Methanomicrococcus blatticola* gen. nov., sp. nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach *Periplaneta americana

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A small irregular coccoid methanogenic bacterium (PA^T) was isolated from the hindgut of the cockroach *Periplaneta americana*. Fluorescence microscopy and transmission electron microscopy of the hindgut of *P. americana* suggest that the organism occurs abundantly in the microbiota attached to the hindgut wall. The strain produces methane by the reduction of methanol and methylated amines with molecular hydrogen. Acetate, coenzyme M, yeast extract, tryptic soy broth and vitamins are required for growth. The cells lack a rigid cell wall and lyse immediately in buffers of low ionic strength. Maximum rate of growth (specific growth rate, 0.22 h⁻¹) occurs in a rich medium at 39 °C, at a pH range of 7.2–7.7 and at a salt concentration below 100 mM NaCl. Sequence analysis of the small-subunit rDNA indicates that strain PA^T is related to the family *Methanosarcinaceae* but does not belong to any previously described genus. Therefore, it is proposed that strain PA^T be classified in a new genus, related to the *Methanosarcinaceae*, as *Methanomicrococcus blatticola* (type strain PA^T = DSM 13328^T).

Keywords: arthropod hindgut, methanogenesis, methanol, *Methanomicrococcus blatticola*

INTRODUCTION

Methanogenic archaea inhabit anoxic environments such as freshwater sediments, tundras, bogs, wastewater treatment plants, landfills and the gastrointestinal tracts of many vertebrates. Methanogens are also found in arthropods (Cruden & Markovetz, 1987), but an extensive screening has revealed that their presence is restricted to four higher taxa: millipedes (*Diplopoda*), termites (*Isoptera*), cockroaches (*Blattoidea*) and rose chafers (*Scarabeidae*) (Hackstein & Stumm, 1994). The hindgut of arthropods is substantially different from the mammalian gastrointestinal tract. Since the volume of insect hindguts is in the range of 1 µl to 1 ml, the surface to volume ratios

are markedly different from those of mammalian systems and, therefore, oxygen diffusion across the surface of the epithelium has a substantial effect on the microbiota of the insect hindgut (Brune, 1998). Therefore, it is likely that methanogenic archaea living in arthropod guts will be notably different from those in the digestive tracts of vertebrates. Recently, three novel species belonging to the genus *Methanobrevibacter* were isolated from the hindgut of the termite *Reticulitermes flavipes* (Leadbetter & Breznak, 1996; Leadbetter *et al.*, 1998), and small-subunit (SSU) rDNA sequence analysis demonstrated the presence of an uncultured *Methanobrevibacter* sp. in the hindgut of the termite *Reticulitermes speratus* (Ohkuma *et al.*, 1995). Two types of methanogenic archaea have also been enriched from the hindgut of the cockroach *Eublaberus posticus* (Cruden & Markovetz, 1987). The first one, a short rod that grows on formate and on hydrogen plus carbon dioxide, was putatively identified as a *Methanobrevibacter* species. The second one was a coccoid organism that utilized hydrogen and

Abbreviations: HS-CoM, 2-mercaptoethanesulfonate; LSU, large subunit; SSU, small subunit.

The EMBL accession number for the SSU rDNA sequence of *Methanomicrococcus blatticola* strain PA^T is AJ238002.

methanol. On the basis of its antigenic properties, it was concluded that the organism was closely related or identical to *Methanospira stadtmanae*, a common inhabitant of mammalian digestive tracts. Here, the isolation, physiological characterization and phylogenetic analysis of a new methanogenic archaeon from the hindgut of the cockroach *Periplaneta americana* are reported. The organism belongs to the order *Methanosarcinales* and grows exclusively on methanol or methylamines with hydrogen as the electron donor.

METHODS

Chemicals. 2-Bromoethanesulfonic acid, 2-mercaptoethanesulfonate (coenzyme M, HS-CoM), penicillin G and kanamycin sulfate were purchased from Sigma, and yeast extract and tryptic soy broth were from Gibco-BRL. Oligonucleotide primers, and sequencing and PCR reagents were purchased from Eurogentec. All reagents were of analytical grade. Gases were supplied by Hoek-Loos. To remove traces of oxygen, H₂-containing gases were passed over a BASF RO-20 catalyst at room temperature; N₂ was passed over a pre-reduced BASF R3-11 catalyst at 150 °C. The catalysts were a gift from BASF Aktiengesellschaft, Ludwigshafen, Germany.

Source of strain PA^T. Strain PA^T was isolated from *Periplaneta americana* var. Amsterdam, which was caught in Artis Zoo (Amsterdam, The Netherlands). A population of several thousand individuals has been maintained in our laboratory in a 100 l metal container since 1992. A single female weighing 1.6 g that produced 170 nmol methane (g fresh wt)⁻¹ h⁻¹ at 29 °C was selected for the isolation of methanogenic archaea from the hindgut. The hindgut was carefully dissected and placed in a drop of sterile 10 mM Tris/HCl buffer (pH 6.8) containing 183 mM KCl and 47 mM NaCl. The hindgut was punctured and its contents were immediately transferred to the anaerobic sterile enrichment medium.

