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Detection of methanogens and proteobacteria from a single cell of rumen ciliate protozoa

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Rumen ciliate-associated bacteria and methanogenic archaea were analyzed by a 16S rRNA gene retrieved from a single cell of *Polyplastron multivesiculatum*, *Isotricha intestinalis*, and *Ophryoscolex purkynjei*. Rumen fluid was taken from a ruminally fistulated goat to prepare a ciliate fraction. Ciliate mixtures were incubated under mixtures of antibiotics for 48 h to eliminate extracellular bacteria. Individual cells of rumen ciliates were selected under microscopic observation after fixation with ethanol. Bacterial and archaeal 16S rRNA gene sequences were retrieved from each cell of three genera of ciliate. Two archaeal sequences related to *Methanobrevibacter smithii* were distributed to nearly all ciliate cells tested. These two methanogenic archaea were likely to be endosymbiotic methanogens commonly carried by the rumen ciliate, although some other sequences similar to the other genera were detected. A range of proteobacteria was retrieved from cells of *P. multivesiculatum*. Some sequences showed similarities to the previously known endosymbiotic proteobacteria. However, there were no proteobacteria that were carried by all the ciliate cells tested.

Key Words——methanogen; proteobacteria; 16S rRNA gene; rumen ciliate

Introduction

Anaerobic protozoa often harbor intracellular symbiotic bacteria, also called endosymbionts (Embley and Finlay, 1993; van Hoek et al., 1998), but protozoa also have episymbiotic associations with bacteria (Hackstein and Vogels, 1997). Rumen ciliates contribute to the greater part of ruminal methanogenesis (Jouany, 1991; Ushida et al., 1997) via hydrogen supply to the endosymbiotic and episymbiotic methanogens (Finlay et al., 1994; Vogels et al., 1980). We have predominantly detected *Methanobrevibacter smithii*-like 16S rRNA gene sequences from buffer-washed rumen ciliates (Chagan et al., 1999; Tokura et al., 1999) and also successfully isolated *M. ruminantium* from similar samples (Tokura et al., 1997). In these experiments, we had used well-washed ciliate preparations virtually free from extracellularly attached methanogens. However, *Methanobrevibacter* spp. are the predominant free-living methanogenic archaea in the rumen of sheep and cattle (Sharp et al., 1998) with a level usually as high as 10^8 cells/ml (by the MPN method) in the rumen fluid (Ushida et al., 1995), constituting one of the major functional bacterial groups in rumen. It is still possible that the detected sequence or isolated *M. ruminantium* originated from free-living methanogens that had been simply engulfed by ciliates because rumen ciliates actively prey on free-living bacteria as well as those attached to the feed particles (Coleman, 1975). Therefore, we prefer to use the term "ciliate-associated methanogens" rather than endosymbiotic

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methanogens (Chagan et al., 1999; Tokura et al., 1997, 1999).

Multiple antibiotic treatments with penicillin, streptomycin, chloramphenicol, tetracycline, erythromycin, polymyxin B, ampicillin, isoniazid, rifampicin, or gentamycin failed to eliminate endosymbiotic bacteria from *Acanthamoeba* sp. (Hall and Voelz, 1985). Later, they were identified as *Chlamydiaceae* (Amann et al., 1997) and unknown species of phylum *Cytophaga-Flavobacterium-Bacteroides* (Horn et al., 2001) by molecular techniques. Accordingly, antibiotic treatment may be helpful to eliminate extracellularly associated bacteria. Indeed, such treatments have been routinely used in the studies of intracellular pathogenic or opportunistic pathogenic bacteria such as *Listeria monocytogenes* (Glomski et al., 2003) and *Staphylococcus aureus* (Hess et al., 2003; Seral et al., 2003). Accordingly, a combination antibiotic treatment may eliminate temporary resident intracellular bacteria. Incubation for 48 h may be effective to digest intracellular bacteria and archaea because bacteria are rapidly digested by ciliate (Coleman, 1975; Coleman and Sandford, 1979) and ruminal *Methanobrevibacter* sp. can be rapidly digested by ciliates at the rate of 2 to 7% h^{-1} (Newbold et al., 1996).

We tried to extract bacterial DNA from a single cell of ciliate instead of using a mixed population (Tokura et al., 1997, 1999) or a cell preparation from a monocontaminated rumen system (Chagan et al., 1999; Newbold et al., 1996; Ushida and Jouany, 1996). In this manner, we worked on each cell of a specific species of ciliate, which originated from a mixed ciliate population found in the rumen fluid of a normal goat. If we could detect the same sequence of methanogenic archaea or bacteria throughout these single cells of ciliate, the sequences would be determined to be rumen ciliate-specific or, at least, belonging to the ciliate genus-specific endosymbionts.

