Sterolibacterium denitrificans gen. nov., sp. nov., a novel cholesterol-oxidizing, denitrifying member of the β -*Proteobacteria*

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A bacterial strain (Chol-1S^T) that is able to oxidize cholesterol to CO₂ and reduce nitrate to dinitrogen was enriched and isolated from an upflow sludge bed (USB) anoxic reactor that treats sanitary landfill leachate from the city of Montevideo, Uruguay. Cells of strain Chol-1S^T were Gram-negative, rod-shaped to slightly curved, measured 0.5–0.6 × 1.0–1.3 µm and were motile by a single polar flagellum. Strain Chol-1S^T grew optimally at 30–32 °C and pH 7.0, with a doubling time of 44–46 h when cholesterol was used as the sole carbon and energy source. The metabolism of strain Chol-1S^T was strictly respiratory, with oxygen or nitrate as the terminal electron acceptor. The presence of ubiquinone Q-8 as the sole respiratory lipoquinone indicated that strain Chol-1S^T belonged to the β -subclass of the *Proteobacteria*. Phosphatidylethanolamine was the predominant polar lipid and the G + C content of the DNA was 65.3 mol%. The fatty acid profile of strain Chol-1S^T, cultivated under denitrifying conditions by using a defined mineral medium supplemented with cholesterol, was characterized by the following major components: summed feature 4 (C_{16:1}ω7c and/or iso C_{15:0} 2-OH), C_{16:0}, C_{18:1}ω7c and hydroxy acid C_{10:0} 3-OH. Minor components included C_{10:0}, C_{11:0}, C_{12:0}, C_{14:0}, C_{15:0}, C_{19:0}, C_{19:0} 10-methyl and hydroxylated acids C_{8:0} 3-OH and C_{16:0} 3-OH. Analysis of the 16S rDNA sequence showed that strain Chol-1S^T represents a separate lineage within the *Thauera*, *Azoarcus*, *Zoogloea* and *Rhodocyclus* assemblage of the β -*Proteobacteria*. Strain Chol-1S^T had highest sequence similarity (96.5 %) with strain 72Chol, a denitrifying β -*Proteobacterium*. On the basis of polyphasic evidence, strain Chol-1S^T (= DSM 13999^T = ATCC BAA-354^T) is proposed as the type strain of *Sterolibacterium denitrificans* gen. nov., sp. nov.

Cholesterol and related sterols are natural recalcitrants in anaerobic treatment of the highly pollutant effluent produced by the wool-scouring process (Gutiérrez *et al.*, 1999). This can be attributed to the low number of functional groups (a carbon–carbon double bond and a hydroxyl group), its low solubility and the complexity of its spatial conformation. Aerobically, various genera of bacteria can degrade cholesterol completely to carbon dioxide by employing mono- and dioxygenases (Kieslich, 1985). However, less is known about the mechanisms that operate in the absence of oxygen. The best-studied anaerobic reaction so far is the reduction of cholesterol to coprostanol by intestinal fermentative bacteria (Freier *et al.*, 1994).

Due to their facultative metabolism, denitrifying bacteria are more versatile than other groups of bacteria and can serve as potential sources for novel biotransformations of natural substances. Previous work has demonstrated that denitrifying conditions can support the mineralization of cholesterol (Taylor *et al.*, 1981). A denitrifying bacterial strain, 72Chol, which oxidizes cholesterol to carbon dioxide in the absence of molecular oxygen, was isolated from a leaf-covered ditch (Harder & Probian, 1997).

A denitrifying post-treatment step of anaerobically pre-treated wastewater can be envisaged to complement the removal of organic matter, in order to comply with environmental discharge standards. The purpose of this investigation was to determine whether organisms capable of anaerobic mineralization of cholesterol were present in different anaerobic man-made ecosystems that treat wool-scouring effluent and sanitary landfill leachate. The latter ecosystem has high contents of readily degradable carbon but also of recalcitrant organic compounds, such as

Published online ahead of print on 6 December 2002 as DOI 10.1099/ijs.0.02039-0.

