetate, isovalerate and isobutyrate, together with H_2 and CO_2 were produced from peptone fermentation, but acetate was the only fatty acid produced from glucose metabolism. In this respect, this bacterium is an acetogen. Its inability to use H_2 as the electron donor and CO_2 as the electron acceptor to produce acetate indicated that it was not a homoacetogenic bacterium (Ljungdahl *et al.*, 1989). Analysis of the 16S rRNA gene sequence of this isolate indicated that it is a member of the order *Clostridiales*, cluster XII, as defined by Collins *et al.* (1994). This cluster comprises *Clostridium* species (e.g. *C. hastiforme*, *Clostridium acidurici*, *Clostridium purinilyticum* and *Clostridium ultunense*), *Eubacterium angustum*, *Tissierella* species and the recently described thermophilic halophile *Thermohalobacter berrensis* (Cayol *et al.*, 2000). Interestingly, this cluster includes acetogenic bacteria (e.g. *C. acidurici*, *C. purinilyticum* and *E. angustum*) that produce acetate, ammonium and CO_2 from purines but, in contrast to strain Lup 33^T, these micro-organisms are described as asaccharolytic (Beuscher & Andreesen, 1984; Ljungdahl *et al.*, 1989). Similarly, the closest relatives of strain Lup 33^T, *C. hastiforme* (similarity of 92·7%) and *Tissierella praeacuta* (similarity of 92·5%), are unable to use sugars (Cato *et al.*, 1986; Farrow *et al.*, 1995; Hippe *et al.*, 1992). Strain Lup 33^T is a moderately thermophilic micro-organism but, unlike *Thermohalobacter berrensis*, it does not require saline conditions and does

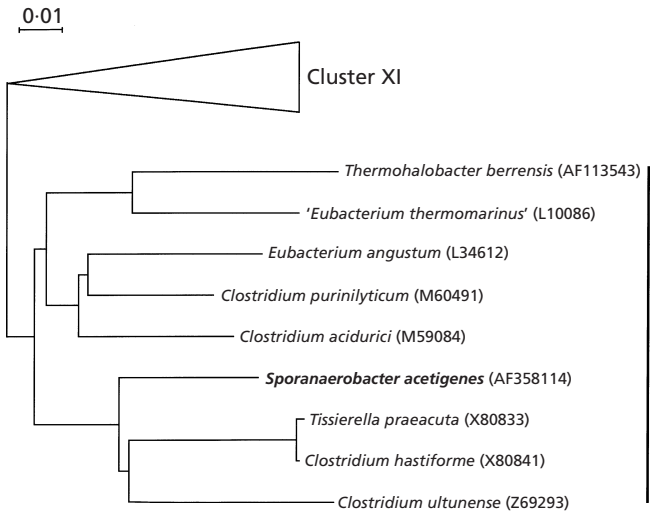

Fig. 3. Phylogenetic dendrogram based on 16S rDNA sequence data indicating the position of strain Lup 33^T amongst cluster XII of the order *Clostridiales* and related bacteria. The clusters are defined on the basis of the guidelines described by Collins *et al.* (1994). Accession numbers of 16S rDNA gene sequences of reference organisms are given. Bar, 1 nucleotide substitution per 100 nucleotides.

not grow at temperatures higher than 50 °C, while *Thermohalobacter berrensis* exhibits optimum growth at 65 °C (Cayol *et al.*, 2000). Strain Lup 33^T differs from *C. ultunense* (Schnürer *et al.*, 1996) in its ability to reduce elemental sulfur and the range of sugars and amino acids used. In contrast to strain Lup 33^T, *C. ultunense* produces formate as well as acetate from glucose fermentation. Strain Lup 33^T therefore represents a distinct phenotypic and phylogenetic lineage within cluster XII.

Strain Lup 33^T facultatively uses elemental sulfur as a terminal electron acceptor, producing sulfide. The ability to use inorganic sulfur-containing compounds (e.g. thiosulfate and/or elemental sulfur) has been reported for several members of the *Clostridiales*. They include species of *Thermoanaerobacter* (cluster V) and *Thermoanaerobacterium* (cluster VII) described as using thiosulfate as an electron acceptor (Schink & Zeikus, 1983; Lee *et al.*, 1993; Fardeau *et al.*, 1993, 1994; Faudon *et al.*, 1994). *Thermoanaerobacterium* species reduce thiosulfate to elemental sulfur, whereas *Thermoanaerobacter* species reduce both thiosulfate and elemental sulfur to sulfide. Other fermentative bacteria also belonging to the *Clostridiales* that have the ability to reduce thiosulfate and/or elemental sulfur to sulfide have been isolated successfully, in particular from oilfield environments (Fardeau *et al.*, 1993; Magot *et al.*, 1997; Ravot *et al.*, 1997, 1999) but also from freshwater sediments (Hermann *et al.*, 1987). None of these micro-organisms belongs to cluster XII. Therefore, they differ phylogenetically from strain Lup 33^T. These results also suggest that thiosulfate and/or sulfur reduction might be a quite widespread metabolic trait within members of the order *Clostridiales*, which gives them a metabolic advantage through their ability to oxidize amino acids, as reported already for other micro-organisms belonging to this order (Faudon *et al.*, 1994; Fardeau *et al.*, 1997; Magot *et al.*, 1997).

