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Isolation and Characterization of "*Methanosphaera cuniculi*" sp. nov.

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A nonmotile, gram-positive, spherical organism was isolated from the intestinal tracts of rabbits. Both hydrogen and methanol were required for growth. No methane was produced from hydrogen-carbon dioxide, formate, acetate, methylamines, ethanol, or isopropanol. The optimum pH was 6.8, and the optimum temperature was 35 to 40°C. The DNA G+C content is 23 mol%. The pseudomurein cell wall contained serine. These characteristics and the immunological fingerprinting results are consistent with its placement in the genus *Methanosphaera* as a new species.

Methanogenic bacteria inhabit the rumens and the intestinal tracts of a variety of animals (12–15). Most species isolated from these habitats use H_2-CO_2 for growth and methane production. *Methanosphaera stadtmaniae*, isolated from human feces, requires H_2 to reduce methanol to methane (13). Here, we describe the isolation and characterization of a H_2-oxidizing, methanol-reducing, spherical methanogen isolated from the intestinal tract of rabbits. We propose the name "*Methanosphaera cuniculi*" sp. nov. and designate 1R7 as the type strain.

MATERIALS AND METHODS

Enrichment and isolation. The serum bottle modification of the Hungate anaerobic technique (11) was used for enrichments, isolations, and growth studies, except that serum tubes (1) replaced bottles. The medium used for enrichments and isolations was medium 1 (1) with the following modifications (final concentrations per liter): $NaHCO_3$, 3 g; yeast extract (Difco Laboratories, Detroit, Mich.), 1 g; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 2 g; cysteine, 0.3 g; $Na_2S \cdot 9H_2O$, 0.3 g; $NiCl_2 \cdot 6H_2O$, 0.2 mg. Other modifications of the medium included the following (final concentrations per liter): 0.5 ml of a 1 mM solution of Na_2SeO_3; methanol, 5 ml; and vancomycin, 100 mg. The final pH after sterilization and pressurization to 202.6 kPa with H_2-CO_2 or N_2-CO_2 (4:1, vol/vol) was 7.0. The medium was inoculated with the contents of the stomachs, duodena, caeca, colons, and rectums of 10 rabbits. The animals were hybrids of a New Zealand White female rabbit and a California male rabbit. The dry matter percentages of the commercial diet were, by weight: crude protein, 18.5; crude lipid, 2.5; crude fiber, 17.5; N-free extract, 51.5; and ash, 10.0. Before isolation, the cultures were transferred twice into fresh medium in which vancomycin was replaced first by 6.7 and 1.7 μg of clindamycin and cephalothin, respectively, per ml and then by penicillin (40 μg/ml). The enrichment cultures were diluted and inoculated into agar (2%) roll tubes (3) for isolation. The procedure was repeated with isolated colonies to ensure the purity of the cultures.

Maintenance and growth studies. Isolates were maintained at −80°C in biphasic cultures (7) containing 5 ml of double-strength solid (3% agar) medium and 1 ml of liquid medium as suggested by Miller and Wolin (13). A solution (0.1 ml) of anaerobic, sterile cryoprotectant was added to the culture before the culture was frozen. The cryoprotectant contained the following: glycerol, 10% (vol/vol); Na_2S, 0.05% (wt/vol); and cysteine, 0.05% (wt/vol). When cells from biphasic cultures were prepared for freeze-drying, a cryoprotectant with the following composition was used (final concentration per liter): skim milk, 100 g; lactose, 30 g; yeast extract, 3 g; $NaHCO_3$, 3 g; Na_2S, 0.03%; and cysteine, 0.03%.

The medium used for growth studies was the same as was used for the enrichment culture, except antibiotics were omitted and substrates were added as indicated. Media at various pH values were obtained by adding 1 M HCl to obtain pH values ranging from 7 to 5.5. *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; 0.1 M) replaced sodium carbonate to produce pH values ranging from 7 to 8.

