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Clostridium thiosulfatireducens sp. nov., a proteolytic, thiosulfate- and sulfur-reducing bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor

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A strictly anaerobic, Gram-positive, sporulating rod (0·5–0·6 × 2·0–4·0 μm), designated strain Lup 21^T, was isolated from an upflow anaerobic sludge blanket (UASB) reactor treating cheese-factory wastewater. Strain Lup 21^T was motile by means of peritrichous flagella, had a G+C content of 31·4 mol% and grew optimally at 37 °C, pH 7·4, in the absence of NaCl. It is a heterotrophic micro-organism, utilizing proteinaceous compounds (gelatin, peptides, Casamino acids and various single amino acids) but unable to use any of the carbohydrates tested as a carbon and energy source. It reduced thiosulfate and elemental sulfur to sulfide in the presence of Casamino acids as carbon and energy sources. Acetate, butyrate, isobutyrate, isovalerate, CO_2 and sulfide were end products from oxidation of gelatin and Casamino acids in the presence of thiosulfate as an electron acceptor. In the absence of thiosulfate, serine, lysine, methionine and histidine were fermented. On the basis of 16S rRNA similarity, strain Lup 21^T was related to members of the low-G+C *Clostridiales* group, *Clostridium subterminale* DSM 6970^T being the closest relative (with a sequence similarity of 99·4%). DNA–DNA hybridization was 56% with this species. On the basis of phenotypic, genotypic and phylogenetic characteristics, the isolate was designated as a novel species of the genus *Clostridium*, *Clostridium thiosulfatireducens* sp. nov. The type strain is strain Lup 21^T (= DSM 13105^T = CIP 106908^T).

Keywords: *Clostridium thiosulfatireducens*, thiosulfate reduction, sulfur reduction, taxonomy, anaerobe

INTRODUCTION

Members of the genus *Clostridium* are ubiquitous chemo-organotrophic micro-organisms. Most of them use carbohydrates and/or proteinaceous compounds as energy sources (Cato *et al*., 1986; Hippe *et al*., 1992). They are not known to dissimilate sulfate (Cato *et al*., 1986). This latter trait clearly differentiates them from another related spore-forming genus, *Desulfotomaculum* (Cato *et al*., 1986; Collins *et al*., 1994). Despite their inability to reduce sulfate, *Clostridium* species produce sulfide when grown on peptone/yeast extract/glucose medium, from the fermentation of cysteine (Cato *et al*., 1986; Holdeman *et al*., 1977). Thermophilic species, including *Clostridium thermohydrosulfuricum* (Hollaus & Sleytr, 1972; Klaushofer & Parkkinen, 1965) and *Clostridium thermosaccharolyticum* (Hollaus & Sleytr, 1972; Matteuzzi *et al*., 1978), also produce sulfide by thiosulfate reduction, whereas *Clostridium thermosulfurigenes* reduces thiosulfate to elemental sulfur (Schink & Zeikus, 1983). However, because of phylogenetic considerations (e.g. 16S rRNA gene sequence analysis studies), the assign-

Abbreviation: UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Lup 21^T is AF317650.

ment of these three thermophiles to the genus *Clostridium* has been re-evaluated (Lee *et al*., 1993). While *C. thermohydrosulfuricum* has been reclassified within the genus *Thermoanaerobacter*, *C. thermosaccharolyticum* and *C. thermosulfurigenes* have been reclassified within the genus *Thermoanaerobacterium* (Lee *et al*., 1993). It is only recently that a mesophilic *Clostridium* species, *Clostridium peptidivorans*, has been reported to use thiosulfate as an electron acceptor (Mechichi *et al*., 2000).

In this study, we report the isolation of a novel proteolytic, thiosulfate-reducing bacterium with phenotypic, genomic and phylogenetic characteristics consistent with its placement within the genus *Clostridium* as a novel species, *Clostridium thiosulfatireducens* sp. nov.