Culture methods and culture media. The enrichment medium contained (per l deionized water): NaHCO₃, 7.5 g; sodium acetate, 2 g; sodium formate, 2 g; methanol, 10 ml; yeast extract, 2 g; tryptic soy broth, 2 g; NaCl, 1.35 g; NH₄Cl, 0.45 g; KH₂PO₄, 0.9 g; boiled and centrifuged ovine rumen fluid, 40 ml; 1 ml each of isobutyric, isovaleric, valeric and α -methylbutyric acids; 0.4 g of both L-leucine and L-isoleucine; MgSO₄·2H₂O, 0.18 g; CaCl₂·2H₂O, 0.12 g; vitamin solution medium DSM141 (Sowers & Schreier, 1995), 10 ml; trace elements solution (Schönheit *et al.*, 1979), 10 ml; sodium resazurin, 1 mg; HS-CoM, 1.6 mg; Na₂S·3H₂O, 0.5 g; cysteine·HCl, 0.5 g. Pure cultures of strain PA^T are presently maintained in a modified enrichment medium, which will be referred to as 'standard medium'. The latter medium contained 89 mM NaHCO₃, 200 mM methanol, 30 mM sodium acetate, 25 mM NaCl, 2 g yeast extract l⁻¹, 2 g tryptic soy broth l⁻¹, 6.6 mM KH₂PO₄, 10 mM NH₄Cl, 1 mM MgCl₂, 1 mM CaCl₂, 10 ml vitamins solution l⁻¹, 10 ml trace elements solution l⁻¹, 0.25 mg sodium resazurin l⁻¹, 10 μ M HS-CoM, 4 mM cysteine and 4 mM Na₂S. After adjustment to pH 7.5, 25 ml aliquots of the media were dispensed into 120 ml serum bottles that were closed with butyl rubber stoppers and aluminium-crimped seals. The bottles were subsequently gassed with 80% hydrogen/20% carbon dioxide (enrichment medium) or 100% hydrogen (standard medium), pressurized to 110 kPa, and autoclaved for 15 min at 121 °C.

After cooling, the bottles were aseptically pressurized to 200 kPa with the respective gases. Inoculation was performed by transferring 0.25 ml of a growing culture to fresh medium using a 1 ml syringe that had been flushed with anoxic, sterile gas. Unless stated otherwise, bottles were horizontally incubated in a rotary-shaking incubator at 125 r.p.m. and 34 °C.

For cultivation on solid media, bacterial suspensions were rapidly mixed with an equal volume of molten anaerobic standard medium that contained 2% (w/v) agarose or 2% (w/v) agar and that was kept at 42 °C in crimp-sealed reagent tubes or in 10 ml serum bottles. After solidification, bottles were incubated under 100% hydrogen at 34 °C.

Isolation procedure. The cockroach hindgut suspension was incubated in enrichment medium for 3 d. Subsequently, a 0.25 ml aliquot of the culture was transferred to fresh medium and incubated. The procedure was repeated every 3–4 d. After approximately 20 transfers, the culture was inoculated in enrichment medium containing 200 μ g ml⁻¹ of both kanamycin sulfate and penicillin G. The culture obtained was serially (10²- to 10¹⁰-fold) diluted in enrichment medium without the antibiotics. Cultures from the highest dilutions showing growth and methane formation were microscopically homogeneous and were subsequently subcultured in standard medium. PA^T was obtained in pure culture by the isolation of individual colonies from deep agar. For this purpose, a 10-fold dilution series in agar medium was prepared in duplicate from a culture grown for 48 h in standard medium. Subsurface microcolonies developed in the course of a 2 week incubation. Both agar cultures from the organism that had been diluted 10⁸-fold were selected for colony isolation; the bottles each contained about ten well-separated and morphologically identical colonies. While inspected under a dissecting microscope, individual colonies were withdrawn from the closed bottles into a 0.8 × 50 mm sterile needle connected to a 1 ml syringe and transferred to fresh standard medium. Methane formation and growth of all isolates resumed immediately and were completed within a 2 to 3 d incubation. By routine, cultures were tested for the absence of contamination by incubation in enrichment medium containing 5 g glucose l⁻¹ and 10 mM 2-bromoethanesulfonic acid, a specific inhibitor of methanogenic archaea.

Presence of *Methanomicrococcus blatticola* in the hindgut of *P. americana*. The dissected hindgut (55 μ l) of an *P. americana* larvae that produced 62 nmol methane (g fresh wt)⁻¹ h⁻¹ was vigorously suspended in anoxic standard growth medium (25 ml) lacking methanol and HS-CoM. The homogenate was diluted (10¹- to 10¹⁰-fold) in standard medium (10 ml) containing 200 μ g ml⁻¹ of both kanamycin sulfate and penicillin G; parallel dilution series were incubated under different growth conditions. Incubations took place in the presence and absence of methanol, with or without HS-CoM, as well as under hydrogen or nitrogen atmospheres. In addition, a control dilution series was prepared in standard medium lacking the antibiotics. Growth was followed by measuring methane formation and by investigating culture preparations under the phase-contrast and epifluorescence microscope. The procedure of serial dilution and incubation under the different growth conditions was subsequently repeated for the highest dilution of each series that had shown growth and methane formation.

