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ISOLATION AND CHARACTERIZATION OF A NEW THERMOPHILIC *METHANOSARCINA* STRAIN (STRAIN MP)

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SUMMARY

A thermophilic *Methanosarcina* strain was isolated from a digester fed with water hyacinths and inoculated with ground termites from the Congo. Optimal growth temperature was 55° C. Methane production was at its optimum between pH 6.5 and 7.0. The bacterium grew on acetate, methanol and methylamines in the absence of growth factors, but could not use H_2-CO_2 or formate. H_2-CO_2 inhibited acetate utilisation. Yeast extract and vitamins stimulated growth.

KEY-WORDS: Methanogenesis, Thermophily, *Methanosarcina;* Acetate, New strain.

INTRODUCTION

During the past decade, thermophilic bacteria producing methane have received increased attention. The first thermophilic methanogen was obtained in pure culture in 1972 [25]. This bacterium, named *Methanobacterium thermoautotrophicum*, grew up to 75° C and used only H_2-CO_2 as the carbon and energy source. In 1979, Zinder and Mah [26] reported the isolation of a thermophilic strain of *Methanosarcina* from an anaerobic sludge digester. This organism could use methanol, methylamines and acetate in the presence of an unknown factor, but was unable to grow on H_2-CO_2.

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The organism has been deposited with the DSM (German Collection of Microorganisms) under the collection number DSM 2980.

An extremely thermophilic rod-shaped methanogen *(Methanothermus fervidus)*, which grew between 65° C and 97° C on H_2-CO_2, was described in 1981 [22]. More recently, Huber *et al.* [7] have isolated, from geothermally heated sediments, a coccoid methanogen related to the genus *Methanococcus*: the only two substrates for methanogenesis were formate and H_2-CO_2. The same substrates were used by two rods belonging to the genus *Methanobacterium* [16].

Two other thermophilic coccoid methanogens *(Methanogenium* sp.) were isolated from marine sediments [20] and from an anaerobic kelp digester [5]. Another acetoclastic rod-shaped bacterium *(Methanothrix* sp.) growing up to 65° C was described in 1982 [19]. The last thermophilic isolate was isolated from a submarine hydrothermal vent [9]. It belonged to the genus *Methanococcus*.

In the present paper, we describe the isolation and characterization of a new thermophilic strain of *Methanosarcina* (strain MP) which grows on acetate in the absence of organic compounds.

MATERIALS AND METHODS

Gases.

Gases and gas mixtures were purchased from « Airgaz » (Marseille). H_2-CO_2 (80-20%) and N_2-CO_2 (80-20%) were used during this work.

Inoculum.

A total of 800 g of crushed water hyacinths were diluted with 800 ml of water and inoculated with 1.25 g of ground termites *(Cubitermes* sp.) from the Congo. The experiment was performed by the Laboratoire d'Énergétique Électrochimique et Biochimique (University of Paris Val-de-Marne). The anaerobic process was carried out in a digester under thermophilic conditions (55° C) for 45 days. Methane was detected 30 days after inoculation. The percentage of methane in the biogas was 53%.

Culture media.

The anaerobic technique of Hungate [8] was used for this work. The methanogenic bacterium was cultivated on a complex medium containing: NH_4Cl, 1.0 g; K_2HPO_4, 0.4 g; $MgCl_2.6H_2O$, 0.2 g; cystein-hydrochloride, 0.5 g; sodium acetate, 5.0 g; yeast extract (Difco Lab., Detroit, MI), 0.1 g; resazurin, 0.001 g; distilled water, 1,000 ml; mineral solution n° 2 [1], 50 ml; trace mineral solution [1], 10 ml.