METHODS

Sample collection and sample source. Enrichments were performed from the sludge of two reactors. One reactor was an 80 m^3 upflow anaerobic sludge blanket (UASB) reactor treating the wastewater of the cheese factory 'Caperucita', located near the city of Queretaro in Mexico (Monroy *et al*., 2000). The mean temperature and pH of the reactor were 23 °C and 7·3. The other one was a UASB reactor treating the wastewater of the 'Unipack' factory, manufacturing cardboard from recycled paper and located in the city of Cuernavaca in Mexico. The reactor temperature was 35 °C and its pH was 6·9 (Monroy *et al*., 2000). The reactors were 300 km apart and were inoculated with sludge from different sources. The sludges were collected from the two reactors by completely filling 500 ml sterile plastic bottles that were maintained at room temperature until use. *Clostridium subterminale* DSM 6970^T, DSM 758 and DSM 2636 were obtained from the DSMZ.

Enrichment and isolation. The basal medium contained (l^{-1}) 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, 10 ml of the trace mineral element solution of Balch *et al*. (1979) and 1 mg resazurin. The pH was adjusted to 7·0 with 10 M KOH. The medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Aliquots of 5 or 40 ml were dispensed under a stream of N_2 gas respectively into Hungate tubes or serum bottles. The vessels were then sealed and autoclaved for 45 min at 110 °C. Prior to inoculation, $Na_2S . 9H_2O$ was injected from sterile stock solutions to a final concentration of 0·04 % (w/v). Enrichment was performed in 120 ml serum bottles. Peptone (5 g l^{-1}) and thiosulfate (20 mM) were added to the basal medium as electron donor and acceptor, respectively. The serum bottle was inoculated with 4 ml sludge, corresponding to 10 % of the final liquid volume (40 ml). After inoculation, the serum-bottle atmosphere was changed to H_2 at a final pressure of 203 kPa to inhibit growth of fermentative bacteria unable to use thiosulfate as an electron acceptor. The bottles were incubated at 35 °C in a controlled-temperature room for 2–3 weeks. After several transfers (10 %, v/v), the enrichment cultures were serially diluted by using the roll-tube technique. Roll tubes were prepared by adding 2 % agar (Difco) to the medium. Isolation was performed in the same medium with N_2 instead of H_2 in the gas phase.

pH, temperature and NaCl ranges for growth. Growth experiments were performed in duplicate, using Hungate tubes containing the basal medium modified as follows: (i) KH_2PO_4 and K_2HPO_4 concentrations were reduced to 0·3 g l^{-1}; (ii) a N_2/CO_2 gas mixture (80 : 20 %, v/v) was used as the gas phase; (iii) prior to inoculation, $NaHCO_3$ was injected from a sterile stock solution to a final concentration of 0·2 % (w/v). For pH growth experiments, the media in Hungate tubes were adjusted to different pH values by injecting $NaHCO_3$ or Na_2CO_3 from 10 % (w/v) sterile anaerobic stock solutions. The temperature range for growth was determined using the culture medium adjusted to the optimum growth pH. For studies on NaCl requirements, NaCl was weighed directly into Hungate tubes and the culture medium was dispensed into the tubes as described above. The strain was subcultured at least once under the same experimental conditions prior to inoculation for each growth experiment.

Tests for sporulation. The presence of spores was determined by microscopic examination of the culture at different phases of growth.

Utilization of substrate and electron acceptors. Substrate utilization was tested in basal medium containing 1 g yeast extract l^{-1} (Difco) and no cysteine. The incubation time was at least 10 days. 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 20 mM sodium thiosulfate. To test for sulfur-containing electron acceptors, sodium thiosulfate (20 mM), sodium sulfate (20 mM), sulfite (2 mM) and elemental sulfur (2 % w/v) were added, as carbon and energy sources, to the growth medium containing peptone. Nitrate (10 mM) and nitrite (10 mM) were also tested as potential electron acceptors. Thiosulfate dismutation was checked for in the presence of sodium acetate (2 mM) as the only carbon source in the absence of yeast extract in the culture medium.

Light and electron microscopy. Light and electron microscopy were performed as described previously (Cayol *et al*., 1994; Fardeau *et al*., 1997a).

Analytical techniques. Growth was measured by inserting Hungate tubes directly into a model UV-160A spectrophotometer (Shimadzu) and measuring the OD_{580}. Sulfide was determined photometrically as colloidal CuS by 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 at the CNRS (IBMS, Marseilles) by the ninhydrin method (Moore *et al*., 1958).