Taking into account its phenotypic and phylogenetic characteristics, we propose to assign strain Lup 33^T as a novel species of a new genus of the order *Clostridiales*, cluster XII, *Sporanaerobacter acetigenes* gen. nov., sp. nov.

Description of *Sporanaerobacter* gen. nov.

Sporanaerobacter [Spo.ra.nae.ro.bac′ter. N.L. n. *spora* spore; Gr. pref. *an* not; Gr. n. *aer* air; N.L. *anaero* not (living) in air; N.L. n. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod, staff; N.L. masc. n. *Sporanaerobacter* a spore-forming anaerobic rod].

Cells are strictly anaerobic rods, occurring singly or in pairs and motile by a few laterally inserted flagella. Gram-positive-type cell wall. Mesophilic and moderately thermophilic, growing at up to 50 °C. Neutrophilic. Heterotrophic. Yeast extract is required for growth on sugars. Ferments peptides and amino acids in the presence of yeast extract. Glucose is fermented to acetate, H_2 and CO_2 as the major end products of metabolism. Performs the Stickland reaction using isoleucine as electron donor and glycine or serine as electron acceptors. Uses elemental sulfur but not sulfate, thiosulfate, sulfite, nitrate or nitrite as an electron acceptor. The G+C content is 32 mol%. The type and only species is *Sporanaerobacter acetigenes*.

Description of *Sporanaerobacter acetigenes* sp. nov.

Sporanaerobacter acetigenes (a.ce.ti.ge′nes. L. n. *acetum* vinegar; Gr. v. *gennano* to produce; N.L. adj. *acetigenes* producing acetate).

In addition to the properties listed in the genus description, the following properties are reported. Cells are 0·4–0·5 × 3–5 μm. Electron microscopy of sections of cells reveals a 33-nm-thick, layered Gram-positive-type cell wall, composed of three dense layers (two thick layers and a middle thinner layer) separated by two light spaces. Grows at temperatures ranging from 25 to 50 °C, with optimum growth at 40 °C. Grows in the presence of NaCl at concentrations

Table 1. End products of glucose metabolism by strain Lup 33^T

Values are concentrations in mM. Control medium is BM containing 2 g yeast extract l^{-1}. The amount of glucose consumed was 2·1 mM.

Treatment	Acetate	Isobutyrate	Isovalerate	H_2	CO_2
Control	2·0	11·7	2·5	3·4	2·0
Glucose	6·2	11·1	1·8	10·3	6·2

ranging from 0 to 40 g l^{-1}, with optimum growth in the absence of NaCl. The optimum pH for growth is around 7·5 and growth occurs between pH 5·5 and 8·5. Heterotrophic. Gelatin and casein are not used as energy sources, but peptone, bio-Trypcase and Trypticase soy are fermented. The following amino acids are used as energy sources in the presence of yeast extract (2 g l^{-1}): arginine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan and valine. In the presence of elemental sulfur as a terminal electron acceptor, the use of the amino acids cited above, with the exception of arginine, is improved. Lysine is only oxidized in the presence of elemental sulfur. Valine is oxidized to isobutyrate, leucine to isovalerate, isoleucine to methyl 2-butyrate, phenylalanine to phenyl acetate, lysine to acetate and histidine to acetate and an unidentified compound. Peptone is fermented to acetate, H_2 and CO_2 as the major end products of metabolism. Traces of isobutyrate and isovalerate are also detected. Pyruvate is converted to acetate, H_2 and CO_2. Utilizes D-glucose, D-ribose and fumarate, but not D-arabinose, D-fructose, D-galactose, maltose, D- or L-xylose, acetate, propionate, butyrate, valerate, ethanol, n-butanol, n-propanol, malate or succinate. Acetate is the only fatty acid produced from glucose metabolism (Table 1). Adverse effects on animals and humans are not known. Because of its ability to degrade amino acids and peptides, the possibility of harmful effects cannot be excluded. Cautious handling and autoclaving of cultures before disposal is recommended. The G+C content is 32·2 mol% (HPLC). Isolated from a UASB reactor in Mexico.

The type strain is strain Lup 33^T (= DSM 13106^T = CIP 106730^T).

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