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Anaerobic mineralization of cholesterol by a novel type of denitrifying bacterium

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Abstract A novel denitrifying bacterium, strain 72Chol, was enriched and isolated under strictly anoxic conditions on cholesterol as sole electron donor and carbon source. Strain 72Chol grew on cholesterol with oxygen or nitrate as electron acceptor. Strictly anaerobic growth in the absence of oxygen was demonstrated using chemically reduced culture media. During anaerobic growth, nitrate was initially reduced to nitrite. At low nitrate concentrations, nitrite was further reduced to nitrogen gas. Ammonia was assimilated. The degradation balance measured in cholesterol-limited cultures and the amounts of carbon dioxide, nitrite, and nitrogen gas formed during the microbial process indicated a complete oxidation of cholesterol to carbon dioxide. A phylogenetic comparison based on total 16S rDNA sequence analysis indicated that the isolated micro-organism, strain 72Chol, belongs to the β2-subgroup in the Proteobacteria and is related to *Rhodocyclus*, *Thauera*, and *Azoarcus* species.

Key words Anaerobic bacteria · Nitrate reduction · Steroids · Hydrocarbon · Anaerobic degradation · *Azoarcus* · *Thauera*

Introduction

Cholesterol is present in the membranes of all eukaryotes. Biodegradation of cholesterol is hindered by its complex chemical structure and low solubility in water: heterogeneous micelles are formed at a critical concentration of 3×10^{-8} M (Gilbert et al. 1975). Consequently, cholesterol and related biogenic and catagenic transformation products are ubiquitous and important biomarkers in organic geochemistry (Mackenzie et al. 1982; Peters and Moldowan 1993).

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The oxidation and mineralization of cholesterol by aerobic bacteria has been studied in great detail. Cholesterol oxidase, the first enzyme in the degradation pathway, requires molecular oxygen as a cosubstrate to form 4-cholesten-3-one (Li et al. 1993). The side chain is oxidized, and then the ring system is cleaved and degraded. Monooxygenases transform 4-cholesten-3-one into 4-cholesten-27-ol-3-one, and 1,4-androstadien-3,17-dione into 1,4-androstadien-9-ol-3,17-dione. Ring A (Fig. 1) is transformed into a phenol that is split by a dioxygenase using the meta-cleavage pathway. Overall, four transformations that require molecular oxygen as a cosubstrate (Kieslich 1985) occur during the mineralization of cholesterol.

The most frequent alteration of cholesterol at oxic-anoxic boundaries and in anoxic habitats is a biogenic reduction of the carbon-carbon double bond of cholesterol yielding coprostanol (Schoenheimer 1931; Wakeham 1989). Many intestinal fermenting bacteria are able to catalyze this reaction (Groh et al. 1993; Freier et al. 1994), and cholesterol reductase activity has recently been assayed in resting cells of *Eubacterium coprostanoligenes* (Li et al. 1995). Studies of lake sediments have revealed – besides the reduction of sterols to stanols – that the total sterol content decreases faster than the total organic carbon pool, but more slowly than the pool of linear, long-chain aliphatic alcohols and fatty acids (Meyers and Ishi-

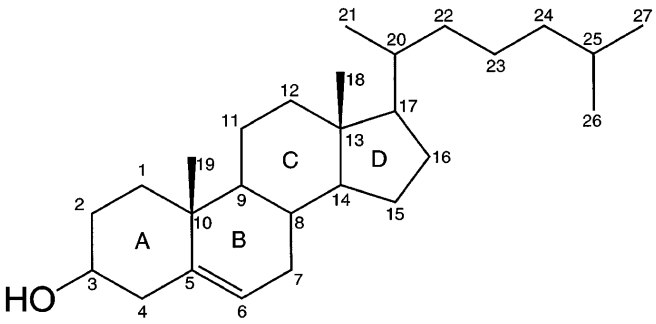

Fig. 1 Structural formula of cholesterol. The nomenclature of carbon atoms and rings is presented

watari 1993). Taylor et al. (1981) have provided initial evidence for the mineralization of cholesterol in anoxic habitats by establishing denitrifying enrichment cultures in which 2–5% of the added 4-^{14}C-cholesterol was recovered as ^{14}C-carbon dioxide.

This study describes the first isolation of an anaerobic, denitrifying bacterium grown on cholesterol as sole organic carbon source and electron donor.