Determination of G+C content and DNA–DNA hybridization studies. The G+C content of DNA was determined and DNA–DNA hybridizations were performed at the DSMZ. The DNA was isolated and purified by chromatography on hydroxyapatite (Cashion *et al*., 1977) and the G+C content was determined using the HPLC method described by Mesbah *et al*. (1989); unmethylated lambda DNA (Sigma) was used as the standard. DNA–DNA hybridization was performed as described by De Ley *et al*. (1970) and modified by Huß *et al*. (1983) and Escara & Hutton (1980), using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the program TRANSFER.BAS (Jahnke, 1992).

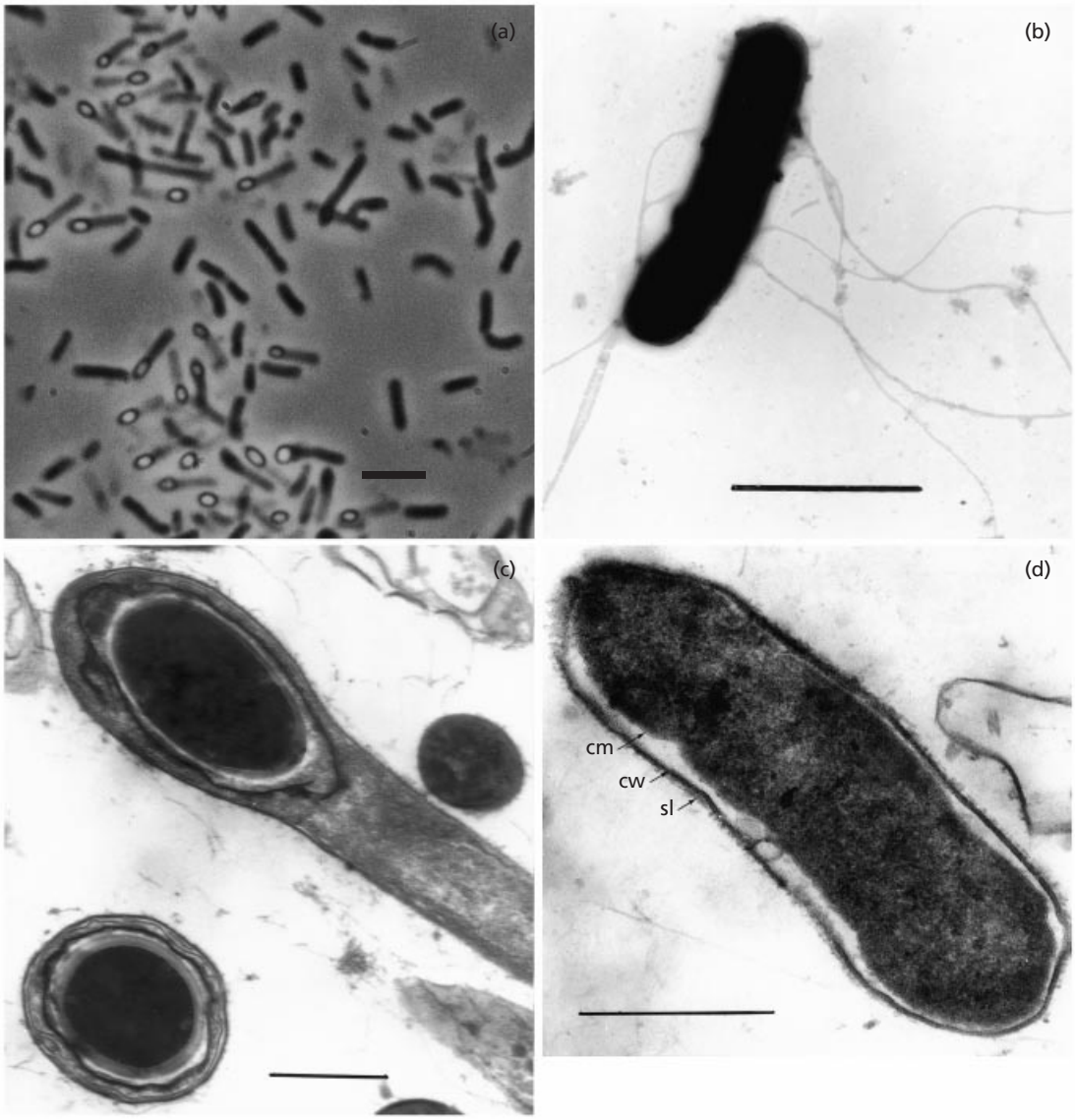

Fig. 1. (a) Phase-contrast micrograph of strain Lup 21^T. Bar, 2 μm. (b) Electron micrograph of a negatively stained culture of strain Lup 21^T, showing peritrichous flagella. Bar, 2 μm. (c) The terminal position of a spore in a cell. Bar, 0·5 μm. (d) The cell-wall ultrastructure. cm, Cytoplasmic membrane; cw, cell wall; sl, S-layer. Bar, 0·5 μm.

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 was aligned automatically with reference sequences of various members of *Clostridium* (cluster I) by using the program CLUSTAL X (Thompson *et al.*, 1997). Reference sequences were obtained from the Ribosomal Database Project (Maidak *et al.*, 1996) and the 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 package (Felsenstein, 1993). Pairwise evolutionary distances based on 1400 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. Bootstrap analyses were used to evaluate the tree topology by performing 100 resamplings (Felsenstein, 1993).

RESULTS

Enrichment and isolation

Enrichment cultures (under anaerobic conditions) of the sludges from the two reactors were regarded as positive after incubation at 35 °C for 2–3 weeks when

copious H_2S was produced (around 40 mM). Microscopic examination revealed the presence of various bacterial morphotypes in each case, including spore-forming micro-organisms. Colonies (1 mm in diameter) appeared after 1 day of incubation at 37 °C in the basal medium containing 5 g peptone l^{-1} and 1·6% agar. They were arborescent and translucent. Single colonies were picked and reinoculated twice before the culture was considered pure, and several axenic cultures were obtained using the serial dilution technique (Magot *et al.