For roll tubes, agar (2.0%) was added to a liquid medium. The thermophilic acetoclastic bacterium was routinely cultivated in a medium containing 0.1% yeast extract. The medium was adjusted to pH 7.0 with KOH 10 M and boiled under O_2-free N_2. After cooling, 20 ml of medium were transferred into 60-ml serum bottles stored in an anaerobic glove box (La Calhene, Bezons, France). The bottles were stoppered with black butyl rubber closures (Bellco Glass Inc., Vineland, NJ) and outgassed with N_2-CO_2; 0.3 ml Na_2CO_3 (10% w/v) and 0.4 ml Na_2S (1% w/v)

TMA = trimethylamine.
UV = ultraviolet.

were added to the medium. When H_2-CO_2 was added as substrate and energy source, the amount of Na_2CO_3 (10% w/v) was increased to 0.4 ml.

For roll tube preparation, agar was supplemented after boiling; 4.5 ml of medium were dispensed inside the anaerobic glove box in Hungate tubes (Bellco Glass Inc.); 0.08 ml Na_2CO_3 (10%) and 0.1 ml Na_2S (1%) were added to the medium after autoclaving. For solid or liquid media, 0.02% (v/v) methanol was injected before inoculation. Stock solutions of Na_2CO_3, Na_2S and methanol were prepared anaerobically under N_2 in 120-ml serum bottles and were sterilized (110° C, 35 mn).

Analytical techniques.

In each case, experiments were repeated twice on duplicate cultures. The temperature of incubation was 55° C. The gas phase was analysed by gas chromatography (Varian Aerograph 2700) equipped with a flame ionization detector and a stainless steel column (2 m × 1/8'') containing « Porapak Q » 80-100 mesh. The carrier gas was N_2 (17 ml/min). The temperature of the column was 190° C. The injector and detector temperature were 115° C and 250° C, respectively. Methane production was quantified by sampling 0.1 ml of the vial gas phase.

Microscopy.

A « Nikon » microscope equipped with a « Nikon Fx35 » camera (Nikon, Japan) was used to take phase photomicrographs with « Ilford HP5 » film (ASA 400). Samples for electron microscopy were fixed with glutaraldehyde (1% v/v) and osmium (1% w/v) and mounted in Epon-araldite resin [18]. Ultrathin sections were examined with the Jeol Jem 100 U electron microscope.

RESULTS

Enrichment and isolation.

Samples of the thermophilic digester were inoculated in the medium previously described containing 0.1% yeast extract and 5% (v/v) rumen fluid. The enrichment procedure was carried out on H_2-CO_2, acetate, trimethylamine (TMA) and methanol. CH_4 was produced after 3 days on H_2-CO_2. After one week, appreciable amounts of methane were detected on the other substrates. Enrichment on H_2-CO_2 showed a coccoid form closely resembling *Methanogenium* sp. morphologically and physiologically. This bacterium isolated on H_2-CO_2 was analysed for further characterization.

We report results concerning the acetoclastic organism. This organism was found on acetate as well as on TMA or methanol enrichments. The UV fluorescence technique of Edwards and McBride [4] as modified by Doddema and Vogels [3] facilitated the identification of methanogenic bacteria in enrichments. After a few transfers of this enrichment, samples were diluted in roll tubes. Purification was unsuccessful using this rich complex medium. Only 0.01% yeast extract was added to the basal medium. In these conditions, the microorganism was isolated by picking and diluting colonies from a roll tube into other roll tubes until the culture was found to be pure.

Purity was established through direct microscopic examination of liquid cultures, examination of colonies in roll tube dilutions and by inoculation in a complex medium containing glucose, yeast extract and biotrypcase (Biomérieux, Lyon).

Colony and cell morphology.

After 4-day incubation at 55° C, granular, white-to-yellow-coloured colonies about 1 mm in diameter became visible in roll tubes. During growth, the organism formed large clumps (fig. 1) like *Methanosarcina* TM1 [26]. Tetrades and single cell bodies were sometimes seen in the young culture. In older cultures, clumps could be broken up either mechanically or spontaneously into irregular coccoid elements (fig. 1C).