Growth experiments. In a number of tests it was established that the optical densities of the bacterial cultures were

linearly related to the amount of methane accumulated in the headspace of the culture bottles for a major period of time. The linear relationship especially held for the exponential growth phase and most parts of the subsequent linear growth stage. Therefore, growth was routinely monitored by measuring methane formation. Growth rates were calculated from the exponential parts of the methane versus time curves. Under the experimental conditions (25 ml cultures incubated in 125 ml serum bottles at 125 r.p.m.), an exponential increase could only be observed for the short period of time in which the rate of methane formation was below 25–50 $\mu\text{mol h}^{-1}$. Above this rate, methanogenesis became linear as the result of hydrogen mass transfer limitations.

To investigate the potential substrates, cells were incubated in standard medium in the presence or absence of methanol under 80% $\text{N}_2/20\%$ CO_2 or 80% $\text{H}_2/20\%$ CO_2 . The ability to utilize sodium formate (2 g l^{-1}), ethanol (10 mM), pyruvate (10 mM), lactate (10 mM), malate (10 mM) or succinate (10 mM) was tested in standard medium under 80% $\text{N}_2/20\%$ CO_2 , both in the presence and absence of methanol (200 mM). The use of methylated substrates other than methanol was investigated in a series of 10 ml standard media containing a limiting amount of methanol (10 mM) and 50 mM of either monomethylamine, dimethylamine, trimethylamine, betaine, sarcosine, syringate (4-hydroxy-3,5-dimethoxybenzoate), vanillate (4-hydroxy-3-methoxybenzoate) or 3,4,5-trimethoxybenzoate. Media were inoculated (1%, v/v) with a culture grown on methanol. If methane was formed above the background level derived from methanol reduction, organisms were subsequently subcultured for at least three transfers on the particular methylated substrate (50 mM) and in the absence of methanol.

For the determination of the optimum NaCl concentration for growth, NaCl (0–800 mM) was added to modified standard medium that contained only 10 mM NaHCO_3 and 1 g l^{-1} of both yeast extract and tryptic soy broth. To determine the pH range for growth, appropriate amounts of sterile anoxic 1 M KH_2PO_4 (from pH 6.5 to 7.5) or 1 M Tris (from pH 7.5 to 8.5) were added to the modified standard medium; pH values were checked in a series of uninoculated bottles. The dependence for growth on the NaCl concentration, pH and temperature was tested with inoculates that had been precultured at the specific conditions.

To investigate the growth requirements of strain PA^T, components were omitted from the enrichment medium. Depleted media were inoculated with 0.5% (v/v) of a culture grown in the complete enrichment medium. If methane had accumulated to more than 2 mmol, 0.125 ml culture was transferred to fresh medium lacking the respective component(s). In case of normal growth after the second transfer, the component(s) were considered to be dispensable and were omitted in subsequent tests. If growth did not continue, the omitted component was added to determine if growth was restored.

Analytical methods. Methane was measured by GC using ethane as an internal standard according to Gijzen *et al.* (1991) with a Pye Unicam GCD chromatograph equipped with a Poropak Q 100–200 mesh column and FID. To quantify acetate consumption, 1 ml culture was centrifuged for 2 min at 19000 g and 50 μl formic acid was added to the supernatant. The precipitate was removed by brief centrifugation and 1 μl aliquots of the supernatant were analysed for acetate on a Hewlett Packard HP 5890 series II plus

chromatograph equipped with a capillary column (HP-INNOWax) and FID (Cazemier *et al.*, 1997b).

For dry weight determination, cell cultures were centrifuged at 12000 g and 4 °C for 10 min. The pellet was collected in a microcentrifuge tube and washed in standard medium. After centrifugation and removal of the supernatant, the cell pellet was dried under vacuum in the microcentrifuge tube at 80 °C for 12 h. Catalase and oxidase tests were performed by mixing small amounts of a concentrated bacterial suspension on a microscope slide with equal volumes of aqueous solutions of 10% H_2O_2 and 1% tetramethyl-*p*-phenylenediamine.HCl, respectively (Skerman, 1967). Gram-staining took place according to established methods (Skerman, 1967).

Microscopy. Methanogenic bacteria and dissected insect hindguts were studied by phase-contrast and epifluorescence microscopy as previously described (Doddema & Vogels, 1978; Gijzen *et al.*, 1991). For transmission electron microscopy, an aqueous solution of glutaraldehyde was added to an exponentially growing culture of strain PA^T to a final concentration of approximately 2.5% (w/v). The dissected hindgut of *P. americana* was fixed as described by Cazemier *et al.* (1997a). Samples were post-fixed with $\text{OsO}_4/\text{K}_3\text{Fe}(\text{CN})_6$ and embedded in Epon 812. Thin sections were made on a Reichert OM U2. The sections were stained with lead citrate and uranyl acetate, and investigated with a Zeiss 109 T electron microscope (Cazemier *et al.*, 1997a).

Osmotic lysis of bacterial cells in buffers of low ionic strength.