*, 1997a, b; Ravot *et al.*, 1997). Culture purity was checked by microscopic examinations. Two sporulating bacteria, designated strain Lup 34 ('Unipack' sludge) and strain Lup 21^T ('Caperucita' sludge), were obtained by this method. They were subsequently found to be nearly identical genotypically (the DNA–DNA hybridization value between the two strains was 98·8%). Strain Lup 21^T was characterized further.

Morphology

Strain Lup 21^T was a spore-forming rod. The cells were 0·5–0·6 μm wide and 2–4 μm long and occurred singly or in pairs (Fig. 1a). Cells possessed peritrichous flagella (Fig. 1b). Spores were terminal and deformed the cells (Fig. 1c). Electron microscopy of thin sections of strain Lup 21^T revealed an atypical Gram-positive cell wall with two dense inner layers and a surface flocculent layer (Fig. 1d).

Optimum growth conditions

Strain Lup 21^T did not grow in oxidized medium (oxidation was indicated by the pink colour of the resazurin) and was therefore strictly anaerobic. It grew at temperatures ranging from 18 to 45 °C, the optimum being at 37 °C (Fig. 2a). The optimum pH for growth was 7·4, and growth occurred between pH 6·0 and 9·8 (Fig. 2b). The isolate grew in the presence of NaCl at concentrations ranging from 0 to 60 g l^{-1}, optimum growth being in the absence of NaCl (Fig. 2c).

Substrates used for growth

Strain Lup 21^T fermented casein, gelatin, peptone, bio-Trypcase, Trypticase soy, Casamino acids, histidine, lysine, methionine, serine and pyruvate. Other substrates used for growth are listed in the species description below.

Effect of added electron acceptors

Strain Lup 21^T used only thiosulfate and sulfur, and not sulfate, sulfite, nitrate or nitrite, as an electron acceptor. It did not perform thiosulfate disproportionation. The use of thiosulfate increased the range of amino acids oxidized and increased growth on peptone (data not shown). The type strain of *C. subterminale*, DSM 6970^T, did not use thiosulfate as an electron acceptor.