Substrates for methanogenesis.

The isolate used methanol, methylamines and acetate as energy and carbon source. No growth was obtained even after 1 month of incubation with H_2-CO_2 or formate. When methanol was added to a medium containing acetate, methanogenesis started earlier. Methane production from acetate after 5 days was identical whether methanol was present or not (fig. 2). Addition of methanol had no effect when TMA was the substrate for methanogenesis (fig. 2). H_2-CO_2 inhibited acetoclasty (fig. 3).

Growth requirements.

The isolate grew without vitamins or organic compounds (fig. 4). Under these conditions, the *Methanosarcina* strain could be transferred three times, but growth was slow. Addition of vitamins or yeast extract greatly stimulated growth (fig. 4). Biotrypcase had no effect on methane production (fig. 4). Although rumen fluid was not required for methanogenesis, it improved growth (fig. 5).

Optimal growth temperature and pH.

The optimal temperature for growth was 55° C, with a lower limit of 30° C and an upper limit of 60° C (fig. 6). Optimal pH was found to be between 6.5 and 7.0 ; pH near 6.0 inhibited methanogenesis (fig. 7).

FIG. 1. — *Electron photomicrographs of the thermophilic* Methanosarcina *strain (MP) grown in a liquid medium containing methanol (40 mM) ; serum bottles were incubated at 55° C. Bar, 10 μm.*

A) A clump of *Methanosarcina* strain (MP). B) Cells of *Methanosarcina* strain (MP) inside the clump. C) Phase-contrast photomicrograph of coccoid cells released from mature clumps.