Aliquots (0.5 ml) from actively growing cultures were centrifuged at 19000 g for 2 min. The supernatants were removed and the pellets were washed twice in 200 μl 10 mM Tris/HCl buffer (pH 8.0) containing 200 mM NaCl. Hereafter, the pellets were resuspended in 200 μl of either demineralized water, 10 mM Tris/HCl buffer (pH 8.0), Tris buffer containing 200 mM NaCl or culture supernatant. Cell lysis was assessed by the following criteria: clearance of the suspension, epifluorescence microscopic observation (formation and disappearance of sphaeroplasts; release of fluorescent coenzyme F_{420} into the medium) and the detection of nucleic acid in the washed cell supernatants by means of agarose gel electrophoresis. To examine the osmotic lysis of methanogens attached to the hindgut wall, a small piece of a freshly dissected hindgut of *P. americana* was squeezed between a cover-slip and a microscope slide in a drop of 10 mM Tris/HCl buffer (pH 6.8) containing 183 mM KCl and 47 mM NaCl. While inspected under the epifluorescence microscope, several drops of demineralized water were drawn through the preparation by capillary action. As above, the release of coenzyme F_{420} and the intermediary formation of sphaeroplasts were indicative of osmotic lysis.

Nucleotide sequence analysis of SSU rDNA. A culture of strain PA^T (25 ml; 62 mg wet wt) was centrifuged, and the pellet was lysed in 100 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Next, 1 ml extraction buffer containing 10 mM Tris/HCl buffer (pH 8.0), 100 mM NaEDTA, 0.5% (w/v) SDS and 100 μg proteinase K ml^{-1} was added, and the mixture was incubated at 50 °C for 3 h. After two extraction steps with equal volumes of phenol:chloroform:isoamylalcohol (25:24:1) and two steps with chloroform:isoamylalcohol (24:1), nucleic acids were precipitated by addition of 0.5 vol. 7.5 M ammonium acetate and 0.9 vol. 2-propanol. The pellet was washed twice in 70% ethanol, dissolved in 100 μl TE buffer and stored at –20 °C.

Fig. 1. Transmission electron micrographs of strain PA^T (a) and thin section of the hindgut of *Periplaneta americana* (b). In (b), the hindgut lumen containing numerous prokaryotic cells including cells with a same morphology and size as PA^T (marked by arrows) are shown; the intestinal epithelium, which is separated from the lumen as a result of the fixation and embedding, is visible in the left part of the picture. Bars, 1 μ m.

Approximately 300 bp of the SSU rDNA of strain PA^T were PCR-amplified (40 cycles; annealing temperature, 58 °C) from 200 ng template with the heterologous primer MG1200 (5'-CGATAATTTCGGGGCATGCTG) and the reversed primer ARC915 (AGGAATTGGCGGGGAGCAC) (Raskin *et al.*, 1994). The amplified fragment was cloned and sequenced as described below. Based on the obtained sequence information, primer PA947f (AGTCTTCAGCTTGACCTACAT) was designed, which is complementary to the SSU rDNA sequence of PA^T at positions 947–967 (*Methanosarcina barkeri* numbering). PCR amplification of the SSU rDNA from 200 ng template yielded a 950 bp fragment with primers ARC007f and PA947r (25 cycles; annealing temperature, 58 °C) and an 1130 bp fragment

with primers ARC915f and ARC23S28r (40 cycles; annealing temperature, 60 °C). Primer ARC007f (GTTG-ATCCTGCCAGAGC) and ARC23S28r (CCCBGGGCT-TATCGCAGCTT) are complementary to positions 7–23 of archaeal SSU rDNA and reversed complementary to positions 28–47 of archaeal large subunit (LSU) rDNA, respectively (Leffer *et al.*, 1987; Maidak *et al.*, 1997). Both PCR products were cloned using the TA cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was isolated with the Flexi prep kit (Pharmacia) and clones containing an insert of the expected size were sequenced in two directions with internal and vector-directed primers on a Pharmacia Automated Laser Fluorescent DNA sequencer using a T7 cycle sequencing kit.

The procedure yielded double-stranded sequence information from position 18 on the SSU rDNA to position 28 on the LSU rDNA. Database homology searches, sequence retrieval, alignment and phylogeny reconstruction were done using the CAOS-CAMM centre facilities at Nijmegen, the Netherlands (www.caos.kun.nl). The SSU rDNA sequence of strain PA^T was compared to sequences present in the GenBank (Benson *et al.*, 1997) and EMBL sequence databases by BLAST searches. For further analyses, a selection was made from the databases. The selection comprised the nearly complete SSU rDNA sequences of 19 cultured methanogens (all belonging to the *Methanosarcinaceae*) that gave the highest similarity scores with respect to strain PA^T, two species belonging to the *Methanosaetaceae* and eight representatives of the *Methanomicrobiales*. An initial alignment was constructed with PILEUP (Devereux *et al.*, 1984) and checked manually. An evolutionary distance matrix was calculated with DNADIST (Felsenstein, 1991) using both the Kimura two-parameter model (Kimura, 1980) and the Jukes–Cantor model (Jukes & Cantor, 1969) for nucleotide substitution. Distance trees were calculated with the NEIGHBOR and FITCH algorithms and a parsimony tree was constructed with DNAPARS (Felsenstein, 1991). One hundred bootstrap resamplings of the data set, constructed from the original alignment with SEQBOOT (Felsenstein, 1991), were used to construct a consensus parsimony tree with DNAPARS and CONSENSE (Felsenstein, 1991), and a consensus distance tree with DNADIST and CONSENSE for each of the above-mentioned distance methods. The maximum-likelihood approach (Felsenstein, 1981) used the facility available at the Ribosomal Database Project server (Maidak *et al.*, 1997).

