CURRENT MICROBIOLOGY Vol. 29 (1994), pp. 7–12

Current Microbiology
An International Journal

Isolation and Characterization of *Methanobrevibacter oralis* sp. nov.

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Abstract. A new coccobacillary, nonmotile, Gram-positive, methane-producing organism was isolated from human subgingival plaque. Both hydrogen and carbon dioxide were required for growth. No methane was produced from acetate, formate, or methanol. The optimum pH was 6.9–7.4, and the optimum temperature was 36–38°C. Fecal extract was required for growth, and a volatile fatty acid mixture was highly stimulatory. The DNA G+C content was 28 mol%. On the basis of these characteristics, DNA-DNA hybridization studies, and electrophoretic analysis of cellular proteins, the isolate was considered a new species and named *Methanobrevibacter oralis*.

The methanogenic bacteria, microorganisms that belong to the Archaeobacteria, are usually found in the gastrointestinal tract of animals and man. The only species found in human intestine are *Methanobrevibacter smithii*, which is present in all subjects with high levels of methanobacteria [15, 17] and which uses H_2 and CO_2 for growth and methane production, and *Methanosphaera stadtmanae*, which is present only in a few individuals and at extremely low concentration and which requires H_2 to reduce methanol to methane [16]. Methanobacteria have been recently found also in the human oral cavity, and we detected them also in periodontal pockets and saliva [7, 8]. With immunological methods, some methanobacteria strains, isolated from human periodontal plaque, have been identified as *M. smithii* [3]. We herein describe the isolation and characterization of a new species of *Methanobrevibacter* isolated from human periodontal plaque. We propose the name *Methanobrevibacter oralis*, sp. nov. for the species.

Materials and Methods

Operating conditions and growth medium. The anaerobic growth medium (MB) used was a modification of Medium 1 of Balch et al. [1], and contained: KH_2PO_4, 0.3 g/L; K_2HPO_4, 0.3 g/L; NH_4Cl, 1 g/L; NaCl, 0.61 g/L; $CaCl_2 \cdot 2H_2O$, 9 mg/L; $CoCl_2 \cdot 6H_2O$, 1 mg/L; $NiCl_2 \cdot 6H_2O$, 0.25 mg/L; $MgSO_4 \cdot 7H_2O$, 0.16 mg/L; $MnSO_4 \cdot 2H_2O$, 5 mg/L; $ZnSO_4 \cdot 7H_2O$, 1 mg/L; $CuSO_4 \cdot 5H_2O$, 0.1 mg/L; $FeSO_4 \cdot 7H_2O$, 2 mg/L; $AlK(SO_4)_2$, 0.1 mg/L; H_3BO_3, 0.1 mg/L; $Na_2WO_4 \cdot 2H_2O$, 1 mg/L; Na_2SeO_3, 0.2 mg/L; $Na_2MoO_4 \cdot 2H_2O$, 0.4 mg/L; $NaHCO_3$, 5 g/L; $(NH_4)_2SO_4$, 0.3 g/L; sodium formate, 2.5 g/L; sodium acetate, 2.5 g/L; $Na_2S \cdot 9H_2O$, 0.25 g/L; cysteine-hydrochloride monohydrate, 0.5 g/L; yeast extract, 1 g/L; trypticase, 1 g/L; nitrilotriacetic acid, 15 mg/L; resazurin, 0.1%, 1 ml/L; human fecal extract [2] at 10% (vol/vol); and 8 ml/L of a mixture of volatile fatty acids (VFA) [9]. The medium was assembled into an anaerobic chamber (Forma Scientific, mod 1024, Marietta, Ohio, USA) in an atmosphere of 5% CO_2: 10% H_2:85% N_2 and dispensed in 10-ml portions into 30-ml serum bottles, then sealed with butyl rubber stoppers and sealed with aluminum crimp seals. Vial head spaces were washed with an atmosphere of 20% CO_2: 80% H_2. Final pH was 7.0. The serum bottle modification of the Hungate anaerobic technique [14] was used for enrichments, isolation and growth studies, except that vials replaced bottles, and microbiological manipulations were performed inside the anaerobic chamber.