Materials and Methods

Collection of rumen fluid and preparation of a ciliate fraction. Preparation of ciliate protozoa from a ruminally fisturated goat, which harbored an A-type mixed ciliate population (Eadie, 1962), was mostly the same as that reported previously (Tokura et al., 1999), except for the use of a goat.

Axenic cultures of ciliates. A mixed protozoa suspension (1 ml) was inoculated into test tubes with 9 ml of a Coleman Simplex buffer containing 0.5 ml cell-free rumen fluid, 10 mg cellobiose, 50 mg soluble starch, 50 mg trypticase, 50 mg yeast extract, and 1 ml of an antibiotic mixture. Two antibiotic mixtures were used; mixture (a) contained penicillin G potassium (1 mg/ml, Wako Pure Chemical, Osaka, Japan), streptomycin sulfate (1 mg/ml, Sigma, St. Louis, MO, USA), kanamycin sulfate (1 mg/ml, Wako Pure Chemical), and 5-fluorocytosine (0.5 mg/ml, Wako Pure Chemical), and mixture (b) contained chloramphenicol (0.032 mg/ml, Wako Pure Chemical) in addition to mixture (a). Mixture (a) was used to eliminate extracellular bacteria from the ciliate culture and the addition of chloramphenicol was done to eliminate extracellular methanogenic archaea. Ciliates were anaerobically incubated at 39°C for 48 h in the presence of O_2-free CO_2 with either antibiotic mixture (a) or (b). For the control, cultures without antibiotics were also done. Four tubes were allotted to each incubation. Methane and hydrogen concentration in the head space gas was determined by gas-chromatography (Ushida et al., 1982).

The number of ciliate protozoa was estimated by microscopic observation with a counting chamber before and after the cultivation. Bacterial and archaeal growth was controlled by epifluorescence microscopy during the incubation with antibiotic mixtures (a) and (b). Cases of no PCR amplification of bacterial or archaeal 16S rRNA gene from the culture supernatant after centrifugation at 500×*g* for 5 min was checked for the final confirmation of axenic status in the cultures. DNA extraction and amplification of the gene were conducted according to the method described below.

Isolation of single cell of Polyplastron multivesiculatum, Isotricha intestinalis, and Ophryoscolex purkynjei. Ciliates were collected after incubation by centrifugation (500×*g*, 5 min at ambient temperature), fixed with 95% (v/v) of ethanol, and kept at −20°C until required. Ethanol was replaced with sterile distilled water (DW), and single *P. multivesiculatum*, *I. intestinalis*, or *O. purkynjei* cells were picked up by serial transfer to a droplet of sterile DW using a Pasteur pipette under a binocular at ×80 magnification. Individual cells were finally transferred separately into Eppendorf tubes. They were then washed by centrifugation (500×*g*, 5 min at ambient temperature) three times in sterile distilled water. After addition of 50 µl Chelex-100 (5% w/v), the cells were frozen and stored at −20°C. A cell

of each of three ciliate species was prepared from an incubation tube, then in total eight cells each were subjected to the further elucidation.

P. multivesiculatum cells were also collected from a mixed protozoa suspension before incubation to analyze 16S rRNA gene of predominant ciliate-associated bacteria in the rumen.