RESULTS

Isolation of *Methanomicrococcus blatticola*

Epifluorescence microscopy of squashed preparations from the hindgut of *P. americana* var. Amsterdam revealed the presence of at least three morphotypes of free-living micro-organisms showing the typical blue autofluorescence of methanogens, notably small irregular cocci, motile spirilla and short rods occurring singly or in chains. The small coccoid methanogens were highly enriched during subsequent transfers in the enrichment medium, whereas the short rods and spirilla were lost. Initially, a minor contamination (< 0.1 %) by non-fluorescent, rod-shaped bacteria was present, but after ten subsequent transfers (1% inoculum volume) these bacteria were no longer observed in microscopic preparations and a homogeneous culture of blue fluorescent cocci, designated strain PA^T, was obtained. The culture was grown in enrichment medium containing 200 µg ml⁻¹ of both kanamycin sulfate and penicillin G. Hereafter, a dilution series was prepared in enrichment medium without the antibiotics. The highest dilutions (10⁶- and 10⁸-fold) that showed both bacterial growth and methane formation were tested for the absence of contamination in enrichment medium supplemented with glucose and 2-bromoethanesulfonic acid. No bacterial growth could be observed, even after prolonged incubation. Strain PA^T was isolated in pure culture from single subsurface microcolonies grown

in agar medium by the procedure outlined in the Methods.

Morphology and properties of strain PA^T

Subsurface colonies in solid media (< 1% agar or agarose) were lens-shaped, yellowish to brown and reached a maximal diameter of ca. 0.3 mm. The colonies showed a faint blue autofluorescence under long-wave ultraviolet light. The organisms were obligately anaerobic, catalase-positive and oxidase-negative. Gram-staining was hampered by the fact that cells were completely lysed during the staining procedure. Cells of strain PA^T appeared as irregular cocci under the phase-contrast microscope. They occurred singly, although aggregates could be observed at high cell densities. Under the epifluorescence microscope, cells of PA^T showed the blue autofluorescence of F₄₂₀. Cells were, however, clearly less fluorescent as compared to those of *Methanobacterium thermoautotrophicum* strain ΔH^T (DSM 1053^T) or *Methanosarcina barkeri* strain Fusaro (DSM 804). Electron microscopy disclosed that the methanogens were of an irregular, more or less rectangular shape showing a cell width of 1.3 ± 0.2 µm (Fig. 1a). A considerably smaller cell diameter (0.8 ± 0.1 µm), however, was calculated from differential interference contrast and phase-contrast micrographs taken from unfixed cells. It may be noted that, under those conditions, cells of *Methanosphaera stadtmanae* DSM 3091^T measured 1.4 ± 0.2 µm, which is in the range of the published value of approximately 1 µm (Miller & Wolin, 1985).

Cell lysis

When the cell pellet obtained from an actively growing culture was suspended in demineralized water or in buffer of low ionic strength, the turbid suspension rapidly clarified and became highly viscous. The intermediate formation of sphaeroplasts could be observed by phase-contrast microscopy. Epifluorescence microscopy revealed that clarification was accompanied by release of F₄₂₀ into the medium (data not shown). Apparently, the hypotonic shock caused cell lysis, since the phenomenon did not occur when cells were resuspended in the culture supernatant or in buffer containing 200 mM NaCl. Agarose gel electrophoresis of lysed cell supernatants confirmed the release of DNA. The same hypotonic treatment did not cause lysis of *Methanosphaera stadtmanae* DSM 3091^T, *Methanobacterium thermoautotrophicum* DSM 1053^T or *Methanosarcina barkeri* DSM 804.

Presence of *Methanomicrococcus blatticola* in the cockroach gut

As mentioned above, three morphotypes of free-living autofluorescent micro-organisms could be observed by epifluorescence microscopy in the hindgut of *P.*

Fig. 2. Requirements for hydrogen (a) and methanol (b) in the process of methanogenesis by strain PA^T. Strain PA^T was cultured on 25 ml standard medium as described in Methods. (a) Cultures were pressurized to 200 kPa with the following $N_2/H_2/CO_2$ mixtures (% v/v): 80/0/20 (0 mmol H_2) (□); 65/15/20 (1.35 mmol H_2) (▲); or 0/80/20 (7.15 mmol H_2) (■). (b) Incubations took place under H_2/CO_2 (80:20; 230 kPa) in the presence of 0 mmol (□) or 5 mmol (■) methanol.

americana, viz. short rods, motile spirilla and irregular cocci. The coccoid methanogens could be enriched from a hindgut suspension that had been diluted up to 5×10^8 -fold in the standard growth medium established for strain PA^T. The cocci showed the same size and morphology, and had the same autofluorescence and lytic properties as pure cultures of strain PA^T. Like PA^T (see below), growth of the cocci was strictly dependent on both methanol and hydrogen, and the presence of tryptic soy broth, yeast extract and acetate also were required for enrichment of the cocci. Unlike PA^T, however, growth was independent on the methanogenic cofactor HS-CoM. When antibiotics were omitted from the rich dilution and incubation medium, a wealth of micro-organisms developed counting as high as 10^{12} cells hindgut ml⁻¹. Fluorescent short rods and motile spirilla were only observed at low dilutions (up to approximately 10^4 -fold); these organisms did not grow any further on the growth medium employed.