Sampling and isolation. Subgingival samples from ten healthy subjects, collected in sterile curettes according to Socransky et al. [21], were tested. Samples were immediately placed in vials containing prereduced dilution blank [10] and maintained in Gaspak under anaerobic conditions. Within 1 h, the samples were introduced into an anaerobic cabinet, and the contents of each vial was inoculated into the vials containing MB and incubated at 37°C for 10 days. For enrichment, the cultures were inoculated biweekly in fresh MB medium with a series of dilutions of 10 to 10^6; for successive passages only the most diluted cultures in which methanobacteria grew were used. After 3 months, the enriched cultures were transferred to MB medium initially containing 6.7 μg of cephalotine and 1.7 μg of clindamycin, and then 15 μg of kanamycin and 100 μg of vancomycin; all antibiotics were supplied by Sigma-Aldrich, (Milan, Italy).

Such a culture, serially diluted into MB, was plated onto agarized medium, 1.5% Noble agar (Difco Laboratories, Detroit, Michigan, USA). The agar plates were prepared inside the anaerobic chamber, then dried overnight and, after streaking, introduced into a stainless steel anaerobic jar appropriate to tolerate high pressure. The jars were flushed and pressurized at 202.6 kPa with 80% H_2:20% CO_2 and incubated at 37°C. Represen-

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tative colonies from agar plates were transferred into vials containing MB inside the anaerobic chamber. Methanogenic broth cultures were serially diluted and plated for colony picking. This procedure was repeated several times until culture purity was established. To assess culture purity, the strains were transferred into complex media containing yeast extract, tryptone, and glucose (Difco Laboratories).

Maintenance and inoculum. Stock cultures of methanogenic isolates were maintained in MB at pH 7; they were transferred every 8–12 days into fresh media. The stock culture vials were repressurized (202.6 kPa) every 4–5 days by injection of 80% H_2:20% CO_2. Unless otherwise stated, the inoculum for tests consisted of 1% (vol/vol) of a 6-day-old culture in MB. All incubations were static. All the tests were done in triplicate.

Bacterial strains. Studies were carried out with all known species of the Methanobrevibacter genus. The following strains were used: *Methanobrevibacter ruminantium*, type strain DSM number 1093; *Methanobrevibacter smithii*, type strain DSM number 861; *Methanobrevibacter arboriphilicus*, type strain DSM number 1125.

Growth characteristics. The optimum temperature for growth was determined in MB at pH 7 by incubating preparations at 15°–55°C. The growth of the methanobacteria present in the different vials at various temperatures was followed daily by evaluating the number of microorganisms by use of a counting chamber. The vial headspaces were repressurized (202.6 kPa) every 3–4 days of incubation by injecting 80% H_2:20% CO_2 to assure adequate substrate availability.

The pH range and the optimum for growth at 37°C were determined by evaluating the growth of microorganisms in the media at different pHs. The tests were carried out with MB broth adjusted to various pH values by adding 1 M HCl to obtain pH values ranging from 5.5 to 7; *N*-Tris(hydroxy-methyl)methyl-2-aminoethanesulfonic acid (TES; 0.1 M) (Sigma-Aldrich, Milan, Italy) replaced sodium carbonate to produce values ranging from 7 to 8.4. The pH in the test vials was verified during and after growth. The pH was maintained at ± 0.1 units during incubation.

Susceptibility to lysis, NaCl optimum, and tolerance. Cells from the mid-exponential growth phase were used for tests of susceptibility to lysis. Lysis was determined by visual and microscopic observation of exposed cultures in comparison with unexposed controls. The tests were performed with sodium dodecyl sulfate and distilled water [6]. Tests to establish the optimal NaCl concentration and the range of tolerance were carried out by adding to the MB medium increasing quantities of NaCl (from 0.01 to 0.5 M) and evaluating growth with time in comparison with a control culture.