Growth was determined by methane production and A_{550}. The A_{550} of cultures was determined with a Bausch & Lomb, Inc., Spectronic 20 spectrophotometer. Methane was quantitated with a model HRGC 5300 Mega gas chromatograph equipped with a thermal conductivity detector and a PQS 80:100 column (Carlo Erba, Milan, Italy). The oven and injector temperatures were both 50°C. N_2 was used as the gas carrier at a flow rate of 15 ml/min, which resolved methane, hydrogen, and CO_2. Methane was also quantitated with a Varian model 3700 gas chromatograph as previously described (17).

Microscopy. Cells for electron microscopy were post-fixed with 0.1 M cacodylate buffer (pH 7) containing 1.5% each of ruthenium red and osmium tetroxide to add electron density. Phase-contrast photomicrographs were taken with a Zeiss microscope equipped with a 35-mm camera.

DNA analysis. The organisms were grown in 4 liters of modified medium 1 in 10-liter carboys sealed with neoprene stoppers. The headspace of H_2-CO_2, at ambient pressure, was flushed daily. After 7 days, the cells were harvested and suspended in 20 ml of saline-EDTA buffer (0.15 M NaCl, 0.01 M sodium EDTA, pH 8.0) and then were broken by passage through a French pressure cell at 137 MPa.

DNA was isolated by a variation (5) of the Marmur procedure (9). The G+C mole percent of DNA was determined from thermal melting points (10). DNA-DNA homol-

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ogies were determined by the S1 procedure described by Johnson (4). Fragmented denatured DNA was labeled with the Prime Time biosystem (International Biotechnology, Inc., New Haven, Conn.) except that [^{32}P]dCTP was replaced with [^{35}S]dCTP. The reassociation mixtures consisted of 10 μl of labeled DNA (0.03 μg), 50 μl of unlabeled DNA (20 μg), 25 μl of 5.15 M NaCl, and 25 μl of 10 mM Tris–1 mM EDTA, pH 8. The S1-resistant material in the labeled DNA preparations was measured by replacing the unlabeled DNA with 50 μl (20 μg) of sheared native salmon sperm DNA. The mixtures were reassociated at 55°C for 22 h. After precipitation, fragments resistant to the S1 nuclease were collected on filters of glass fiber paper and counted.

Polyacrylamide gel electrophoresis. Electrophoretic analysis of soluble proteins obtained from 10-ml log phase cultures was performed by using the procedure of Moore et al. (16).

RESULTS

Methane was detected only in enrichment cultures derived from rabbit caeca, colons, or rectums. After serial transfer in media containing antibiotics, gram-positive cocci became the predominant organisms. Only methane-producing cocci were isolated from these enrichment cultures. One of the rectal isolates, strain 1R7, was studied further. Microscopically pure cultures failed to grow in complex media without methanol or antibiotics. Surface colonies were circular and reached approximately 1 mm in diameter after 10 days of incubation at 37°C. Single cells of the spherical isolate were ca. 0.6 to 1.2 μm in diameter (Fig. 1) and occurred in pairs or tetrads. Thin-section electron micrographs (Fig. 2) revealed a thick (14 to 16 μm), electron-dense cell wall. Cells were resistant to lysis by lysozyme (1 mg/ml), sodium dodecyl sulfate (2% [wt/vol]), proteinase K (1 mg/ml), and achromopeptidase (1 mg/ml). Autolysis was frequent.