Inspection under the epifluorescence microscope of different sections of the cockroach hindgut demonstrated that blue fluorescent cocci highly resembling PA^T were quite specifically and abundantly present

along the hindgut epithelium. These cocci were the only blue fluorescent cells that lysed like the cells from pure cultures of strain PA^T. Also, transmission electron microscopy revealed the presence near the hindgut epithelium of numerous cocci showing a remarkable morphological similarity to strain PA^T (Fig. 1b).

The above data indicate that the isolate described here, or its close relatives, represents a major methanogenic organism in the *P. americana* hindgut, finding a specific niche near the hindgut epithelium.

Methanogenic substrates

Strain PA^T is specialized in its use of methanogenic substrates. Methane was formed from methanol but only in the presence of hydrogen (Fig. 2). Incubation of cells with known amounts of hydrogen and excess methanol demonstrated that one mole of methane was formed per mole of hydrogen added. Similarly, with excess hydrogen, cell suspensions produced approximately 1 mole methane per mole of methanol added (data not shown). Apparently, the organism derives its energy for growth from the conversion of hydrogen and methanol according to the following reaction: $H_2 + CH_3OH \rightarrow CH_4 + H_2O$. Fig. 2(a and b) also shows that methane formation did not occur from acetate or hydrogen plus carbon dioxide. Formate, ethanol, pyruvate, lactate, malate and succinate did not serve as sole substrates for methanogenesis, nor did these compounds substitute for hydrogen as an electron donor in methanol reduction.

Growth also took place on monomethylamine, dimethylamine or trimethylamine, but only under a hydrogen atmosphere. Methane was formed showing a stoichiometry of one mole methane per mole of methyl group in the substrate. Other *N*- and *O*-methylated compounds tested, including betaine, sarcosine, vanillate, syringate and 3,4,5-trimethoxybenzoate, were not utilized. Methanol was the preferred substrate, when media containing a mixture of methanol and mono-, di- or trimethylamine were inoculated with cells that had been precultured on methanol. The conversion of the methylated amines only started after the consumption of methanol. Once adapted to the particular methylated amine, mono-, di- and trimethylamine were converted without a lag; growth rates and growth yields were as found for growth on methanol. Cell yields on methanol and the different methylated amines varied between 3.5 and 6.0 g dry weight per mole of methane formed, exponentially growing cells showing higher yields than cells collected from the final stages of growth.

Growth requirements

Although acetate did not function as a methanogenic substrate for strain PA^T, its presence in the medium appeared to be indispensable. Acetate was used for growth in quantities amounting to 0.8 g acetate con-

Fig. 3. HS-CoM requirement for growth of strain PA^T. Cells were inoculated in standard medium containing 0 nM (□) or 61 nM (■) HS-CoM. At the time indicated by the arrow, 61 nM HS-CoM was added.

sumed per g dry weight formed. When tryptic soy broth and yeast extract, or vitamin solution were omitted from the standard medium, cells did not grow. Growth, however, instantaneously resumed when the compounds were subsequently added. Remarkably, HS-CoM was strictly required for growth of strain PA^T (Fig. 3). In the absence of the methanogenic cofactor, methane was formed at a low linear rate. Addition of the cofactor in very low concentrations resulted in the immediate increase in methanogenesis and the concomitant increase in optical density. Moreover, the presence in the medium of an appropriate reducing agent (sodium sulfide, cysteine or both) was strictly required for growth. Other components present in the enrichment medium, such as ovine rumen fluid, sodium formate, isobutyric acid, isovaleric acid, valeric acid, α -methylbutyric acid, L-leucine and L-isoleucine, proved to be dispensable.

Temperature, pH and salt optima

The specific growth rate of strain PA^T, as determined from the rate of exponential methane production, steadily increased in the temperature range between approximately 20 and 40 °C (Fig. 4a). Growth was not observed at 15 or 45 °C. At the optimal growth temperature (39 °C), the specific growth rate was 0.22 h⁻¹ (doubling time, 3.1 h). Strain PA^T grew between pH 6.7 and 8.2 (optimum pH, 7.0–7.5) (Fig. 4b). The NaCl concentration range that permitted growth was between 0 and 300 mM (Fig. 4c). At 400 mM NaCl, methane was only formed at a linear rate, but growth could not be observed; at 800 mM, methanogenesis was inhibited as well. A specific requirement for sodium ions was not investigated, since minimal standard medium contained approximately 40 mM sodium ions derived from salts other than NaCl.

Fig. 4. Growth rates of strain PA^T as a function of temperature (a), pH (b) and in the presence of NaCl (c). Strain PA^T was cultured in standard (a) or modified standard (b and c) medium as described in Methods. Specific growth rates were calculated from the exponential parts of the methane production versus time plots. The data points represent the mean of triplicate experiments; error bars are indicated when they exceed the size of the symbols. In (b) and (c), incubations were performed at 32 °C.