DNA extraction, PCR amplification, and SSU rRNA gene sequencing. DNA extraction was performed according to van Hoek et al. (1998). Briefly, a proteinase K solution was added to the frozen sample. Samples were vortexed for half a minute. After incubation for 4 h at 56°C, the homogenates were heated at 95°C for 10 min in order to inactivate the proteinase K. They were then chilled on ice and centrifuged at 13,000 rpm for 10 min at 4°C. Aliquots (20 µl) of the supernatants were used for the amplification of intracellular archaeal 16S rRNA gene for *O. purkynjei* and *I. intestinalis* and both archaeal and bacterial 16S rRNA gene in the case of *P. multivesiculatum* with *ExTaq* DNA polymerase (TaKaRa, Kyoto, Japan) according to manufacturer's directions. The PCR primers used were the bacterial universal primers 907F (5′-AAACTCAAATGAATTGACGGG-3′) (Lane et al., 1985) and 1388R (5′-ACGGGCGGTGTGTACAAG-3′), which amplify the regions from V6 to V8 of the 16S rRNA gene. Twenty-five temperature cycles were performed at 52°C for 1 min, 72°C for 1 min, and 94°C for 1 min with an initial denaturation at 94°C for 4 min and a final extension at 72°C for 5 min. For the archaeal 16S rRNA gene amplification, primers ArcF915 (5′-AGGAATTGGCGGGGGAGCAC-3′) and ArcR1326 (5′-TGTGTGCAAGGAGCAGGGAC-3′) were used (van Hoek et al., 2000). PCR with 25 thermal cycles was performed at 60°C for 1 min, 72°C for 1 min, and 94°C for 1 min with an initial denaturation at 94°C for 4 min and a final extension at 72°C for 5 min. For the identification of ciliates, eukaryotic 18S rRNA gene was amplified using primers CilF (5′-CCGTTCTTAGTTGGTGGAGTG-3′) and EkyB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin et al., 1988). The amplified products were purified by electrophoretic size-fraction on 1.5% agarose with a QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's directions. The purified products were ligated into a pGEM-T vector (Promega, Madison, WI, USA) according to the manufacturer's directions. *Escherichia coli* JM109 (TaKaRa) was transformed by the cloning vector, and the transformants were selected by blue-white selection on LB plates containing ampicillin (100 µg/ml), 5-bromo-4-chloro-3-indoryl-β-D-galactoside (1 mg/plate), and isopropyl-β-D-thiogalactopyranoside (2.38 mg/plate). For practical reasons, 96 transformants were picked and subjected to further analysis. The appropriate insert was amplified and confirmed by the direct colony PCR technique with the primers (TaKaRa) that correspond to both sides of the cloning site on the vector. Amplified ribosomal DNA restriction analysis (ARDRA) was conducted using a previously reported method (Inoue and Ushida, 2003) with *Sau*3AI, *Rsa*I, and *Hha*I (Toyobo, Tokyo, Japan). Two to three clones were randomly selected to be sequenced from an ARDRA clone group if it consisted of more than 10 clones. Otherwise one clone was randomly selected and sequenced. Sequencing of the insert region of plasmid DNA wasdone by the Shimadzu Genomic Research Laboratory (Shimadzu, Kyoto, Japan). Sequence data were aligned by CLUSTAL X ver. 1.8 (Thompson et al., 1994) with a selection of reference sequence of proteobacteria that was obtained from the nucleotide sequence libraries (GenBank, DDBJ, and EMBL) and the Ribosomal Database Project (Maidak et al., 1997). Phylogenetic trees were constructed by the maximum-parsimony method, maximum-likelihood method, and neighbor-joining method with the MEGA ver. 2.1 (Kumar et al., 2001). The CHECK-CHIMERA program in the Ribosomal Database Project was used to evaluate the formation of chimeras.

Results and Discussion

Growth of ciliates during incubation (Table 1)

The population level of ciliates was maintained, although it did not increase during the 48 h culture. Antibiotic treatments successfully eliminated free-living bacteria as evidenced by no amplification of 16S rRNA genes and no microscopical detection of bacteria and archaea, but virtually unaffected ciliate cell growth. As a consequence of the treatment, methane production was nearly zero. Addition of chloramphenicol further decreased the methane emission from ciliate cells. The ciliate cells isolated after incubation were *P. multivesiculatum*, *O. purkynjei*, and *I. intestinalis* according to their 18S rDNA partial sequences (>98% match with U57767, U57768, and U57770, respectively).

During the 48 h incubation, intracellular bacteria engulfed by ciliates as food were likely to be rapidly

Table 1. Relative number of rumen protozoa (%) and their apparent gas production (pmol/cell/h) during 48 h incubation with or without antibiotics.

Treatment		Relative protozoa number (%)	Hydrogen	Methane
T_0		100[a]	—	—
T_{48}	Control	107±6.9	1.4±0.23	5.6±0.44
	(a)	96±4.7	13.7±0.38	1.5±0.04
	(b)	91±3.9	18.9±0.03	0.7±0.02

Values are means±SD (n=4).

Antibiotics used and the final concentration of them in the medium were as follows: Treatment (a), penicillin G potassium (100 μg/ml), streptomycin sulfate (100 μg/ml), kanamycin sulfate (100 μg/ml) and fluorocytosine (50 μg/ml); Treatment (b), penicillin G potassium (100 μg/ml), streptomycin sulfate (100 μg/ml), kanamycin sulfate (100 μg/ml), fluorocytosine (50 μg/ml) and chloramphenicol (3.2 μg/ml).