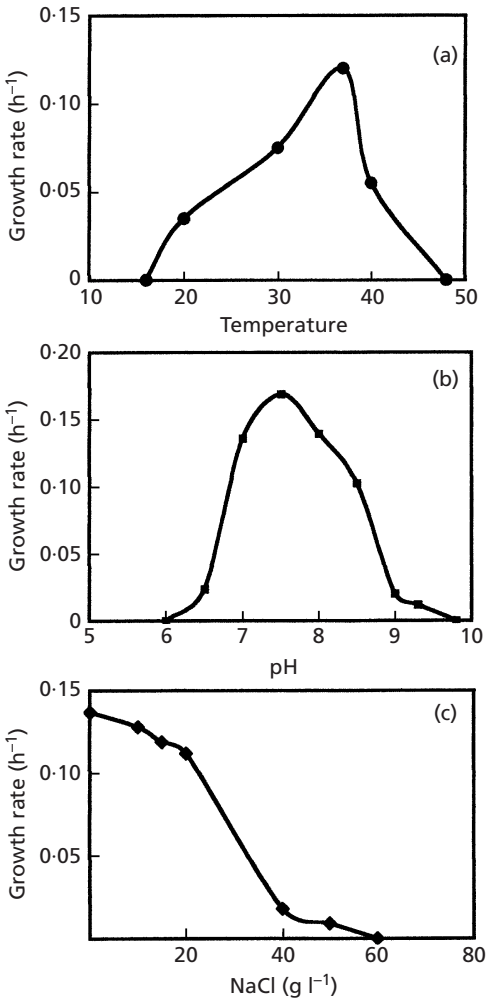

Fig. 2. Effects of temperature (pH 7·4, no NaCl) (a), pH (37 °C, no NaCl) (b) and NaCl (37 °C, pH 7·4) (c) on growth of strain Lup 21^T. Experiments were performed in the presence of peptone (5 g l^{-1}) as the carbon and energy source.

G+C content and DNA–DNA hybridization analysis

The G+C content of strain Lup 21^T was 31·4 mol% (HPLC). Strain Lup 21^T showed low hybridization values with *C. subterminale* DSM 6970^T (= ATCC 25774^T) (56%), DSM 758 (28%) and DSM 2636 (41%).

16S rRNA sequence analysis

Analysis of the 16S rRNA sequence showed that strain Lup 21^T was a member of the low-G+C Gram-positive bacteria and a member of cluster I of the *Clostridium* subphylum. Phylogenetic analysis indicated that *C. subterminale* DSM 6970^T (sequence

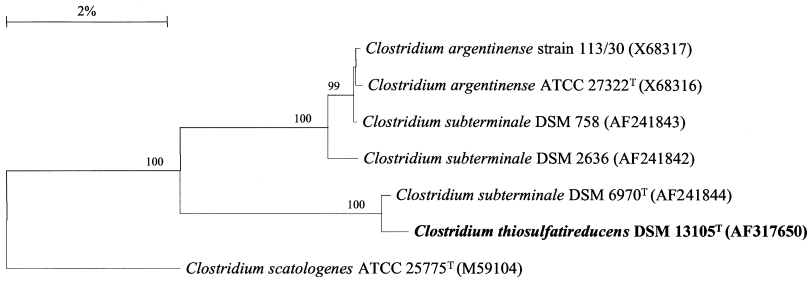

Fig. 3. Unrooted phylogenetic dendrogram based on 16S rRNA sequence data, indicating the position of *C. thiosulfatireducens* sp. nov. Lup 21^T (= DSM 13105^T) within the radiation of representative members of the genus *Clostridium* (cluster I) according to Collins *et al.* (1994). Numbers at branching points refer to bootstrap values > 90 %. Accession numbers of 16S rRNA gene sequences are given in parentheses. Bar, 2 nucleotide substitutions per 100 nucleotides.

similarity of 99 %) was the closest relative of strain Lup 21^T. Fig. 3 is a dendrogram generated by the neighbour-joining method (Felsenstein, 1993) from the Jukes–Cantor evolutionary similarity matrix (Jukes & Cantor, 1969).