A H_2-CO_2 (4:1, vol/vol) atmosphere, together with methanol and acetate in the medium, was required for growth. Cultures typically reached an A_{550} of 0.8 after 4 days of incubation at 37°C (Fig. 3). No significant growth or methanogenesis occurred with H_2-CO_2 alone or with methanol or acetate in media after 30 days of incubation under a N_2-CO_2 atmosphere. These results indicate that H_2 was required to provide electrons for the reduction of methanol to methane. The inability of acetate to support methanogenesis suggests that it is required as a carbon source when cultures are grown on H_2 plus methanol. No growth or methanogenesis was obtained after 30 days of incubation under a H_2-CO_2 or a N_2-CO_2 atmosphere when ethanol, isopropanol, methylamines, or formate replaced methanol in the media. Maximum growth occurred in media containing 3.5 to 5% methanol; higher concentrations were inhibitory. The optimum pH for maximum growth was 6.8. The optimum growth temperature at pH 7 was 35 to 40°C; no growth occurred at 25 or 45°C. The DNA base composition was 23 ± 1 mol% G+C. Antigenic fingerprinting by the indirect immunofluorescence technique (2, 8) showed that strain 1R7 reacted completely (4+) with the anti-*M. stadtmaniae* MCB-3 S

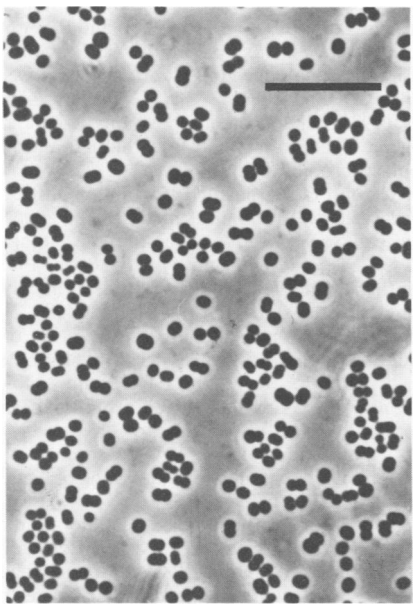

FIG. 1. Phase-contrast photomicrograph of strain 1R7 cells from a culture in exponential phase. Bar = 10 μm.

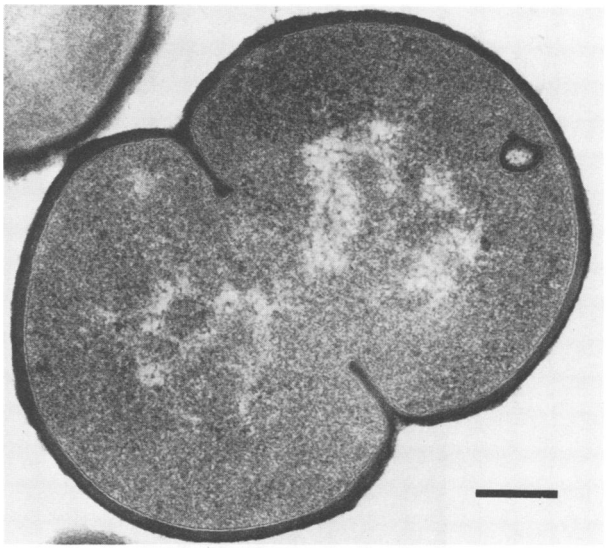

FIG. 2. Electron micrograph of a thin section through a dividing cell of strain 1R7. Bar = 0.1 μm.

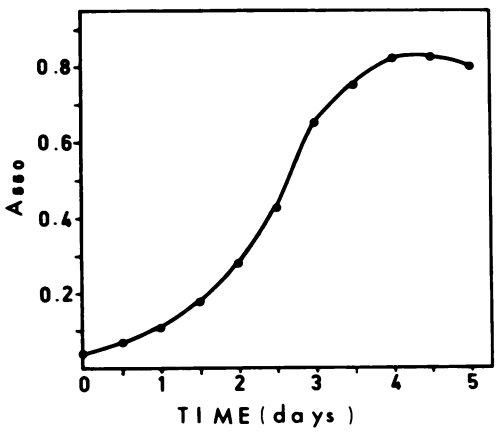

FIG. 3. Typical time course for growth of strain 1R7 in medium containing methanol under an atmosphere containing H_2. The tube contained 10 ml of culture.