Materials and methods

Sources of micro-organisms and chemicals

The enrichment culture was prepared by inoculation of 350 ml of anoxic medium containing 10 mM nitrate and 400 mg cholesterol with 100 ml of a water-mud mixture sampled in December 1993 from a ditch in a mixed forest near Bremen, Germany (Harder and Probian 1995). The ditch contained black mud covered by fallen autumn leaves; the leaves were mainly beech and larch leaves. The chemicals used were of analytical grade. The cholesterol (from gallstones, purity > 99%) used was obtained from Fluka (Neu-Ulm, Germany). AC broth was from Difco (Detroit, Mich., USA).

Media and culture conditions

Anoxic media and cultivation techniques used in this study were described by Widdel and Bak (1992) and Harder and Probian (1995). Strain 72Chol was enriched and isolated in the medium described by Harder and Probian (1995). Dilution of the sample in the liquid dilution series was performed in steps of 1:20 between 1 and 1×10^{-8}, and at lower concentrations in steps of 1:2. All pure-culture experiments were done with a defined medium modified from that of Rabus and Widdel (1995) as follows: 50 mM sodium hydrogen carbonate and 0.2 mM copper sulfate (pH 7.0). Maintenance of the isolate and growth experiments were performed in 21-ml culture tubes containing 15 ml anoxic medium with 2 mM cholesterol and 10 mM nitrate. The cultures were shaken at 60 rpm at 28° C in the dark. The temperature optimum of growth was determined from growth curves taken at nine different temperatures (10–50° C). The pH optimum of growth was measured in media buffered with 20 mM potassium phosphate and 30 mM sodium hydrogen carbonate at 15 different pH values (2.8–9.8). Cholesterol particles settled to the bottom of culture vessels within 30 min. The bacteria were not attached to the particles and remained in suspension. Thus, bacterial growth could be monitored by turbidimetry at 660 nm even in the presence of cholesterol particles.

To quantify the catabolic process (Fig. 4, Table 1), 200 ml anoxic medium was used in an Erlenmeyer flask with a round photometer side-arm and a thread (Glasgerätebau Ochs, Bovenden, Germany). Turbidimetric determinations at 660 nm in the side-arm and in standard cells (1 cm light path) yielded identical values. The flask was closed with a black butyl rubber stopper kept in place with an open-top screw cap. A N_2-CO_2 atmosphere (90:10, v/v) filled the headspace volume (120 ml). The flasks were equilibrated overnight at 28° C on a rotary shaker (75 rpm). The culture medium was inoculated with 200 µl taken from a recently grown preculture. The gas pressure was equilibrated to atmospheric pressure with a thin needle (0.65 × 30 mm). Microbial growth was followed by photometric measurements. At the end of the experiment, produced gas was measured volumetrically with a syringe. A sample of the gas phase was analyzed for methane and dinitrogen oxide content. From the culture, four 1-ml samples were taken from the culture for determination of the optical density in a standard cell, the protein content, the ammonium concentration, and nitrite/nitrate analysis. Larger samples were taken for analysis of cholesterol. Formation of inorganic carbon (CO_2, HCO_3^-, and CO_3^{2-}) was assayed in cultures that were prepared without bicarbonate, contained a nitrogen atmosphere, and were buffered by 20 mM potassium phosphate at pH 7.0. Nitrogen formation was measured by gas chromatography in cultures containing a He-CO_2 atmosphere (90:10, v/v).

Chemical analyses

Free cholesterol was analyzed enzymatically. Ten milliliters of culture sample including bacterial cells were mixed with 10 ml of isopropanol and shaken overnight at 250 rpm and 60° C. An aliquot was assayed using the Cholesterol Kit according to the manufacturer's protocol (Boehringer Mannheim, Germany). Chemical analyses of free cholesterol and cholesterol esters was performed using the *o*-phthaldialdehyde procedure (Rudell and Morris 1973). A 1-ml culture sample was hydrolyzed with 3 ml KOH (50%, mass/vol.) in the presence of 3 ml ethanol for 15 min at 60° C. The saponified samples were extracted with 5 ml hexane and 1 ml distilled water by shaking for 1 h at room temperature. One ml of the hexane phase was freed of hexane in a stream of nitrogen. The residue was dissolved in 2 ml glacial acetic acid containing 500 mg *o*-phthaldialdehyde l^{-1}. After 10 min, 1 ml concentrated sulfuric acid was added and immediately mixed with the sample. Ten minutes later, the absorbance was determined at 590 nm. The assay was standardized using a solution of 1 mg cholesterol dissolved in 1 ml methanol. To confirm the photometric detection of cholesterol, thin-layer chromatography was performed with samples from the aforementioned hexane phase by application of a method developed for food lipids (Macherey-Nagel 1992). Routinely, 5 ml of the hexane phase was dried, and the residue was dissolved in 100 µl of hexane. Samples (1 µl) were developed with dichloromethane:toluene [5:2 (v/v) freshly prepared] on Polygram Sil G silica-coated polyester sheets (100 mm high; Macherey-Nagel, Düren, Germany). Cholesterol was detected by spraying the plate with a solution of 50 mg molybdatophosphoric acid dissolved in 10 ml ethanol, followed by a short period of heating with an air dryer (approximately 120° C) (Macherey-Nagel 1992).