Details, see MATERIALS AND METHODS.

[a] Protozoal number at the beginning of incubation (7.8×10^4/cell). Protozoal numbers relative to T_0 are indicated.

Gas production was measured by syringe. Collected gas was analyzed by GC to obtain the concentration of hydrogen and methane.

The production rate of each gas was calculated using the final number of protozoa.

digested according to Coleman (1975), Coleman and Sandford (1979), and Newbold et al. (1996). Since the free-living bacteria were eliminated during incubation, a further engulfment of free-living bacteria was unlikely to occur. Moreover, antibiotic treatment helped to eliminate the extracellularly attached bacteria. Therefore, the sequences detected in this study are likely some specific bacteria or archaea for ciliates, although FISH analyses are still required to conclude this.

Ciliate-associated archaeal sequences

Methanobrevibacter spp. were the archaea detected predominantly from single cells of ciliate protozoa (Table 2). *Methanomicrobium mobile* was detected from some cells of *O. purkynjei* (Oa1, Oa3, Ob4) and *I. intestinalis* (Ib1, Ib3). The sequence homologies with known taxa were relatively high; hence, most of the sequences detected showed an identity higher than 97%. Unidentifiable archaeal sequences related to the genus *Thermoplasma* (Ia4-m1, Oa1-m4, Ob3-m3, and Oa4-m2) were detected from a cell of *I. intestinalis* and three cells of *O. purkynjei*.

The sequences of Pb1-m1, Pb4-m1, Ia1-m1, Ib1-m3, Ib2-m1, Ia3-m1, Ib3-m1, Ia4-m3, Ib4-m1, Oa1-m5, Ob1-m1, Oa2-m1, Ob2-m1, Oa3-m1, Ob3-m2, Oa4-m1, and Ob4-m2 were the same and highly similar to *Methaobrevibacter* sp. SM9 (AJ009958). This sequence was detected in more than half of the cells tested (17/24): 8 cells over 8 for *O. purkynjei*, 7 cells over 8 for *I. intestinalis*, and 2 cells over 8 for *P. multivesiculatum*. The sequences of Pa1-m1, Pb2-m1, Pa4-m1, Ia1-m2, Ia2-m1, Ia3-m3, Ib4-m2, Oa1-m3, Ob1-m2, Ob2-m3, Ob3-m1, and Ob4-m1 were the same and highly similar to uncultured rumen methanogen M6 (AB034185). This sequence was detected in half of the cells tested (12/24): 5 cells over 8 for *O. purkynjei*, 4 cells over 8 for *I. intestinalis*, and 3 cells over 8 for *P. multivesiculatum. Methanobrevibacter* sp. SM9 is related to *M. smithii*, and uncultured rumen methanogen M6 is related to *M. gottschalkii* MSU 55239, which is a neighbor of *M. smithii* and is separated from *M. ruminantium* (Fig. 1). Indeed, both *M. gottschalkii* and *M. smithii* are predominantly hindgut methanogens (Miller and Lin, 2002; Miller et al., 1986). As indicated above, these two sequences accounted for most of the individual cells of ciliates in this experiment. The sequences Pb3-m1, Pb4-m2, Ia2-m2, Ia3-m2, Ia4-m2, Ib1-m1, Ib2-m2, Oa1-m1, Oa2-m2, and Ob2-m2 were the same and related to *M. wolinii* SH (U55240). Since this sequence showed only a 94% identity with known taxa, this methanogen is an unknown *Methanobrevibacter* species.

As indicated previously (Chagan et al., 1999; Tokura et al., 1997, 1999), archaeal sequences detected in freshly prepared rumen ciliates were limited to those belonging to the families of *Methanobacteriaceae*, *M. ruminantium*, *M. smithii*, or *Methanosphaera stadtmaniae*. It is likely, therefore, that these methanogens re-

Table 2. Clones harboring fragments of 16S rRNA gene of methanogens retrieved from ruminal ciliate single cell of *Polyplastron multivesiculatum* (P), *Isotricha intestinalis* (I), and *Ophryoscolex purkynjei* (O).