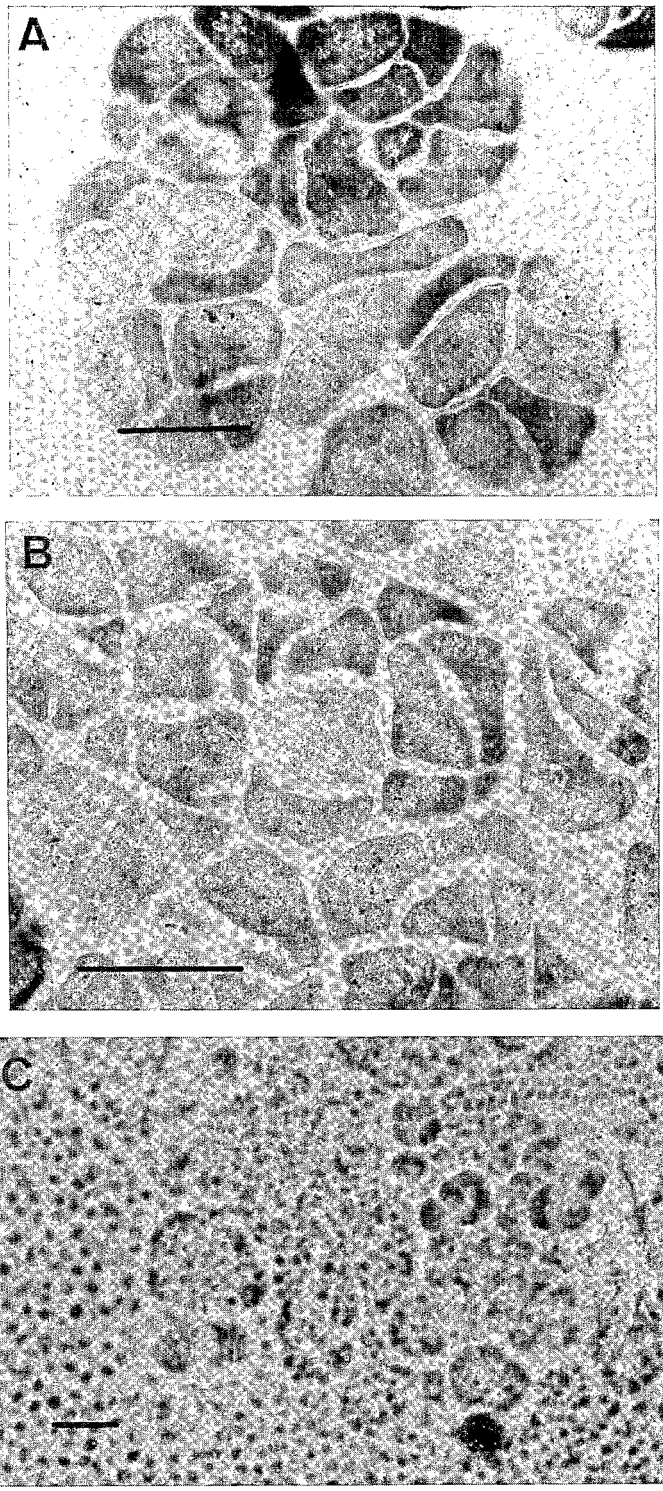

FIG. 1

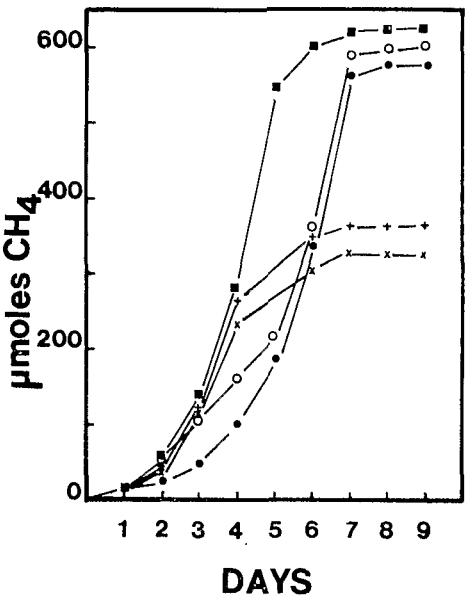

FIG. 2. — *Effect of methanol addition on methanogenesis from acetate or TMA by the thermophilic* Methanosarcina *strain (MP).*

Data are given in micromoles for 20-ml medium. Serum bottles were incubated at 55° C. Basal medium contained 0.1 % (w/v) yeast extract. Symbols: methanol, 40 mM, ■ ; 5 mM methanol +36 mM acetate, ○ ; acetate, 36 mM, ● ; 5 mM methanol+10 mM TMA, + ; trimethylamine, 10 mM, ×.

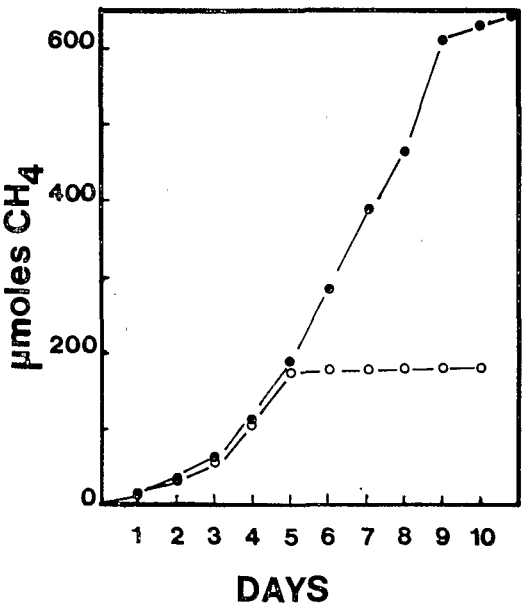

FIG. 3. — *Effect of H_2/CO_2 on acetate degradation by the thermophilic* Methanosarcina *strain (MP).*

Data are given in micromoles for 20-ml medium. Serum bottles were incubated at 55° C. Basal medium contained 0.1 % yeast extract. H_2/CO_2 (0.5 bar) was injected 5 days after inoculation on basal medium (see « Materials and Methods ») containing 0.1 % yeast extract. Symbols: control, ● ; addition of H_2/CO_2, ○.