SSU rDNA analysis

Strain PA^T was sequenced from position 18 on the SSU rDNA to position 28 on the LSU rDNA. From the sequence information, a G + C content of 55 mol % was calculated. Sequence analysis of the SSU rDNA indicated a relationship of strain PA^T with the *Methanosarcinaceae*. The organism was, however, only

Fig. 5. Phylogenetic tree of strain PA^T and representatives of the families *Methanosarcinaceae* and *Methanosaetaceae*. The tree was constructed with the FITCH algorithm (Felsenstein, 1991) and used 1195 unambiguously aligned nucleotide positions of the SSU rDNA sequence. The construction of the tree is based on 29 cultured species of methanogens, 12 of which are shown (EMBL accession nos are given in parentheses): *Methanococcoides methylutens*^T (M59127), *Methanococcoides burtonii* (X65537), *Methanohalophilus halophilus* (M59132), *Methanohalophilus mahii*^T (M59133), 'Methanolobus oregonensis' (U20152), *Methanolobus tindarius*^T (X62862), *Methanosarcina thermophila* (M59129), *Methanosarcina barkeri* (MG59144), *Methanohalobium evestigatum*^T (U20149), strain PA^T (AJ238002), *Methanosaeta concilii* (M59146) and *Methanosaeta thermoacetophila* (M59141). In addition, the following sequences were used in tree construction, but were omitted from the figure for the sake of clarity: *Methanolobus vulcani* (U20155), *Methanolobus bombayensis* (U20148), *Methanolobus taylorii* (U20154), *Methanosarcina mazei* (AF028691), *Methanosarcina mazei* (U20151), *Methanosarcina frisia* (M59138), *Methanosarcina* sp. (M59136), *Methanosarcina acetivorans* (M59137), *Methanosarcina siciliae* (U20153), *Methanosarcina barkeri* (AF028692), *Methanospirillum hungatei* (M60880), *Methanocorpusculum parvum* (M59147), *Methanoculleus marisnigri* (AF028693), *Methanogenium marisnigri* (M59134), *Methanoculleus thermophilus* (M59129), *Methanoplanus limicola* (M59143), *Methanogenium cariaci* (M59130), *Methanogenium organophilum* (M59131) and *Archaeoglobus fulgidus* (X05567). The latter organism was defined as outgroup. Bootstrap values (100 resamplings) above 90% are indicated at the left of each node. The bar represents the expected number of changes per sequence position. Maximum-parsimony and neighbour-joining approaches using methods described by Felsenstein (1991) yielded similar topologies and shared all clusters with > 90% bootstrap value with those shown in the figure.

distantly related to other members of this family: the most similar sequence was that of *Methanosarcina mazei* strain Göl (88.6% sequence identity). A phylogenetic tree constructed with the FITCH distance algorithm of strain PA^T and representatives of the families *Methanosarcinaceae* and *Methanosaetaceae* is shown in Fig. 5. The cluster formed by the *Methanosaetaceae* was supported by a bootstrap value of 100%, irrespective of the algorithm used to construct the tree. The cluster formed by strain PA^T and the species belonging to the *Methanosarcinaceae* was also supported by a bootstrap value of 100% with each of these methods. The branching order within this cluster, however, could not be unequivocally resolved. The cluster formed by the exclusion of strain PA^T had a low bootstrap value with both matrix methods (FITCH distance, 47%; NEIGHBOR-JOINING, 84%), and a different branching order was obtained with a maximum-parsimony approach (in this case, the bootstrap value for exclusion of strain PA^T was only 39%).

A maximum-likelihood approach (Felsenstein, 1981) again gave a different branching order. However, all clusters in the tree (Fig. 5) with a bootstrap value above 90% were also strongly supported by maximum-parsimony and neighbour-joining methods.

The SSU rDNA sequence of strain PA^T showed another remarkable feature (Fig. 6). The 'methanosarcina group' (order *Methanosarcinales*) is defined by a signature sequence at positions 234–241 and the (complementary) positions 247–254 (*Methanosarcina barkeri* numbering) (Fig. 6b) (Rouvière *et al.*, 1992). In fact, in all SSU rDNA sequences of the order *Methanosarcinales* known to date, positions 247–254 are occupied by the sequence **TACCTACT**, in contrast to the **GCYYACCA** (Y is either C or T) motif which is characteristic for most other archaea (Fig. 6a). The sequence of strain PA^T, however, differed at three positions from all other representatives of the *Methanosarcinales* (Fig. 6c).

Fig. 6. Signature sequence and specific differences in the region 232–256 of SSU RNA as found in Archaea (a), Methanosarcinales (b) and *Methanomicrococcus blatticola* (c). Differences in the signature sequence having a predicted stem-loop structure (*Methanosarcina barkeri* numbering; Rouvière *et al.*, 1992) between the Methanosarcinales and other Archaea (represented by *Methanococcus vannielii*) are printed in bold; specific base substitutions present in *Methanomicrococcus blatticola* are marked by arrows.

DISCUSSION

A methanogenic archaeon, designated strain PA^T, has been isolated from the hindgut of the cockroach *P. americana*. Both the physiological properties and the phylogenetic analysis of its SSU rDNA gene characterize it as a new species within the order *Methanosarcinales*. Placement of strain PA^T within the order is supported by a high bootstrap value (96–100%) (Fig. 5). The most closely related cultured methanogenic archaea were all representatives of the family *Methanosarcinaceae*. Within the family, the SSU rDNA sequences of species belonging to the same genus are 97±0.65% identical, and a mean SSU rDNA sequence identity of 92±1% is found for species belonging to different genera (Springer *et al.*, 1995). Considering the low SSU rDNA sequence conservation (84.5–88.6%) between strain PA^T and the representatives of the *Methanosarcinaceae*, one may conclude that the organism at least belongs to a distinct genus. The position of strain PA^T within the *Methanosarcinaceae* could not be resolved unequivocally, since the tree topology of the order was only supported by low bootstrap values. Another remarkable feature is that the SSU rDNA sequence of strain PA^T differs from all other members of the order *Methanosarcinales* at three positions within the signature region found at the positions 234–254 (Rouvière *et al.*, 1992) (Fig. 6). Both the high SSU rDNA divergence and the presence of three substitutions in the signature sequence argue for a long-lasting evolutionary isolation of strain PA^T.