For protein determination, 1 ml of culture sample was centrifuged. Pellet and supernatant were examined separately in order to include exoenzymes and released protein from lysed cells. The pellet was dissolved in 1 ml 0.5 M sodium hydroxide and was boiled for 10 min. Protein concentrations were determined in a 200-µl aliquot by the method of Bradford (1976). Concentrations were calculated using bovine serum albumin as a standard and including a correction term for the unusual high binding of Coomassie stain to albumin. One milligram of albumin corresponds to 2 mg of protein (BioRad 1994).

Ammonium was determined photometrically by the indophenol method (Boltz and Taras 1978). Samples (10 ml) were mixed with 1 ml of solution A (3 g phenol and 30 mg sodium nitroprusside in 100 ml water) and 1 ml of solution B [2 ml NaClO solution (13% mass/vol.) and 2 g NaOH in 100 ml of water]. After incubation in the dark at room temperature for 1 h, the concentration of indophenol was measured at 635 nm.

Nitrate and nitrite were separated by HPLC on an A09 micro anion exchange column (3 × 125 mm; Sykam, Gilching/Munich, Germany) at 65° C using 70 mM NaCl at a rate of 1 ml min^{-1} as eluent. The HPLC system was equipped with an S 3200 UV detector (both Sykam) and an autosampler (Jasco, Tokyo, Japan).

Gas analysis was performed with a GC-8A gas chromatograph equipped with a thermal conductivity detector (Shimadzu, Kyoto, Japan) connected to a digital data-analyzing system (Perkin Elmer, Überlingen, Germany). Methane, carbon dioxide, and dinitrogen oxide were separated by using a Poraplot Q column (3 mm × 2 m) at 40° C and nitrogen as carrier gas at a flow rate of 16 ml min^{-1}. Nitrogen and oxygen were separated on a 5-Å molecular sieve column (3 mm × 2 m) at 40° C with helium as carrier gas at a flow rate of 32 ml min^{-1}. Dissolved carbonate was measured by gas chromatography. Ten milliliters of culture was transferred into a serum bottle that was filled with nitrogen. The culture fluid was acidified with 2 ml 2 N HCl, and the carbon dioxide was determined in a sample from the gas phase.

16S rDNA sequence analysis

Extraction of genomic DNA and PCR-mediated amplification of the 16S rDNA were carried out as described by Rainey et al. (1992). PCR products were directly sequenced using the Taq Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Germany) according to the manufacturer's protocol. The Applied Biosystems 373A DNA sequencer was used for electrophoresis of the sequence reaction products. The 16S rDNA sequences were manually aligned with sequences currently available from public databases.

Evolutionary distances were calculated by the method of Jukes and Cantor (1969) and used in the reconstruction of the phylogenetic dendrogram applying the algorithm of DeSoete (1983). Sequence analysis was performed by F. A. Rainey, Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Results

Enrichment and isolation

The enrichment culture with cholesterol formed in 4 weeks twice as much gas as a substrate-free control culture. Selection towards a complete oxidation of cholesterol to carbon dioxide was achieved by establishing a carbon-limited second passage. The cultures contained a large inoculum (20 ml of the first culture to 200 ml medium), 2 mM cholesterol, and initially 10 mM nitrate. Nitrate (10 mM) was added four times upon electron acceptor depletion, which was monitored by gas formation. Isolation from the second culture was attempted in agar dilution series. Gas evolved, but no colony formation was observed. Therefore, liquid dilution series were used for isolation. Bacterial growth in 10-ml liquid cultures from an inoculum of less than 1×10^{-8} ml, calculated via dilution factors, was observed within 2 weeks. In three consecutive liquid dilution series, the highest dilutions of the preculture showing microbial growth were 3×10^{-9}, 1.25×10^{-9}, and 1.5×10^{-10}, respectively. Growth was then established in a liquid culture that was prereduced with ferrous sulfide. The isolation was completed by two passages on nutrient agar plates and one passage on a succinate agar plate under air. The isolate, named 72Chol, formed tiny colonies slowly on plates. The purity of the culture was controlled on AC broth, diluted AC broth (1:10), meat extract (2 g l^{-1}), yeast extract (2 g l^{-1}), and peptone (2 g l^{-1}).