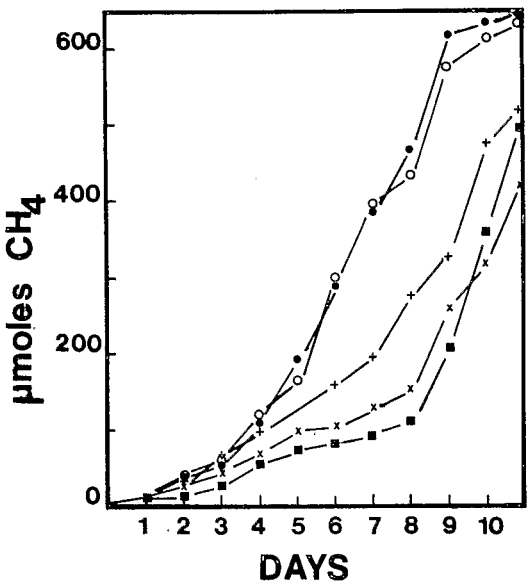

FIG. 4. — *Growth requirements of the thermophilic* Methanosarcina *strain (MP).*

Organic compounds were added at 1 g/l ; 0.2 ml of vitamin solution [1] were added for 20 ml of acetate medium. Basal medium without organic compounds was used for the experiment. Inoculum was a culture of the *Methanosarcina* strain grown on a mineral medium. Data are expressed in micromoles for 20 ml. Serum bottles were incubated at 55° C. Symbols: no addition, ■ ; vitamins, + ; biotrypcase, × ; yeast extract, ○ ; yeast extract+biotrypcase, ● .

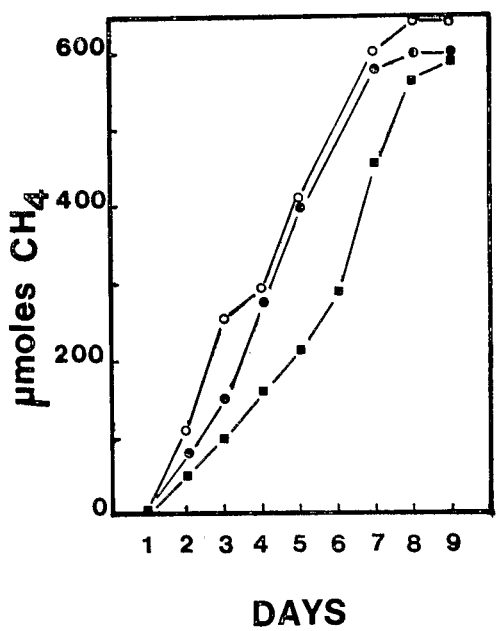

FIG. 5. — *Effect of rumen fluid addition on methane production by the thermophilic* Methanosarcina *strain (MP).*

Data are given in micromoles for 20 ml medium. Serum bottles were incubated at 55° C. Basal medium contained 0.1 % yeast extract. Symbols: no addition, ■ ; rumen fluid 1 % (v/v), ● ; rumen fluid 5 %, ○ .

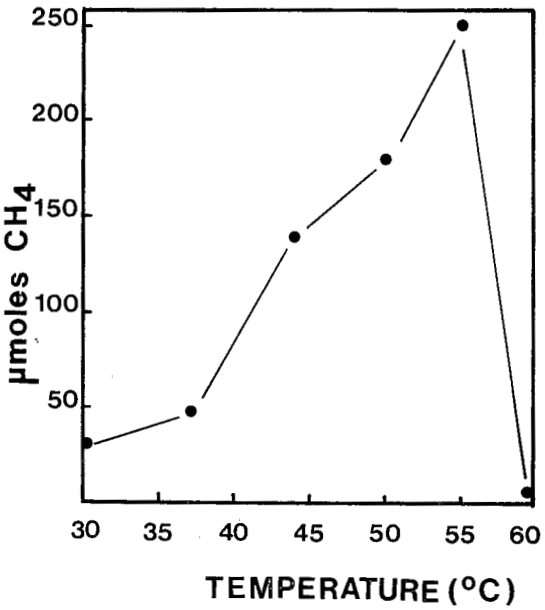

FIG. 6. — *Effect of temperature on methane production by the thermophilic* Methanosarcina *strain (MP).*

