**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 21T, was isolated from an upflow anaerobic sludge blanket (UASB) reactor treating cheese-factory wastewater. Strain Lup 21T 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, CO2 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 21T was related to members of the low-G+C Clostridiales group, Clostridium subterminale DSM 6970T 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 21T (DSM 13105T = CIP 106908T).

**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 dissipilate sulfate (Cato et al., 1986). This latter trait clearly differentiates them from another related spore-forming genus, *Desulfooccus* (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 thermohydrodsulfuricum* (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-
ment of these three thermophiles to the genus Clostridium has been re-evaluated (Lee et al., 1993). While C. thermohydrosulphuricum has been reclassified within the genus Thermoanaerobacter, C. thermosaccharolyticum and C. thermosulfurificans 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 (Mecchi 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 m³ 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 6970T, DSM 758 and DSM 2636 were obtained from the DSMZ.

Enrichment and isolation. The basal medium contained (1 l) 1 g NaCl, 3 g KH₂PO₄, 3 g KH₂PO₃, 0.2 g MgCl₂, 6H₂O, 0.1 g CaCl₂, 2H₂O, 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 O₂-free N₂ gas and cooled to room temperature. Aliquots of 5 or 40 ml were dispensed under a stream of N₂ gas respectively into Hungate tubes or serum bottles. The vessels were then sealed and autoclaved for 45 min at 110 °C. Prior to inoculation, Na₂S . 9H₂O 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 H₂ 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 N₂ instead of H₂ in the gas phase.

\textbf{pH, temperature and NaCl ranges for growth.} Growth experiments were performed in duplicate, using Hungate tubes containing the basal medium modified as follows: (i) KH₂PO₄ and K₂HPO₄ concentrations were reduced to 0.3 g l⁻¹; (ii) a N₂/O₂ gas mixture (80:20%, v/v) was used as the gas phase; (iii) prior to inoculation, NaHCO₃ 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 NaHCO₃ or Na₂CO₃ 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.

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

\textbf{Utilization of substrate and electron acceptors.} Substrate utilization was tested in basal medium containing 1 g yeast extract 1⁻¹ (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 1⁻¹ 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.

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

\textbf{Analytical techniques.} Growth was measured by inserting Hungate tubes directly into a model UV-160A spectrophotometer (Shimadzu) and measuring the OD₅₆₀. Sulphide 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).

\textbf{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 Membabi 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).
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### 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 $\text{H}_2\text{S}$ 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-Tryptase, 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.

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

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**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.
similarity of 99%) was the closest relative of strain Lup 21T. 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 Thermoaeroclostridium 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 21T is a novel anaerobic, spore-forming rod that reduces thiosulfate to sulfide.

The taxonomic assignment of strain Lup 21T 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 6970T is its closest phylogenetic relative. Within the *Clostridiales*, micro-organisms utilizing thiosulfate as an electron acceptor – Fasibacter 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 21T, *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 21T. Strain Lup 21T 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 21T does. In addition, isoleucine is not fermented by strain Lup 21T, as reported for *C. peptidivorans*.

On the basis of 16S rRNA sequence, the closest relative of strain Lup 21T is *C. subterminale* DSM 6970T, 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 21T and *C. subterminale* (DSM 6970T, 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 *C. sticklandii*, strain Lup 21T 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 21T, 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 6970T. Therefore, both genomic and phenotypic characteristics of strain Lup 21T 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 peptidivorans* (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'qens. 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 are round, 1–2 mm in 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 and 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-xylene, D-xylene, 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, CO₂ 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 21T (= DSM 13105T = CIP 106908T).

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