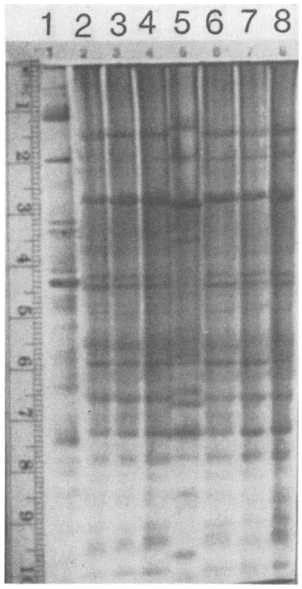

FIG. 4. Denaturing polyacrylamide gel electrophoresis of soluble proteins. Lanes: 1, *Streptococcus faecalis* VPI V4-20; 2 to 4, isolates from rabbit rectums; 5, *M. stadtmaniae* MCB-3; 6 to 8, isolates from rabbit caeca.

probe and showed no reaction with the anti-*Methanobacterium thermoautotrophicum* ΔH and GC1 S probes. The electrophoretic pattern of soluble cell proteins from strain 1R7 and from other rectal isolates was similar to those of methanogenic cocci isolated from the caecum; however, this pattern varied from that of *M. stadtmaniae* MCB-3 (Fig. 4). DNA-DNA homology relationships between *M. stadtmaniae* MCB-3 and strain 1R7 were 26 and 32%, respectively, with the two strains as references.

DISCUSSION

Several characteristics clearly indicate that strain 1R7 belongs to the genus *Methanosphaera*. (i) Methanol was required for growth and methanogenesis in a H_2-CO_2 atmosphere, a feature unique to this genus. (ii) The cell morphology was highly characteristic of the genus. (iii) Like *Methanosphaera* spp. (6), the 1R7 cell wall contains pseudomurein, with an amino acid composition characterized by the presence of serine (H. König, manuscript in preparation). (iv) Indirect immunofluorescence fingerprinting indicated that the isolate was immunologically most closely related to *M. stadtmaniae*, the only species assigned to the genus. However, several characteristics distinguish strain 1R7 from the only described species. (i) Strain 1R7 does not immunologically cross-react with *M. thermoautotrophicum*, whereas *M. stadtmaniae* cross-reacts with that species (6). (ii) Differences in protein profiles between strain 1R7 and *M. stadtmaniae* MCB-3 are evident. (iii) DNA from strain 1R7 contained a mole percent G+C content of 23 ± 1 (T_m), compared with the value of 25.8 mol% (T_m) obtained previously for *M. stadtmaniae* MCB-3 (6). (iv) The average DNA-DNA homology between 1R7 and the *M. stadtmaniae* type strain was 29%. Thus, we propose that strain 1R7 be placed in the genus *Methanosphaera* as a new species, "*Methanosphaera cuniculi*" sp. nov. The species description follows.

Methanosphaera cuniculi sp. nov. (cu.ni'cu.li. L. n. *cuniculus*, rabbit; L. gen. n., cuniculi of the rabbit).

Cells are round, 0.6 to 1.2 μm in diameter, gram positive, nonmotile, and usually occur in pairs. Cell walls are composed of pseudomurein and contain serine. The optimum temperature is 35 to 40°C, and the optimum pH is 6.8. The organism is anaerobic. Colonies are circular or elliptical, yellowish, and ca. 1 mm in diameter and have entire edges. Both hydrogen and methanol are required for growth. No growth occurs with H_2-CO_2 alone or under a N_2-CO_2 atmosphere in media containing methanol or acetate. No growth occurs under a H_2-CO_2 or a N_2-CO_2 atmosphere in media containing ethanol, isopropanol, methylamines, or formate. Acetate is required for growth but does not support methane production. The DNA G+C content is 23 mol%. The type strain, isolated from rabbit rectums, is 1R7 (DSM4103).

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