Morphological and phylogenetic characterization of 72Chol

The slow formation of tiny colonies of strain 72Chol led us to try two different means of maintaining the isolate. One stock culture was kept from July 1994 onwards in anoxic liquid culture on cholesterol and nitrate. After three passages, this culture lost the ability to grow on nutrient agar plates. A second stock culture was initiated with alternate culture conditions: oxic nutrient agar plates and anoxic selective liquid medium. After six cycles, this culture lost the capacity to grow on nutrient agar plates.

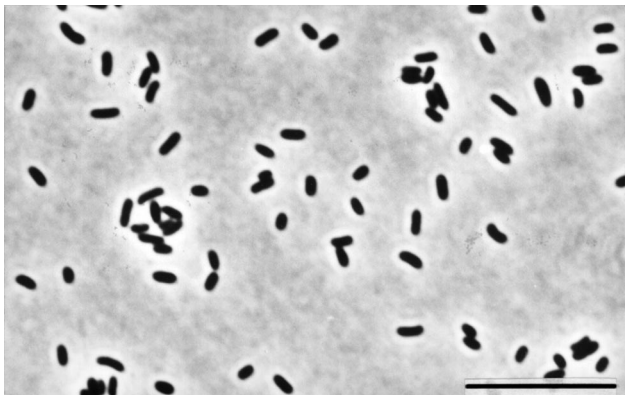

Fig. 2 Phase-contrast photomicrograph of the novel isolate 72Chol grown with cholesterol (1 mM) and nitrate (10 mM) (*Bar* 10 μm)

Cells of strain 72Chol were gram-negative, nonmotile small rods, 1.0–2.2 μm × 0.5–0.75 μm in size (Fig. 2). Strain 72Chol grew aerobically and anaerobically with cholesterol as sole carbon and energy source. Growth on cholesterol and nitrate was observed also in media that were not only prepared oxygen-free but also chemically prereduced with either 4 mM ascorbate or 1 mM ferrous sulfide. The temperature range for anaerobic growth on cholesterol was 10–32° C, with an optimum at 32° C. The doubling time at 32° C was 37 h. Neither growth nor nitrate consumption was observed at 35° C. The pH range of strain 72Chol was between 6.0 and 8.2, with an optimum at 7.2. Strain 72Chol grew under anoxic conditions with 10 mM nitrate on cholesterol (5-cholesten-3β-ol) and 5α-cholestan-3β-ol (0.5 mM). Denitrifying growth was not observed on acetate (20 mM), butyrate (10 mM), palmitate (1 and 5 mM), lactate (20 mM), pyruvate (20 mM), fumarate (20 mM), glutamate (10 mM), ascorbate (4 mM), glucose (5 mM), fructose (5 mM), galactose (5 mM), sucrose (5 mM), and cyclohexanol (2–20 mM).

The analysis of the 16S rDNA gene sequence of isolate 72Chol revealed that the strain is a member of the β2-subclass of the Proteobacteria (Woese 1987). Within the subclass, isolate 72Chol represents a new lineage that branches out at the root of a major phylogenetic group including representatives of the genera *Rhodocyclus*, *Zoogloea*, *Thauera*, and *Azoarcus* (Anders et al. 1995; Fig. 3). The nucleotide sequence of the 16S rDNA gene is deposited under EMBL accession no. YO 9967.

Quantification of cholesterol degradation

The heterogeneity of the culture excluded a continuous observation of the cholesterol consumption by isolate 72Chol. Therefore, anaerobic oxidation of cholesterol was quantified with different amounts of cholesterol to demonstrate the effect of carbon limitation on growth (Fig. 4 and Table 1). The 200-ml cultures contained between 0 and 200 μmol cholesterol and 2 mmol nitrate. This amount of