Abbreviations: FAME, fatty acid methyl ester; USB, upflow sludge bed.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Chol-1S^T is AJ306683.

aromatics (Wang & Barlaz, 1998). In the study presented here, we report the isolation and polyphasic characterization of a novel nitrate-reducing bacterium that oxidizes cholesterol to CO_2 .

Source, enrichment and isolation

Enrichment and cultivation were performed in a bicarbonate/ CO_2 -buffered, basal anaerobic mineral medium, modified from that of Tarlera *et al.* (1997) as follows (l^{-1}): 1.2 g K_2HPO_4 , 0.4 g KH_2PO_4 , 1.0 g KNO_3 and 1 ml (25 g $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ l^{-1}) solution. Prior to inoculation, 1 ml vitamin solution (Touzel & Albagnac, 1983), 20 ml (11.6 g $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ l^{-1} , 3.7 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ l^{-1}) solution and substrates were added. Oxidic liquid media did not contain nitrate or bicarbonate and were incubated with agitation. Soluble substrates were added from anaerobic filter-sterilized stock solutions. Insoluble substrates were added either as a solid before autoclaving or as 1% (w/v) solution in 2,2,4,4,6,8,8-heptamethylnonane as the inert carrier phase. All incubations were carried out at 30–32 °C and pH 7.0, except for tests to determine temperature and pH ranges for growth. For quantification studies, acetylene (10%) was added to the culture headspace to block the last step of denitrification ($\text{N}_2\text{O} \rightarrow \text{N}_2$). Total N_2O content was calculated from the headspace concentration as described by Christensen & Tiedje (1988). Growth tests were considered to be positive for substrate utilization when an increase in optical density above that of control tubes with no added substrate was detected and confirmed by nitrate and nitrite quantification.

Denitrification in the presence of oxygen was tested by measuring the reduction of nitrate in anoxic and oxic conditions (incubated with agitation at 80 r.p.m.). Nitrate, nitrite and N_2O were measured as described by Etchebehere *et al.* (2001). Volatile fatty acids, alcohols and sugars were analysed by HPLC as described by Menes & Muxí (2002). For quantification of cholesterol, samples were extracted with ethyl acetate, evaporated under vacuum conditions, redissolved in methanol and analysed by UV (SPD-10AV; Shimadzu)-HPLC (Waters) at 210 nm, by using a C_{18} column with a mobile phase of 60/40% (v/v) acetonitrile/2-propanol at a flow rate of 1.5 ml min^{-1} . Biomass was calculated from the total protein content of the cultures by using a protein per cell dry weight ratio of 50% and assuming the simplified cell formula $\text{C}_4\text{H}_7\text{O}_3$ ($M_r = 103$). Protein determination was performed according to the Lowry method, by using BSA as the standard (Daniels *et al.*, 1994).

Strain Chol-1S^T was isolated from an upflow sludge bed (USB) denitrifying reactor that treats sanitary landfill leachate, after enrichment with cholesterol as the carbon source and nitrate as the electron acceptor (Barrandeguy & Tarlera, 2001). Other enrichments, set up with inocula from anaerobic lagoons and reactors that treat wool-scouring effluent, failed to denitrify cholesterol.

Isolation of a pure culture was attempted from the cholesterol-degrading enrichment by: (a) streaking onto nutrient, brain–heart infusion (BHI) and R2A agar plates under air; (b) streaking onto anoxic plates of mineral medium with 10 mM nitrate and an agar overlay of cholesterol; and (c) anoxic agar dilution and liquid dilution series of mineral medium with 1 mM cholesterol and 10 mM nitrate. Colonies that grew on solid media were transferred to liquid mineral medium with cholesterol as the sole carbon and energy source. None of the isolates was able to grow on cholesterol.