Sources	Antibiotics	Number of clones	Representative clone name of RFLP group	Nearest relative (similarity %)	Accession No.
P 1	a	96	Pa1-m1	Uncultured rumen methanogen M6 (99.4)	AB189855
	b	96	Pb1-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189857
P 2	a	96	Pa2-m1	*Methanobrevibacter smithii* ALI (96.7)	AB189858
	b	96	Pb2-m1	Uncultured rumen methanogen M6 (99.4)	AB189855
P 3	a	96	Pa3-m1	*Methanobrevibacter wolinii* SH (94.1)	AB189856
	b	96	Pb3-m1	*Methanobrevibacter wolinii* SH (94.1)	AB189856
P 4	a	96	Pa4-m1	Uncultured rumen methanogen M6 (99.4)	AB189855
	b	85	Pb4-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189857
		11	Pb4-m2	*Methanobrevibacter wolinii* SH (94.1)	AB189856
I 1	a	96	Ia1-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189859
		96	Ia1-m2	Uncultured rumen methanogen M6 (99.4)	AB189860
	b	76	Ib1-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189861
		3	Ib1-m2	*Methanomicrobium mobile* (100)	AB189862
		17	Ib1-m3	Uncultured rumen methanogen M6 (99.4)	AB189859
I 2	a	94	Ia2-m1	Uncultured rumen methanogen M6 (99.4)	AB189860
		2	Ia2-m2	*Methanobrevibacter wolinii* SH (94.1)	AB189861
	b	94	Ib2-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189859
		2	Ib2-m2	*Methanobrevibacter wolinii* SH (94.1)	AB189861
I 3	a	66	Ia3-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189859
		3	Ia3-m2	*Methanobrevibacter wolinii* SH (94.1)	AB189861
		27	Ia3-m3	Uncultured rumen methanogen M6 (99.4)	AB189860
	b	92	Ib3-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189859
		4	Ib3-m2	*Methanomicrobium mobile* (100)	AB189862
I 4	a	25	Ia4-m1	Uncultured rumen archaeon M1 (97.7)	AB189863
		11	Ia4-m2	*Methanobrevibacter wolinii* SH (94.1)	AB189861
		60	Ia4-m3	*Methanobrevibacter* sp. SM9 (99.4)	AB189859
	b	85	Ib4-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189859
		11	Ib4-m2	Uncultured rumen methanogen M6 (99.4)	AB189860
O 1	a	3	Oa1-m1	*Methanobrevibacter wolinii* SH (94.1)	AB189866
		6	Oa1-m2	*Methanomicrobium mobile* (100)	AB189867
		5	Oa1-m3	Uncultured rumen methanogen M6 (99.4)	AB189865
		2	Oa1-m4	*Thermoplasma* sp. (95.5)	AB189868
		80	Oa1-m5	*Methanobrevibacter* sp. SM9 (99.4)	AB189864
	b	91	Ob1-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189864
		5	Ob1-m2	Uncultured rumen methanogen M6 (99.4)	AB189865
O 2	a	94	Oa2-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189864
		2	Oa2-m2	*Methanobrevibacter wolinii* SH (99.4)	AB189866
	b	85	Ob2-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189864
		5	Ob2-m2	*Methanobrevibacter wolinii* SH (94.1)	AB189866
		6	Ob2-m3	Uncultured rumen methanogen M6 (99.4)	AB189865
O 3	a	92	Oa3-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189864
		2	Oa3-m2	*Methanomicrobium mobile* (100)	AB189867
	b	40	Ob3-m1	Uncultured rumen methanogen M6 (99.4)	AB189865
		54	Ob3-m2	*Methanobrevibacter* sp. SM9 (99.4)	AB189864
		2	Ob3-m3	*Thermoplasma* sp. XT107 (95.5)	AB189868
O 4	a	90	Oa4-m1	*Methanobrevibacter* sp. SM9 (99.4)	AB189864
		6	Oa4-m2	*Thermoplasma* sp. XT107 (95.5)	AB189868

Table 2. continued

Sources	Antibiotics	Number of clones	Representative clone name of RFLP group	Nearest relative (similarity %)	Accession No.
	b	33	Ob4-m1	Uncultured rumen methanogen M6 (99.4)	AB189865
		57	Ob4-m2	*Methanobrevibacter* sp. SM9 (99.4)	AB189864
		6	Ob4-m3	*Methanomicrobium mobile* (100)	AB189867

Source, indicating species of ciliate and the tube number of the incubation.
Antibiotic treatment, see Table 1.
Number of clones, transformants subjected to RFLP analyses.