DISCUSSION

Biological thiosulfate reduction is known to be performed by mesophilic, facultative anaerobes of the family *Enterobacteriaceae* (Barrett & Clark, 1987) and strict anaerobes belonging to domains *Bacteria* and *Archaea* (Barrett & Clark, 1987; Jochimsen *et al.*, 1997; Lee *et al.*, 1993; Le Faou *et al.*, 1990; Stetter *et al.*, 1990). Amongst the anaerobic, non-sulfate-reducing micro-organisms of the domain *Bacteria*, members of the genera *Thermoanaerobacter* and *Thermoanaerobacterium* were first described as using thiosulfate as an electron acceptor (Fardeau *et al.*, 1993, 1994; Faudon *et al.*, 1994; Lee *et al.*, 1993; Schink & Zeikus, 1983). Since then, fermentative bacteria that reduce thiosulfate to sulfide have been isolated (in particular from oilfield environments) and characterized (Fardeau *et al.*, 1993, 1997b; Magot *et al.*, 1997a, b; Ravot *et al.*, 1995a, b, 1997). Strain Lup 21^T is a novel anaerobic, spore-forming rod that reduces thiosulfate to sulfide.

The taxonomic assignment of strain Lup 21^T to the genus *Clostridium* is ascertained by our phylogenetic studies, which indicate that it belongs to cluster I of the order *Clostridiales* (Collins *et al.*, 1994; Stackebrandt & Rainey, 1997). *C. subterminale* DSM 6970^T is its closest phylogenetic relative. Within the *Clostridiales*, micro-organisms utilizing thiosulfate as an electron acceptor – *Fusibacter paucivorans* (Ravot *et al.*, 1999) and *C. peptidivorans* (Mechichi *et al.*, 2000) – have been described only recently. However, phylogenetic analysis of 16S rRNA indicates that *F. paucivorans* belongs to cluster XI of the *Clostridium* subphylum (Collins *et al.*, 1994). Furthermore, in contrast to strain Lup 21^T, *F. paucivorans* is saccharolytic. *C. peptidivorans* is also a proteolytic, thiosulfate-reducing bacterium, but its 16S rRNA possesses only 93·5 % similarity to that of strain Lup 21^T. Strain Lup 21^T also differs from *C. peptidivorans* in its ability to reduce elemental sulfur and in the range of amino acids used. *C. peptidivorans* does not use alanine and threonine, whereas strain Lup 21^T does. In addition, isoleucine is not fermented by strain Lup 21^T, as reported for *C. peptidivorans*.

On the basis of 16S rRNA sequence, the closest relative of strain Lup 21^T is *C. subterminale* DSM 6970^T, with which it shares similar phenotypic properties, including the use of proteins, peptides and amino acids and the inability to use carbohydrates. However, the low DNA–DNA hybridization values (28–56 %) obtained between Lup 21^T and *C. subterminale* (DSM 6970^T, DSM 2636 and DSM 758) indicate that they do not belong to the same species (Stackebrandt & Goebel, 1994). It is known that the use of proteinaceous compounds is a common feature shared by several *Clostridium* species, rendering their taxonomic comparison difficult (Cato *et al.*, 1986; Elsden & Hilton, 1979; Hippe *et al.*, 1992; Mead, 1971). However, the pattern of amino acid utilization can differentiate *Clostridium* species (Elsden & Hilton, 1979; Mead, 1971). Similarly to *Clostridium sticklandii*, strain Lup 21^T can be differentiated from *C. subterminale* as using threonine (Hippe *et al.*, 1992) and having a higher DNA G+C content (31·4 mol % for strain Lup 21^T, as opposed to 28 mol % for *C. subterminale*). In addition, we demonstrated that thiosulfate was not used as an electron acceptor by the type strain of *C. subterminale*, DSM 6970^T. Therefore, both genomic and phenotypic characteristics of strain Lup 21^T support its assignment to a novel species of the genus *Clostridium*, *C. thiosulfatireducens* sp. nov.