However, the isolation of a pure culture was successful after several successive anoxic liquid dilution series, which were subcultured as soon as nitrite production was detected in the highest dilutions. This strategy allowed enrichment of the cholesterol-degrading bacteria with respect to other bacteria. Finally, after consecutive series of dilutions that took place over 1 year, a pure culture that consisted only of straight or slightly curved short rods with rounded ends was obtained. The purity of the culture was tested by subculturing in mineral salt medium amended with volatile fatty acids, amino acids and ethanol (growth substrates commonly used by denitrifiers). In addition, the culture was transferred into complex media [oxic and anoxic TSB (trypticase soy broth), BHI and nutrient broth] and streaked onto different oxic and anoxic agar plates (R2A, TSA and nutrient agar). No growth was observed under the different conditions tested after more than 2 months of incubation. In a previous report, amplified rDNA restriction analysis (ARDRA), performed on the clones from the enrichment, revealed the presence of only two different restriction patterns (Barrandeguy & Tarlera, 2001). The partial 16S rDNA sequence (700 bp) that was representative of the dominant member of the enrichment, according to ARDRA, was identical to that determined later for strain Chol-1S^T. These results allow the conclusion that a pure culture was obtained.

Morphological and physiological characteristics

Cells of strain Chol-1S^T were Gram-negative, straight to slightly curved rods (0.5–0.6 × 1.0–1.3 µm) that were motile by means of a single polar flagellum (Fig. 1a) and had lateral fimbria-like appendages (Fig. 1b).

Physiological characterization showed that strain Chol-1S^T used only a limited number of substrates, including sterols and saturated long-chain fatty acids. Detailed physiological characteristics are listed in the formal species description below. Briefly, anaerobically grown cultures showed a lag phase before they started to grow with oxygen as the electron acceptor. Simultaneous reduction of oxygen and nitrate was not observed. The co-respiration of nitrate and oxygen under oxygen-saturated conditions is noted only for bacteria isolated from aerobic habitats, such as soil and activated sludge (Patureau *et al.*, 1998; Rainey *et al.*, 1999; Scholten *et al.*, 1999).

Fig. 1. Transmission electron micrographs of strain Chol-1S^T, showing its morphology and flagellar position (a) and fimbria-like appendages (b). Bars, 0.3 µm (a); 50 nm (b).

Oxidation of cholesterol

Strain Chol-1S^T was not able to grow with cholesterol as the electron donor, with sulphate, thiosulphate, fumarate or Fe(III) as electron acceptors or under fermentative conditions. Growth tests in an ammonium-free medium were likewise negative. After 10 weeks incubation, incomplete nitrate reduction (but no visible growth) was observed on acetate and propionate. Partial reduction (2 mM) of nitrate to nitrite was observed on lithocholic acid. Noticeably, strain Chol-1S^T has a narrow substrate-utilization profile that is specific for non-polar, hydrophobic compounds as carbon and energy sources.

To determine whether complete degradation of cholesterol was taking place, the oxidation of cholesterol and reduction of nitrate were quantified. The disappearance of 0.42 mM cholesterol was accompanied by the consumption of

9.7 mM nitrate and the synthesis of 120 mg cell dry mass l⁻¹. Nitrite accumulated temporarily during growth, but was further consumed after near-depletion of nitrate (Fig. 2). More than 80 % of nitrate reduced was recovered as dinitrogen oxide in experiments with an acetylene block, whereas only traces of dinitrogen oxide were detected in vials with no added acetylene. Also, as an increase in growth yield accompanied nitrate and nitrite reduction, a respiratory denitrification process could be ascertained. Time-course analysis of growing cultures by HPLC showed no accumulation of short-chain fatty acids or alcohols in the culture broth of strain Chol-1S^T. Synthesis of biomass (120 mg l⁻¹; Fig. 2) can account for the consumption of 0.17 mM cholesterol and 1.29 mM nitrate based on the assimilation equation:

Initial and final cholesterol concentrations were 1 and 0.58 mM, respectively. Considering the cholesterol and nitrate consumed for cell synthesis, 0.25 mM cholesterol was dissimilated while 8.41 mM nitrate was consumed and not recovered as nitrite. An electron recovery of 90 % was calculated (the ratio of number of electrons produced by complete oxidation of the dissimilated amount of cholesterol to CO₂ to number of electrons consumed by nitrate reduction to dinitrogen). The complete oxidation of 1 mol cholesterol yields 152 mol electrons; 5 mol electrons are considered to be accepted for 1 mol nitrate to be reduced