Cultures were incubated 6 days. Micromoles of methane are given for 20 ml of medium.

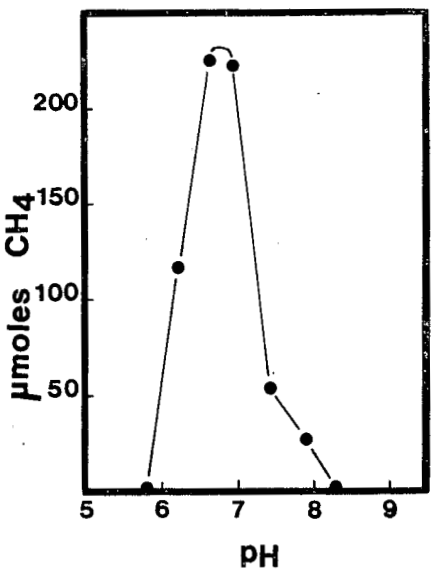

FIG. 7. — *Effect of pH on methane production by the thermophilic* Methanosarcina *strain (MP).*

Cultures were incubated 6 days. Micromoles of methane are given for 20 ml of medium.

DISCUSSION

The isolate presented a growth temperature optimum of 55° C. Methane production was optimal at about 55° with a lower limit of 30° C and an upper limit of 60° C. These results showed that the organism was a moderate thermophile. Its substrate range was similar to other thermophilic acetoclastic strains recently isolated [19, 26]. Although mesophilic members of the genera *Methanolobus* or *Methanococcoides* were described as using methylamines and methanol [10, 21], our isolate differed from these genera by its morphological characteristics and by the ability to use acetate.

These physiological responses coupled with the typical morphology indicated that it belonged to the genus *Methanosarcina.* The inability to use H_2-CO_2 for *Methanosarcina* strain MP is shared by other acetoclastic bacteria [19, 23, 26], but is not observed for mesophilic *Methanosarcina* strains [14, 15], except for *M. mazei,* which slowly metabolizes H_2-CO_2 [12]. Morphologically, the bacterium looked like *Methanosarcina* TM1 [26], but differed from this strain by its ability to use acetate in the absence of complex organic compounds. In the case of TM1, a growth factor present in the supernatant of anaerobic digester sludge was required. Contrary to *Methanosarcina* strain TM1, strain MP was able to release irregular coccoid elements from mature clumps as described for the mesophilic *M. mazei* [12, 13]. Our isolate also showed a different pH range of growth from TM1. Indeed, this organism could grow at pH 5.5 or at pH 8.0. At these pH, growth was inhibited for the MP strain. Finally, optimal growth temperature was slightly different from that of TM1: 55 instead of 50° C.

Growth on acetate in a mineral medium was reported for the mesophilic *Methanosarcina* strain 227 [15]. Yeast extract or vitamins stimulated growth of the new MP isolate, but biotrypcase had no effect. H_2-CO_2 completely inhibited methanogenesis on acetate. The same results were found for *M. mazei* [6]. Inhibition of acetate degradation by H_2 was also observed for *M. barkeri* [17]. Other results indicated a requirement for hydrogen by *M. barkeri* for efficient acetate utilization [11, 24].

Methanosarcina-like clumps were observed in different thermophilic laboratory digesters [26]. Recently, a granular consortium growing at 55° C using acetic acid was described. Inside the consortium, macrocysts and coccoid cells, resembling *Methanosarcina* cells, could be distinguished [2].