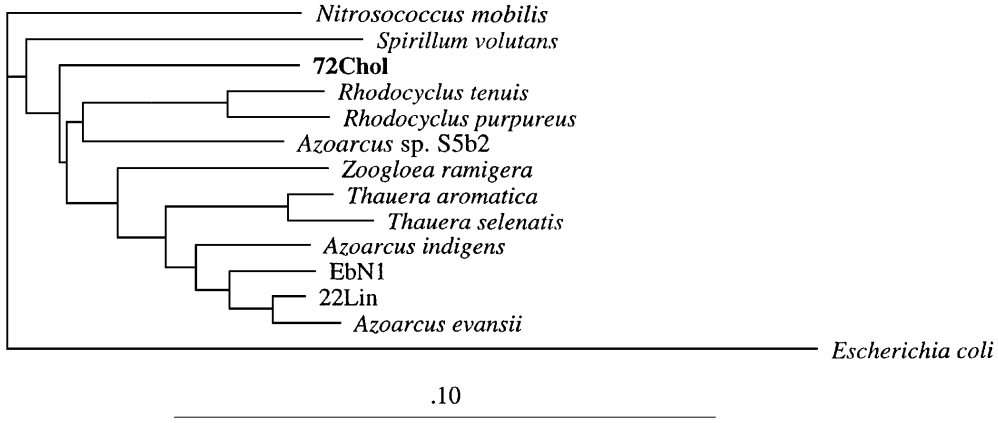

Fig. 3 Phylogenetic dendrogram indicating the position of strain 72Chol. The *scale bar* represents 10 nucleotide substitutions per 100 nucleotides. The root was determined by inclusion of *Escherichia coli* as an outgroup. The phylogenetic analysis was performed by F. A. Rainey (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig)

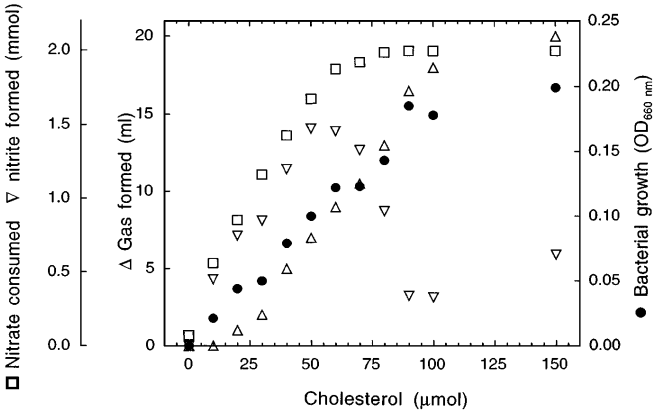

Fig. 4 Cholesterol-limited, denitrifying growth of strain 72Chol on cholesterol and nitrate. Increase in optical density, nitrate consumption, nitrite formation, and gas formation is shown for a set of experiments with increasing amounts of cholesterol at constant nitrate concentrations. See Materials and methods for details

nitrate allows complete oxidation of 65.8 µmol cholesterol according to the stoichiometric equation:

$$C_{27}H_{46}O + 30.4\ NO_3^- + 30.4\ H^+ \rightarrow 27CO_2 + 15.2\,N_2 + 38.2\ H_2O$$

The results of one experiment are summarized in Fig. 4. Since strain 72Chol does not adhere to cholesterol particles, microbial growth was monitored by turbidimetry. A maximum of bacterial density was reached with 90–100 µmol cholesterol. Nitrate reduction to nitrite was preferred over nitrogen gas formation. Nitrite was accumulated up to 7.4 mM. Before complete nitrate depletion, the amount of nitrite decreased. This observation coupled with the increased amount of gas evolved indicates a further reduction of nitrite to nitrogen. Analysis by gas chromatography revealed that the formed gases were not dinitrogen oxide or methane. Traces of dinitrogen oxide were detected only in cultures fed several times with nitrate to follow the transition from a nitrate to a carbon limitation (data not shown).

Ammonium was assimilated at an average of 11 µmol (mg protein)$^{-1}$. In nitrate-limited cultures containing 100, 150, and 200 µmol cholesterol, small amounts of cholesterol (2.0, 4.4, and 5.4 µmol, respectively) were recovered from the grown cultures. The cholesterol was detectable only after alkaline lysis of the cell lipids. A cell dry mass of 292 g was produced in cholesterol-limited cultures per mol cholesterol consumed. Taking cholesterol consumption for cell synthesis into account, the cell yield is 444 g dry wt. per mol cholesterol dissimilated. The calculated electron recovery (Table 1) suggests a complete oxidation

Table 1 Quamtification of cholesterol consumption and nitrate reduction during growth of isolate 72Chol on cholesterol and nitrate