Fig. 2. Growth of strain Chol-1S^T with cholesterol: OD₆₀₀ (●), nitrate consumption (■) and nitrite production (▲). Data shown are representative of repeated experiments with similar results. Data are corrected for the amount of nitrate consumed in controls that lacked cholesterol.

to dinitrogen. These findings are in good agreement with the theoretical stoichiometry for cholesterol mineralization according to the equation:

Chemotaxonomy

Analysis of the respiratory lipoquinones was carried out by using HPLC at 30 °C as described by Tindall (1990). The HPLC system was equipped with a model 510 pump, a model UK6 injector, a model 484 UV detector (all from Waters) and a reversed-phase column (Hypersil ODS RP 18, 250 × 4.6 mm, 5 µm particles; Agilent Technologies). Analysis revealed a single peak that corresponded to ubiquinone-8 (Q-8). This quinone system is a characteristic feature of the *β-Proteobacteria* (Collins & Jones, 1981; Yokota *et al.*, 1992). Two-dimensional TLC of cellular lipids (Denner *et al.*, 2001) indicated only the presence of phosphatidylethanolamine (PE) in strain Chol-1S^T. PE is one of the most widely occurring bacterial phospholipids (Wilkinson, 1988); solely PE was reported for *Alcaligenes faecalis* (Ghanekar & Nair, 1974) and *Azotobacter chroococcum* (Reczek & Burton, 1979). All other species of *β-Proteobacteria* that have been investigated so far have been shown to contain a more complex polar lipid pattern (Wilkinson, 1988; Cox & Wilkinson, 1989; Yabuuchi *et al.*, 1992, 1998; Blumel *et al.*, 2001). Thus, the polar lipid pattern of strain Chol-1S^T is a potentially distinctive trait of this species.

For fatty acid analysis, strain Chol-1S^T was grown on anaerobic mineral medium supplemented with 1 mM cholesterol and 10 mM nitrate, harvested by centrifugation, washed with 20 mM phosphate buffer and freeze-dried. Fatty acid methyl esters (FAMES) were extracted and prepared according to the standard protocol of the Microbial Identification system (MIDI; Microbial ID). FAME extract of strain Chol-1S^T was analysed by GLC as described by Kämpfer & Kroppenstedt (1996). The fatty acid profile of strain Chol-1S^T was characterized by 33.8 % C_{16:1ω7c} and/or iso C_{15:0} 2-OH (summed feature 4), 21.5 % C_{16:0}, 14.5 % C_{18:1ω7c} and 14.0 % C_{10:0} 3-OH. Other fatty acids that were present in significant amounts included C_{12:0} (5.3 %), C_{19:0} 10-methyl (4.0 %) and C_{19:0} (2.3 %). Fatty acids present in minor amounts included hydroxylated fatty acids C_{8:0} 3-OH (0.2 %) and C_{16:0} 3-OH (1.0 %) and saturated fatty acids C_{10:0} (0.9 %), C_{11:0} (0.2 %), C_{14:0} (0.9 %) and C_{15:0} (0.8 %). Some unknown fatty acids were also present in minor amounts (data not shown). Similar fatty acid profiles have been described for the genera *Thauera* and *Azoarcus* (Song *et al.*, 2001) and *Zoogloea ramigera* (Hiraishi *et al.*, 1992); the presence of C_{8:0} 3-OH, C_{16:0} 3-OH and C_{19:0} 10-methyl was not reported (Hiraishi *et al.*, 1992; Song *et al.*, 2001), although a direct comparison of fatty acid profiles may be difficult because of the different cultivation

conditions. However, fatty acid analysis clearly allows differentiation of strain Chol-1S^T from these taxa.