The isolation of a new thermophilic *Methanosarcina* from a laboratory digester fed with water hyacinths and inoculated with ground termites confirm that these bacteria may play an important role in thermophilic digestion, as suggested by Zinder and Mah [26]. However, our results were unable to prove that the new isolate did, in fact, come from the termites.

RÉSUMÉ

ISOLEMENT ET CARACTÉRISATION D'UNE NOUVELLE SOUCHE THERMOPHILE (SOUCHE MP) APPARTENANT AU GENRE « METHANOSARCINA »

Une souche méthanogène thermophile appartenant au genre *Methanosarcina* a été isolée d'un digesteur alimenté en jacinthes d'eau et inoculé avec un broyat de termites du Congo.

La température optimale de croissance se situe vers 55° C. La production de méthane est optimale entre pH 6,5 et 7,0. La bactérie croît sur acétate, méthanol et méthylamines en l'absence de facteurs de croissance, mais ne peut utiliser H_2-CO_2 ou le formiate. L'apport de H_2-CO_2 inhibe l'utilisation de l'acétate. L'extrait de levure ou les vitamines stimulent la croissance.

MOTS-CLÉS : Méthanogenèse, Thermophilie, *Methanosarcina*, Acétate; Souche nouvelle.

ACKNOWLEDGEMENTS

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REFERENCES

[1] BALCH, W. E., FOX, G. E., MAGRUM, L. J., WOESE, C. R. & WOLFE, R. S., Methanogens: re-evaluation of a unique biological group. *Microbiol. Rev.*, 1979, 43, 260-296.

[2] BOCHEM, H. P., SCHOBERTH, S. M., SPREY, B. & WENGLER, P., Thermophilic biomethanation of acetic acid: morphology and ultrastructure of a granular consortium. *Canad. J. Microbiol.*, 1982, 28, 500-510.

[3] DODDEMA, H. J. & VOGELS, G. D., Improved identification of methanogenic bacteria by fluorescence microscopy. *Appl. environ. Microbiol.*, 1978, 36, 752-754.

[4] EDWARDS, T. & MCBRIDE, B. C., New method for the isolation and identification of methanogenic bacteria. *Appl. Microbiol.*, 1975, 29, 540-545.

[5] FERGUSON, T. J. & MAH, R. A., Isolation of an H_2-oxidizing thermophilic methanogen. *Appl. environ. Microbiol.*, 1983, 45, 265-274.

[6] FERGUSON, T. J. & MAH, R. A., Effect of H_2-CO_2 on methanogenesis from acetate or methanol in *Methanosarcina* spp. *Appl. environ. Microbiol.*, 1983, 46, 348-355.

[7] HUBER, H., THOMM, M., KÖNIG, H., THIES, G. & STETTER, K. O., *Methanococcus termolithotrophicus*, a novel thermophilic lithotrophic methanogen. *Arch. Microbiol.*, 1982, 132, 47-50.

[8] HUNGATE, R. E., A roll tube method for cultivation of strict anaerobes, *in* « Methods in microbiology » (R. Norris & D. W. Ribbons), 3B (p. 117-132). Academic Press, New York, London, 1969.

[9] JONES, W. J., LEIGH, J. A., MAYER, F., WOESE, C. R. & WOLFE, R. S., *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. *Arch. Microbiol.*, 1983, **136**, 254-261.

[10] KÖNIG, H. & STETTER, K. O., Isolation and characterization of *Methanolobus tindarius*, sp. nov., a coccoid methanogen growing only on methanol and methylamines. *Zbl. Bakt. Mikrobiol. Hyg. C-Allg.*, 1982, **3**, 478-490.

[11] LAUBE, V. M. & MARTIN, S. M., Effect of some physical and chemical parameters on the fermentation of cellulose to methane by a co-culture system. *Canad. J. Microbiol.*, 1983, **29**, 1475-1480.