Cholesterol provided (µmol)	Amount of cholesterol consumed (µmol)	Amount of nitrate consumed (µmol)	Amount of nitrite formed (µmol)	Amount of cell dry mass formed (mg)[a]	Electron recovery (%)[b]
0	0.0	72	0	0.0	–
30	30.0	1,162	850	7.3	100
60	60.0	1,876	1,458	17.9	89
90	89.6	2,000	340	27.2	101
150	145.6	2,000	618	29.2	60
200	194.6	2,000	518	29.2	46

[a] Calculated via cell density using an experimentally determined conversion factor (1 $OD_{660\ nm}$ = 367 mg protein l^{-1}) and a protein-dry mass ratio of 1:2

[b] Calculated ratio of numbers of electrons recovered as cell dry mass or consumed by nitrate reduction to numbers of electrons available by complete oxidation of the amount of cholesterol consumed. The assimilation equation used was $9\ C_{27}H_{46}O + 21\ H_2O + 76\ NH_4^+ + 61\ CO_2 \rightarrow 76\ C_4H_9O_2N + 76\ H^+$. For nitrate reduction, two electrons were considered to be accepted for each molecule of nitrite formed and five electrons for nitrate molecules that were consumed and not recovered as nitrite

of cholesterol to carbon dioxide in cholesterol-limited cultures. Higher values were obtained in nitrate-limited cultures, indicating an incomplete oxidation of cholesterol. The increased formation of biomass suggests formation of an intracellular storage compound. Further evidence for a complete oxidation of cholesterol was obtained from the measurements of nitrogen formed as the product of nitrate reduction and of carbon dioxide formed during the microbial growth. Nitrogen formation was determined using a helium atmosphere in the culture flasks. In a grown culture, 2 mmol nitrate disappeared and 957 μmol of dinitrogen was detected. The control culture that lacked nitrate contained 172 μmol dinitrogen. A phosphate-buffered medium was used to quantify the carbon dioxide formation. The observed formation of 1.88 mmol carbon dioxide is between expected values of 1.77 mmol (complete oxidation of cholesterol based on numbers of electrons consumed by nitrate consumption) and 2.03 mmol carbon dioxide (complete oxidation based on cholesterol consumption). Cultures without nitrate or cholesterol did not grow and contained 30 or 23 μmol carbon dioxide, respectively. The pH value of the grown culture (7.37) was 0.26 units more alkaline than that of the controls (7.09 and 7.12), in accordance with an expected alkalinization according to stoichiometry.

Discussion

Anoxic enrichments of denitrifying bacteria that utilize natural isoprenoic compounds were attempted with monoterpenes (Harder and Probian 1995) and cholesterol as sole carbon and energy source. Strain 72Chol represents the first bacterium isolated and cultured on cholesterol under strictly anoxic conditions. Quantitative analysis of the degradation balance indicated a complete oxidation of cholesterol to carbon dioxide in cholesterol-limited cultures. Growth in chemically reduced medium, where small traces of oxygen that might be present are reduced to water before inoculation, confirms that cholesterol mineralization is possible in the absence of molecular oxygen. In nitrate-limited cultures, the electron recovery value indicated an incomplete oxidation of cholesterol. Only traces of cholesterol esters were found. In future studies, we plan to characterize the transformation products present in these cultures.

The isolation of strain 72Chol expands our knowledge of the degradative capacities of anaerobic bacteria. The degradation of chemical substances in nature depends on the chemical structure and physical properties that control the bioavailability. Based on sediment and mature oil studies from oxic and anoxic habitats, a sequence of chemical compound classes has been delineated that describes a general order of susceptibility towards biodegradation: sugars, nucleic acids, proteins > low-molecular-weight hydrocarbons > acyclic isoprenoids > steranes and hopanes > aromatic steroids > porphyrins, high-molecular-weight polymers (lignins, tannins, algaenans, cutans, suberans) (Peters and Moldowan 1993; de Leeuw and Largeau 1993). Molecular oxygen is required to initiate the mineralization of many of these compounds. Therefore, such substances were initially considered recalcitrant in anoxic environments. In recent years, the isolation of anaerobic bacteria on alkanes (Aeckersberg et al. 1991; Rueter et al. 1994), aromatic hydrocarbons (Dolfing et al. 1990; Lovley and Lonergan 1990), and monoterpenes (Harder and Probian 1995) as sole carbon and energy sources has shown that anaerobic mineralization of many natural low-molecular-weight carbon compounds previously considered recalcitrant is possible. With strain 72Chol, the range is now extended to cholesterol, a molecule with a medium molecular weight and a complex chemical structure.

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