Microscopy. Cells were routinely observed with a Zeiss phase-contrast microscope equipped with a 35-mm camera to take microphotographs of cells during the exponential growth phase. Fluorescence was observed with a Zeiss phase-contrast microscope equipped for epifluorescence with a 426 filter set and an LP 470 barrier filter. For electron microscopy, samples were fixed with 3% glutaraldehyde in 0.1 cacodylate buffer, pH 6.9, and rinsed in cacodylate buffer. The specimens were postfixed in 1% osmic acid, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with 2% uranyl-acetate and lead citrate [19]. Sections were examined using a Philips 400 T transmission electron microscope.

Growth substrates. The ability of some of the generally reported methanogenic substrates to support growth was determined in MB broth at pH 7 and 37°C. Test substrates were filter-sterilized and stored under N_2 until use. Growth in different substrates was determined through methane production and by evaluating the number of microorganisms with a counting chamber. The suitability of acetate (2.5 g/L), formate (2.5 g/L), or methanol (0.4% vol/vol) to serve as a hydrogen donor was determined after 2 and 4 weeks of incubation in MB medium, 80% N_2:20% CO_2 gas phase, and the capacity to incorporate carbon from the same organic substrates was determined after incubation in the presence of an 80% H_2:20% N_2 gas phase.

Gas chromatography. Methane analysis was performed with a Dani model 3200 HWD gas chromatograph equipped with a column (1m × 4mm) packed with a molecular sieve. The carrier gas was H_2 at 20 ml/min, the oven temperature was 50°C, and a conductivity detector was used.

Electrophoretic analysis. The electophoretic patterns of cellular proteins were obtained and documented by the procedures described by Moore et al. [18] and Biavati et al. [4].

DNA analysis. The cells were harvested and suspended in saline-EDTA buffer (0.15 M NaCl, 0.01 M sodium EDTA, pH 8) and then broken by passage through a French pressure cell at 137 MPa. DNA was isolated by a variation [11] of the Marmur procedure [12]. The guanine-plus-cytosine (G + C) contents of DNA were determined by melting point analysis [13] with a Response series UV-visible spectrophotometer equipped with a Response II thermal programmer. *Escherichia coli* K-12 DNA was used as the standard. The construction of nonradioactive DNA probes by random primed incorporation of digoxigenin-labeled deoxyuridine triphosphate was performed with a nonradioactive DNA labeling and detection kit (Boehringer Mannheim Biochemia, Mannheim, Germany). The labeling of DNA, dot blot hybridization on nitrocellulose membranes, and detection of the hybridized probe by an enzyme-linked immunoassay procedure were performed as suggested by the manufacturer (Boehringer Mannheim Biochemia).

Results

Isolation. Four samples of the ten collected subgingival plaque specimens were positive for methane production within 8 days in enrichment cultures. After transfer in media containing antibiotics, the four subgingival plaque enrichments that produced methane contained high numbers of short Gram-positive coccobacillary cells that occurred mainly in pairs or short chains and showed factor-420 fluorescence by epifluorescence microscopy. Serial dilutions of these cultures were streaked onto agarized MB. Visible colonies were usually observed after 15–20 days of incubation. Colonies were picked and transferred into MB broth, serially diluted, and plated onto the same agarized medium. The procedure was repeated until culture purity was established by colony morphology and microscopic observation of cultures transferred into MB and complex media containing yeast extract, tryptone, and glucose.