[12] MAH, R. A., Isolation and characterization of *Methanococcus mazei*. *Curr. Microbiol.*, 1980, **3**, 321-326.

[13] MAH, R. A. & KUHN, D. A., Transfer of the type species of the genus *Methanococcus* to the genus *Methanosarcina*, naming it *Methanosarcina mazei* (Barker 1936) *comb. nov. et emend.* and conservation of the genus *Methanococcus* (approved lists 1980) with *Methanococcus vannielii* (approved lists 1980) as the type species. *Int. J. syst. Bact.*, 1984, **34**, 263-265.

[14] MAH, R. A. & SMITH, M. R., The methanogenic bacteria, *in* « The prokaryotes » (M. P. Starr, H. Stolp, H. G. Trüper, A. Balows & H. G. Schlegel), I (p. 948-977). Springer Verlag, Berlin, 1981.

[15] MAH, R. A., SMITH, M. R. & BARESI, L., Studies on an acetate-fermenting strain of *Methanosarcina*. *Appl. environ. Microbiol.*, 1978, **35**, 1174-1184.

[16] MARTY, D. G. & BIANCHI, A. J. M., Isolement de deux souches méthanigènes thermophiles appartenant au genre *Methanobacterium*. *C. R. Acad. Sc.* (Paris) *(Sér. D)*, 1981, **292**, 41-43.

[17] MCINERNEY, M. J. & BRYANT, M. P., Anaerobic degradation of lactate by syntrophic associations of *Methanosarcina barkeri* and *Desulfovibrio* species and effect of H_2 on acetate degradation. *Appl. environ. Microbiol.*, 1981, **41**, 346-354.

[18] MOLLENHAUER, H., Plastic embedding mixtures for use in electron microscopy. *Stain Technol.*, 1964, **39**, 111-114.

[19] NOZHEVNIKOVA, A. N. & YAGODINA, T. G., A thermophilic acetate methane-producing bacterium. *Mikrobiologija*, 1982, **51**, 642-649.

[20] RIVARD, C. J. & SMITH, P. H., Isolation and characterization of a thermophilic marine methanogenic bacterium, *Methanogenium thermophilicum* sp. nov. *Int. J. syst. Bact.*, 1982, **32**, 430-436.

[21] SOWERS, K. R. & FERRY, J. G., Isolation and characterization of a methylotrophic marine methanogen, *Methanococcoides methylutens* gen. nov., sp. nov. *Appl. environ. Microbiol.*, 1983, **45**, 684-690.

[22] STETTER, K. O., THOMM, M., WINTER, J., WILDGRUBER, G., HUBER, H., ZILLIG, W., JANECOVIC, D., KÖNIG, H., PALM, P. & WUNDERL, S., *Methanococcus fervidus*, sp. nov., a novel extremely thermophilic methanogen isolated from an icelandic hot spring. *Zbl. Bakt. Mikrobiol. Hyg. C-Allg.*, 1981, **2**, 166-178.

[23] ZEHNDER, A. J. B., HUSER, B. A., BROCK, T. D. & WUHRMANN, F., Characterization of an acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium. *Arch. Microbiol.*, 1980, **124**, 1-11.

[24] ZEIKUS, J. G., WEIMER, P. J., NELSON, D. R. & DANIELS, L., Bacterial methanogenesis: acetate as a methane precursor in pure culture. *Arch. Microbiol.*, 1975, **104**, 129-134.

[25] ZEIKUS, J. G. & WOLFE, R. S., *Methanobacterium thermoautotrophicum* sp. nov., an anaerobic, autotrophic extreme thermophile. *J. Bact.*, 1972, **109**, 707-713.

[26] ZINDER, S. H. & MAH, R. A., Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2-CO_2 for methanogenesis. *Appl. environ. Microbiol.*, 1979, **38**, 996-1008.

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