Phylogenetic position of strain Chol-1S^T

Genomic DNA for 16S rDNA sequencing was extracted and purified by using the Wizard Genomic DNA purification kit (Promega) as described by the manufacturer. PCR amplification and sequencing were performed as described by Menes & Muxí (2002). The primers used for sequencing were: 27F (positions 8–27: 5'-AGAGTTTGATCCTGGCTCAG-3'); ST1F (positions 606–632: 5'-GGCTCAACCTGGGAACT-3'); ST3F (positions 1273–1290: 5'-GAGCCAATCCCAGAAAG-3'); ST4R (positions 1512–1496: 5'-ACGGCTACCTTGTTACG-3'); ST5R (positions 999–983: 5'-GCATGTCAAGGGTAGGT-3); and ST6R (positions 462–445: 5'-CCCAGTCCGTTTCTTCC-3') (*Escherichia coli* numbering). Sequencing was carried out by the DNA Sequencing Core Laboratory at the University of Florida (USA). Phylogenetic analyses were performed with the PHYLIP 3.5c software package (Felsenstein, 1993) as described by Menes & Muxí (2002).

A total of 1531 nt of the 16S rRNA gene of strain Chol-1S^T were determined. Phylogenetic analyses based on a dataset that comprised 1390 unambiguous nucleotides between positions 53 and 1459 (*E. coli* numbering) showed that the sequence of strain Chol-1S^T is most similar to those of species of the *β*-subclass of the *Proteobacteria* (Fig. 3). Comparative evolutionary distance analysis showed that strain Chol-1S^T represents a separate lineage of descent within the *β-Proteobacteria*. The nearest, albeit relatively distant, phylogenetic relatives were species of the genera *Azoarcus*, *Thauera* and *Zoogloea*. 16S rDNA sequence similarities ranged from 91 % (with *Z. ramigera* ATCC 19544^T) to 93 % (with *Thauera linaloolentis* DSM 12138^T). These similarity values are clearly below the usual criterion of 95 % that is applied for genus delineation. The neighbour-joining tree inference clustered the genera *Thauera*, *Azoarcus* and *Zoogloea* at a significant level (86 %) in bootstrap analysis. Also, the branching of genera *Propionivibrio*, *Rhodocyclus*, *Ferribacterium* and *Dechloromonas* was supported by a high bootstrap value (100 %). Highest 16S rRNA gene sequence similarity (96.5 %) was found to the already mentioned denitrifying bacterial strain 72Chol (Harder & Probian, 1997).

Comparative physiological investigations showed that strain 72Chol also uses only a limited number of substrates and degrades cholesterol and other sterols, but not short-chain fatty acids or sugars, under denitrifying conditions. In contrast to strain Chol-1S^T, strain 72Chol has been reported to be non-motile, has a maximum growth temperature of 32 °C, has a doubling time of 37 h on cholesterol and is unable to grow on palmitate (Harder & Probian, 1997).

To summarize, by considering the polyphasic taxonomic data presented in this study, we could identify the novel cholesterol-oxidizing denitrifying bacterial isolate Chol-1S^T as a hitherto unknown taxon of the *β-Proteobacteria*, for

Fig. 3. Phylogenetic tree derived from the analysis of 16S rRNA gene sequences of strain Chol-1S^T and other related β -Proteobacteria. Numbers at nodes indicate levels of bootstrap support, based on data for 100 resamplings. *E. coli* was used as outgroup. Bar, 10 nucleotide substitutions per 100 nucleotide positions.

which we propose the name *Sterolibacterium denitrificans* gen. nov., sp. nov. Key taxonomic characteristics that differentiate the genus *Sterolibacterium* from other related genera are listed in Table 1.

Description of *Sterolibacterium* gen. nov.

Sterolibacterium (Ste.ro.li.bac.te'ri.um. N.L. neut. n. *sterolum* sterol; Gr. dim. neut. n. *bakterion* small rod; N.L. neut. n. *Sterolibacterium* sterol-utilizing small rod).