Pure cultures of methanogens were isolated from

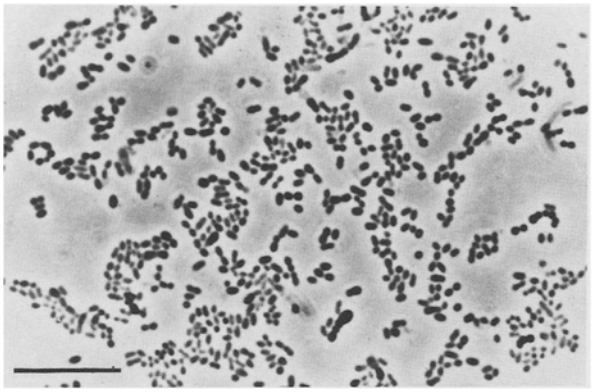

Fig. 1. Phase-contrast micrograph of strain ZR cells during the exponential growth phase. Bar = 10 μm.

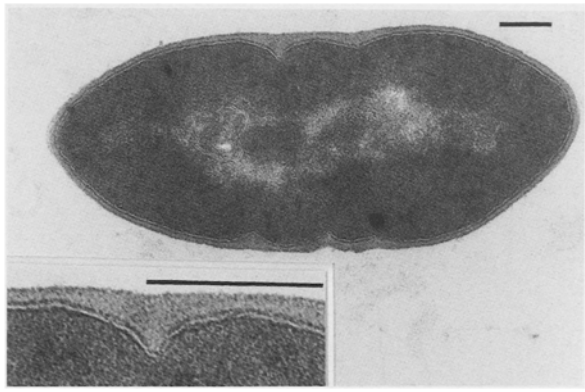

Fig. 2. Thin section of *M. oralis* strain ZR, and thin section through the wall of the same strain. Bar = 0.1 μm.

two of the four positive primary enrichments. For the other two, the purification was incomplete.

Morphology. The isolated strains were morphologically the same short coccobacillary cells observed in the enrichments, and all showed factor-420 fluorescence. Figure 1 shows the typical appearance of the isolates as observed by phase-contrast microscopy. The two isolated strains were identical morphologically and for culture characteristics (pH, temperature, Gram). Therefore, only one of these organisms, strain ZR, was characterized in detail and is described below.

Surface colonies of strain ZR were 0.5–1.0 mm in diameter, were circular, had entire margins, and were creamy to light yellow in color. The cells were short, oval rods or coccobacilli with tapered ends, 0.4 μm in width, and 0.7–1.2 μm in length. Thin-section electron microphotographs (Fig. 2) revealed a thick (0.016–0.020 μm) electron-dense cell wall, composed of three layers and invaginated at multiple septa sides, as previously reported for *M. ruminantium* strain M1 by Zeikus and Bowen [23].

Cell lysis and Gram stain. When cultures were less than 4 days old, all microorganisms were Gram positive. Older cultures were Gram variable. The isolate was nonmotile when examined in hanging-drop preparations. Cells from mid- or late-exponential growth phase did not lyse when they were suspended in a hypotonic solution or in SDS (0.1 g/L).

Optimum growth conditions. The optimal NaCl concentration for growth was between 0.01 and 0.1 M at 37°C. A decrease in growth was evident at a concentration of 0.2 M, and no growth occurred above this concentration. The optimum growth temperature was 36°–38°C. Under 20°C the strains did not grow, and only slight growth was evidenced between 25° and 30°C. Growth was markedly reduced above 39°C and completely inhibited above 40°C. The optimum pH for growth at 37°C was between 6.9 and 7.4. Only slight growth was noted in the pH ranges 6.2–6.9 and 7.4–7.8. The strain was unable to grow below pH 6.0 or above pH 8.0.

A typical time course for growth is shown in Fig. 3. Doubling time of the cells was 15 h.

Substrates and growth requirements. Growth and methanogenesis was obtained only in the previously described MB medium.

Reduced growth and slight methane production was observed even in the absence of the VFA mixture. Fecal extract is not substitutable by CoM, which is used for growth of other nutritionally fastidious methanogens [22]. Fecal extract is only partially substitutable by sludge extract; in the presence of sludge, growth is always weaker and slower than with fecal extract. A very weak and slow growth was obtained only in the complex medium, "Methanobacterium medium," which contains sludge extract from anaerobic digester, proposed by DSM [9]. The type strains used as controls, *M. smithii* (DSM-861), *M. arboriphilicus* (DSM-1125), and *M. ruminantium* (DSM-1093), instead grew in MB broth without fecal extract or VFA mixture. The new strains cannot use acetate, formate, or methanol.