Peptide and amino acid utilization by *C. thiosulfatireducens* is improved in the presence of thiosulfate as an electron acceptor. This improvement was also observed with other thiosulfate-reducing, non-sulfate-reducing anaerobes, including *Dethiosulfovibrio peptidovorans* (Magot *et al.*, 1997b) and *Thermoanaerobacter brockii* (Cayol *et al.*, 1995; Fardeau *et al.*, 1997b; Faudon *et al.*, 1994; Schmid *et al.*, 1986; Zeikus *et al.*, 1979). Growth is particularly stimulated with valine, leucine and isoleucine, which are used by all these organisms via oxidative deamination when reducing thiosulfate to sulfide. Our results indicate, therefore, that *C. thiosulfatireducens* could play a significant role in protein, peptide or amino acid degradation, especially in the presence of thiosulfate, when available in the ecosystem. They also extend the known diversity of micro-organisms involved in amino

acid and peptide degradation, and emphasize the importance of thiosulfate- or sulfur-reducers in the oxidation of peptides and amino acids in various environments, as already reported (Fardeau *et al.*, 1997b; Faudon *et al.*, 1994; Magot *et al.*, 1997b).

Description of *Clostridium thiosulfatireducens* sp. nov.

Clostridium thiosulfatireducens (thi.o.sul.fa.ti.re.du′cens. N.L. n. *thiosulfas* (*-atis*) thiosulfate; L. v. *reduco* to draw backwards, bring back to a state or condition; N.L. part. adj. *thiosulfatireducens* thiosulfate-reducing).

The cells are rods, 0·5–0·6 μm wide and 2–4 μm long, occurring singly or in pairs. Gram staining is positive, and electron microscopy of thin sections reveals an atypical Gram-positive cell wall ultrastructure. The cells are motile with peritrichous flagella. Terminal spores are formed; sporangia are swollen. Colonies (1 mm diameter), appearing after 1 day of incubation in peptone-rich medium at 37 °C, are arborescent and translucent. Growth occurs from 18 to 45 °C, the optimum temperature being 37 °C. It tolerates NaCl concentrations up to 60 g l^{-1}. The optimum pH for growth is 7·4, and growth occurs between pH 6·0 and 9·8. Yeast extract is required for growth on amino acids but not for growth on peptides. Gelatin, peptone, bio-Trypcase and Trypticase soy are used as carbon and energy sources in the absence of thiosulfate as terminal electron acceptor. The following amino acids are used as carbon and energy sources in the presence of thiosulfate: alanine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine and valine. Pyruvate is converted to acetate. The following substrates are not used: arginine, asparagine, aspartate, cysteine, glutamine, glycine, tryptophan, tyrosine, L-arabinose, D-fructose, D-galactose, D-glucose, maltose, mannitol, D-ribose, L-xylose, D-xylose, formate, acetate, butyrate, propionate, valerate, ethanol, *n*-butanol, *n*-propanol, fumarate, lactate, malate and succinate. In the presence of thiosulfate, Casamino acids, gelatin, peptone and Trypticase soy are converted mainly to acetate, butyrate, isobutyrate, isovalerate or 2-methylbutyrate, CO_2 and sulfide. Acetate is the only fatty acid detected from alanine and threonine oxidation, whereas isoleucine is oxidized to 2-methylbutyrate, leucine to isovalerate, valine to isobutyrate and phenylalanine to phenylacetate. In the absence of thiosulfate, acetate is the major end product of the metabolism of proteinaceous compounds (Casamino acids, gelatin, peptone and Trypticase soy). The isolate ferments serine to acetate, lysine to acetate and butyrate, methionine to propionate and histidine to an unidentified product. It performs the Stickland reaction, using alanine as electron donor and methionine and serine as electron acceptors. Thiosulfate and sulfur, but not sulfate, sulfite, nitrate or nitrite, are used as electron acceptors. The use of thiosulfate increases the range of amino acids oxidized and has a beneficial effect on growth on peptone. Adverse effects on animals and humans are not known. Because of the ability of *C. thiosulfatireducens* 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 DNA has a G+C content of 31·4 mol% (HPLC). Isolated from UASB digestors in Mexico treating industrial wastewaters. The type strain is strain Lup 21^T (= DSM 13105^T = CIP 106908^T).

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