Straight or slightly curved, small, rod-shaped, Gram-negative cells. Mesophilic. Strictly respiratory type of metabolism with oxygen or nitrate as terminal electron acceptor. Nitrate is reduced to dinitrogen. Oxidase- and catalase-positive. Chemo-organoheterotrophic. Does not grow on complex media. Cholesterol is completely oxidized to CO₂; this reaction is coupled to nitrate reduction. Q-8 is the sole respiratory lipoquinone. Phosphatidylethanolamine is the predominant polar lipid. Major fatty acids are C_{16:0}, summed feature 4 (C_{16:1} ω 7c and/or iso C_{15:0} 2-OH), C_{18:1} ω 7c and C_{10:0} 3-OH; minor amounts of C_{8:0} 3-OH and C_{16:0} 3-OH are present. DNA G+C content is 65.3 mol% (HPLC). The type species of the genus is *Sterolibacterium denitrificans*.

Description of *Sterolibacterium denitrificans* sp. nov.

Sterolibacterium denitrificans (de.ni.tri'fi.cans. N.L. part. adj. *denitrificans* denitrifying).

Cells are 1.0–1.3 $\times$ 0.5–0.6 μ m in size and are motile by a single polar flagellum. Optimal pH and temperature for growth are 7.0 and 30–32 $^{\circ}$ C, respectively. Temperature and pH ranges for growth are 15–35 $^{\circ}$ C and 5.8–8.0, respectively. Carbon sources used include cholesterol (5-cholesten-3 β -ol),

Table 1. Differential characteristics of *Sterolibacterium* gen. nov. and related taxa

Genera: 1, *Sterolibacterium*; 2, *Thauera*; 3, *Azoarcus*; 4, *Zoogloea*. Data for *Thauera*, *Azoarcus* and *Zoogloea* were compiled from Unz (1984), Hiraishi *et al.* (1997), Song *et al.* (1998, 2001) and Reinhold-Hurek & Hurek (2000). +, Positive; –, negative.

Characteristic	1	2	3	4
Growth on nutrient agar, R2A or BHI agar	–	+	+	+
Utilization as sole carbon source:				
Succinate	–	+	+	+
Acetate	–	+	+	+
Glutamate	–	+	+	+
Major quinone system*	Q-8	Q-8	Q-8	Q-8, RQ-8
Diagnostic cellular fatty acids:				
C _{8:0} 3-OH	+	–	–	–
C _{16:0} 3-OH	+	–	–	–
C _{19:0} 10-methyl	+	–	–	–
DNA G+C content (mol%)	65.3	64–69	64–68	67–69

*Q-8, ubiquinone-8; RQ-8, rhodoquinone-8.

4-cholesten-3-one, 5 α -androstane-3,17-dione, 4-androstene-3,17-dione, 3 β -hydroxy-5 α -cholestane, palmitate (C_{16:0}) and stearate (C_{18:0}). Neither growth nor nitrate reduction was demonstrated after 6 months with glucose, fructose, xylose, ethanol, lactate, isobutyrate, succinate, malate, crotonate, citrate, laurate, oleate, caproate, heptanoate, cyclohexanol, cyclohexanone, glutamate, leucine, benzoate, phenol, cysteine, dimethylmalonate, desoxycholate or 1,4-androstadiene-3,17-dione. Doubling time for growth on cholesterol and nitrate is 44–46 h.

The type strain is Chol-1S^T (= DSM 13999^T = ATCC BAA-354^T). Isolated from a USB denitrifying reactor that treats sanitary landfill leachate in Montevideo, Uruguay.

Acknowledgements

We are grateful to Professor Dr Peter Kämpfer, Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Gießen, Germany, for performing the fatty acid analysis and helpful suggestions. Professor Hans Trüper (University of Bonn, Germany) is acknowledged for advice on Latin usage. S. T. also wishes to thank Lucía Muxí for her encouragement and valuable advice. This study was partly supported by PEDECIBA, a Program for the Development of Basic Sciences in Uruguay and by Universidad de la República (CSIC). Transmission electron microscopy and analysis of DNA G+C content was carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSMZ, Braunschweig, Germany.

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