Cell protein analysis. The electrophoretic patterns of soluble proteins of the type strain of *M. smithii* and strain ZRF, ZR, ZR1 are shown in Fig. 4. Close inspection revealed uniformly distinct bands for the two groups of strains isolated from human feces (ZRF, DSM 861) and from subgingival plaque (ZR, ZR1).

DNA-DNA homology. DNA dot-blot assay (Fig. 5) clearly showed that the strongest signals in the

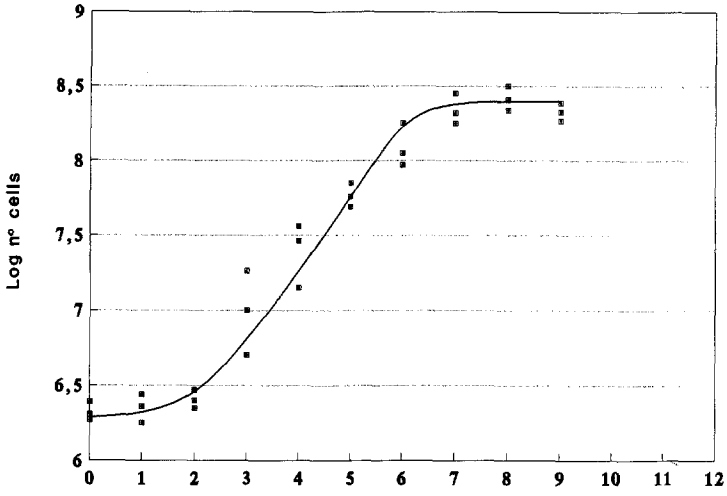

Fig. 3. Typical time course for growth of strain ZR.

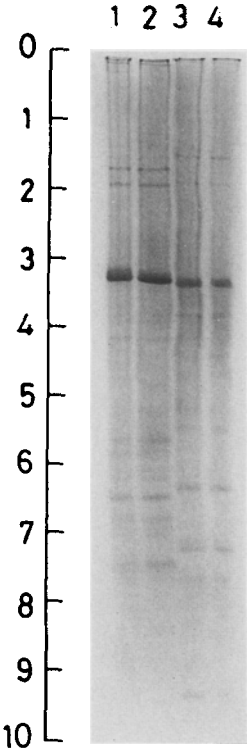

Fig. 4. Electrophoretograms of soluble cellular proteins. Lanes: 1 = ZRF; 2 = DSM 861; 3 = ZR; 4 = ZR1.

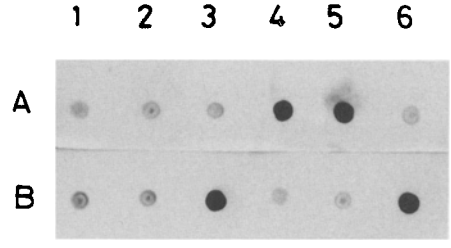

Fig. 5. DNA dot-blot hybridization with nonradioactive genomic DNA probes. Rows: (A) ZR, (B) DSM 861; columns (1) DSM 1125; (2) DSM 1093; (3) ZRF; (4) ZR1; (5) ZR; (6) DSM 861.

hybridization reaction were observed between strains ZR1 and ZR (row A, columns 4 and 5) and strains ZRF and DSM 861 (row B, columns 3 and 6). In all other cases the hybridization reaction was very light. The DNA base composition was 28 mol% G+C.

Discussion

The phenotypic characterization of strain ZR clearly showed that the organism belongs to the genus Methanobrevibacter. In fact, as previously described, the following characters are typical of the genus: strain morphology is coccobacillary, cells are Gram positive, nonspore-forming, and nonmotile. The optimum temperature for growth is 36–38°C, and the strain grows with H_2–CO_2. The mol% G+C of the DNA is 28, and the strain is a very strict anaerobe (Table 1).