The phylogenetic uniqueness of strain PA^T is also reflected in a number of physiological properties that discriminate it from all species of the *Methanosarcinaceae* that have been cultured to date. Like other representatives of the family, strain PA^T is able to convert methanol and methylated amines into methane. However, methanogenesis is strictly dependent on the presence of molecular hydrogen. Growth under laboratory conditions on methanol plus hydrogen has been reported for *Methanosarcina barkeri* strain Fusaro DSM 804 (Müller *et al.*, 1986), but in this case hydrogen utilization is facultative, and it remains to be established whether the organism uses this type of metabolism in its natural habitats. As yet, the specialism of hydrogen-dependent methanol reduction is only shared with *Methanosphaera* species isolated from mammalian digestive tracts (Miller & Wolin, 1985). Being a member of the order *Methanobacteriales*, *Methanosphaera* is phylogenetically unrelated to strain PA^T. In addition, *Methanosphaera* species can not utilize methylated amines, and the organisms are unable to grow at temperatures below 25 °C like PA^T.

Strain PA^T displays a number of specific growth requirements. Acetate, yeast extract, tryptic soy broth and vitamins are indispensable for growth, indicating that the methanogen is rather limited in its biosynthetic capabilities. Moreover, growth of strain PA^T appears to be strictly dependent on the presence of the methanogenic cofactor HS-CoM. Lastly, all species belonging to the family *Methanosarcinaceae* described to date are halophilic or at least halotolerant, and display a relatively broad pH range for growth (Boone & Whitman, 1988; Maestrojuàn *et al.*, 1992). In these respects, strain PA^T is exceptional in its inability to grow at NaCl concentrations above 400 mM and its limited pH range for growth.

Coccoid methanogens showing cellular and nutritional properties that are typical for strain PA^T could be enriched from highly (up to 5×10⁸-fold) diluted hindgut preparations of *P. americana*. Growth of the cocci, however, did not require HS-CoM. This would imply that either strain PA^T is an HS-CoM auxotrophic mutant obtained during the isolation procedure, or that the requirement for the cofactor is an intrinsic strain-related property. The same situation was encountered with respect to *Methanobrevibacter ruminantium* (*Methanobacteriales*) from the bovine rumen. While the organism originally was reported to be HS-CoM dependent, other strains that were subsequently isolated from the rumen did not display such a requirement (Miller *et al.*, 1986).

In conclusion, strain PA^T, isolated from the hindgut of the cockroach *P. americana* var. Amsterdam, is clearly distinct from all described genera of the *Methanosarcinaceae*, both phylogenetically and physiologically. The status of a separate genus related to the family *Methanosarcinaceae* is therefore proposed, *Methanomicrococcus blatticola* (type strain PA^T). Formal genus and species descriptions based on properties of the isolated strain are given below.

Description of *Methanomicrococcus* gen. nov.

Methanomicrococcus (Me.tha.no.mi'cro.coc.cus. N.L. n. *methanum* methane; Gr. adj. *micro* small; M.L. n. *coccus* a sphere; M.L. n. *methanomicrococcus* a small methane-forming sphere).

Methanomicrococcus cells are irregular methane-forming cocci with a mean diameter of 0.8 µm, occurring singly or in clusters. Susceptible to hypotonic lysis, also during Gram-staining. Obligately anaerobic. Catalase-positive, oxidase-negative. Reduce methanol with molecular hydrogen, but not hydrogen plus CO₂ or methanol alone. Tryptic soy broth, yeast extract and acetate are required for growth. The type species is *Methanomicrococcus blatticola* sp. nov.

Description of *Methanomicrococcus blatticola* sp. nov.

Methanomicrococcus blatticola (blat.ti'co.la. L. fem. n. *blatta* cockroach; L. v. *colare* to dwell; M.L. n. *blatticola* inhabitant of the cockroach).

Cells form subsurface, lens-shaped, yellowish to brown colonies with a maximal diameter of ca. 0.3 mm in solid medium containing 1% agar or agarose. Methanol, trimethylamine, dimethylamine and monomethylamine are reduced to methane with molecular hydrogen. Formate, acetate, methanol alone, ethanol, pyruvate, lactate, malate, succinate and glucose are not utilized as methanogenic substrates. Growth occurs between approximately 20 and 40 °C, with an optimum at 39 °C (doubling time 3.1 h); no growth is observed at 15 or 45 °C. Optimal pH is 7.2–7.7 (range, 6.8–8.2). NaCl optimum is < 100 mM (range, 0–300 mM). Growth is dependent on HS-CoM and vitamins. The type strain, *Methanomicrococcus blatticola* PA^T, was isolated from the hindgut of the cockroach *Periplaneta americana* L. var. Amsterdam (*Blattidae*). The type strain has been deposited in the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) as DSM 13328^T.

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