However, results of our study show that strain ZR presents many physiological and genetic differences with respect to the other species of the genus, including *M. smithii*, which is the predominant one in the human intestinal tract. The microorganism is slightly smaller than the type strains of the other species. Its wall is tristratified and with deep invaginations; the latter character has also been observed in *M. ruminantium*. Instead, a monostratified wall has been described for the other two species of *Methanobrevibacter* (*M. smithii* and *M. arboriphilicus*) [5].

In a previous study, methanobacteria from the oral cavity were identified as *M. smithii* on the basis of immunological reactions [3]. In this study we isolated strains ZR and ZRF from two samples (subgingival and feces), both from the same subject. To prove that

Table 1. Comparison of strain ZR and related species of *Methanobrevibacter* genus

Strain	DNA G + C content (mol%)	Optimum growth conditions			Dimensions (mm)	Use of substrates				Cell wall	Susceptibility to lysis		References
		pH	Temp (°C)	NaCl concn. (M)		H_2-CO_2	For-mate	Ace-tate	Meth-anol		SDS	Hypotonic solution	
ZR	28	6.9–7.4	36–38	0.01–0.1	0.4 × 0.7–1.2	+	–	–	–	Three layers invaginated at multiple septa sites	–	–	This study
Methanobrevibacter arboriphilicus	28–32	7.8–8.0	30–37	ND	0.5 × 1–3	+	–	–	–	Single layer	–	–	[5, 24]
Methonobrevibacter ruminantium	31	neutral	37–39	ND	0.7 × 0.8–1.7	+	+	–	–	Three layers invaginated at multiple septa sites	–	–	[5, 20]
Methanobrevibacter smithii	28–31	neutral	37–39	ND	0.5–0.7 × 1.0–1.5	+	+	–	–	Single layer	–	–	[1, 5]

the strain isolated from the feces was different from that isolated from subgingival plaque, we compared them with ZR1 and *M. smithii* DSM 861 by electrophoretic analysis. The results reported in Fig. 4 show that the strains isolated from the feces were different from those of subgingival plaque. The same result was also obtained by dot blot hybridization (compare columns 3, 4, 5, and 6 of Fig 5). Moreover, this experiment showed that the new isolates from subgingival plaque were also different from the other two species of the genus, *M. arboriphilicus* and *M. ruminantium*.

The microorganism has, with respect to the reference type strains, particular nutritional requirements because VFA mixture and fecal extract were necessary for growth, and the latter was only partially substitutable by sludge fluid from anaerobic digester. For these reasons, we believe that strain ZR is a new species of the genus Methanobrevibacter, and we propose the description of this new species, *Methanobrevibacter oralis*, as follows.

Methanobrevibacter oralis sp. nov. (o.ra'lis. M.L. adj. *oralis* of the mouth). Cells are short, oval rods with tapered ends, 0.4–0.5 mm in width and 0.7–1.2 mm in length. Cells occur most frequently in pairs or short chains; they are Gram positive and nonmotile. The organism is a *very* strict anaerobe.

The optimum temperature is 35°–37°C, the temperature range is 25°–39 °C, the optimum pH is 6.9–7.4, and the pH range is 6.2–8. The optimum sodium chloride concentration is between 0.01 and 0.1 M, and there is no growth above 0.2 M.

Fecal extract is required for growth, and VFA mixture is highly stimulatory. Growth occurs under H_2-CO_2 atmosphere. Formate, acetate, and methanol are not required for growth.

The DNA G+C content is 28 mol% G+C. The type strain is ZR (=DSM 7256) isolated from human oral cavity.

ACKNOWLEDGMENTS

The authors thank Prof. Stefania Pellegrini of the Department of Biology, University of Milan, for preparation and interpretation of the ultrathin section. The research was supported by the Italian National Research Council, Special Project RAISA, subproject no. 4, paper no. 1279.

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