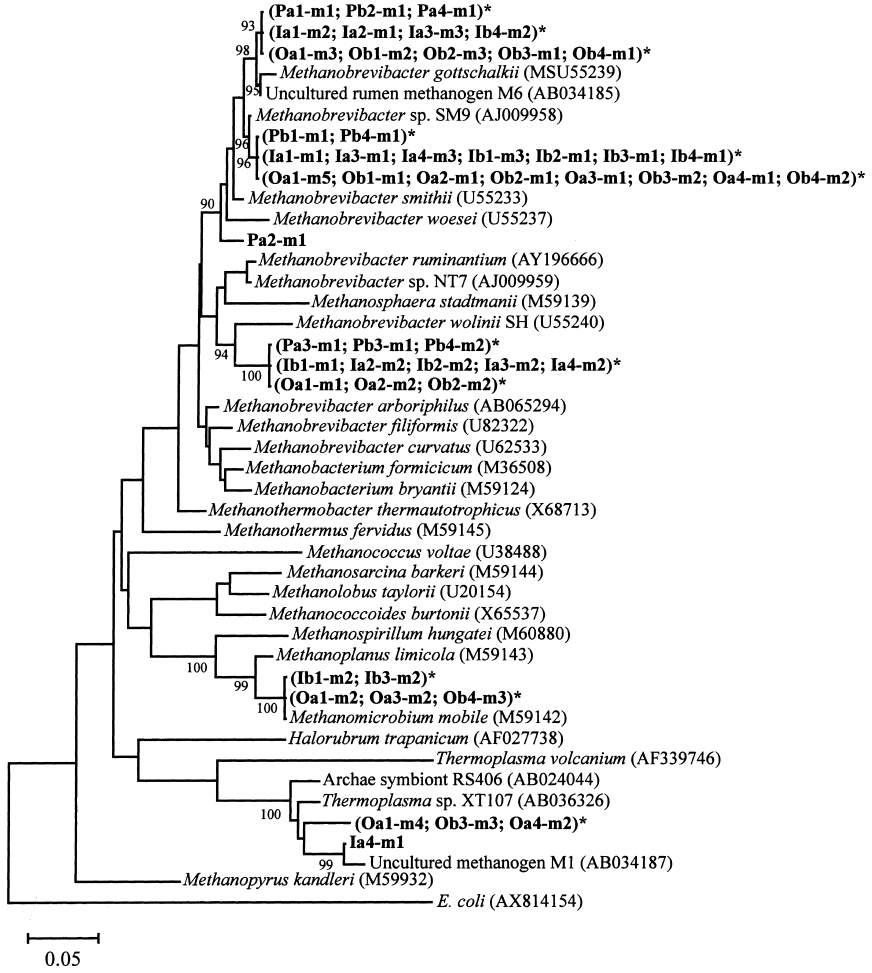

Fig. 1. Phylogenetic placement of archaeal 16S rDNA sequences from the single cell of *Polyplastron multivesiculatum* (P), *Isotricha intestinalis* (I), and *Ophryoscolex purkynjei* (O) cultured with antibiotic treatments (a) and (b).

The database sequences have the GenBank accession numbers in brackets. The *E. coli* sequence is used as the outgroup for rooting the tree. Since the maximum-parsimony method, maximum-likelihood method, and neighbor-joining method showed the same placement, the neighbor-joining tree is presented. The numbers around the nodes are the confidence levels (%) generated from 1,000 bootstrap trials. The scale bar is in fixed nucleotide substitutions per sequence position. Bootstrap values above 80% are given. The name of clone, see Table 2. * The sequences within the same parentheses are identical.

Table 3. Clones harboring fragments of 16S rRNA gene of proteobacteria retrieved from the washed cells before incubation (Pw) and single cell of rumen ciliate *Polyplastron multivesiculatum* after incubation (P).

Sources	Antibiotics	Number of clones	Representative clone name of RFLP group	Nearest relative (similarity %)	Accession No.
Pw	—	33	Pw-e1	*Ruminococcus albus* (98.8)	AB189869
		8	Pw-e2	*Ruminococcus albus* (99.8)	AB189870
		31	Pw-e3	*Streptococcus bovis* (98.4)	AB189871
		2	Pw-e4	*Streptococcus bovis* (99.2)	AB189872
		3	Pw-e5	Uncultured rumen bacterium 3C0d-16 (98.0)	AB189873
		6	Pw-e6	Uncultured rumen bacterium (99.6)	AB189874
		2	Pw-e7	*Mycoplasma iowae* (91.7)	AB189875
		5	Pw-e8	Unidentified rumen bacterium RF10 (99.2)	AB189876
		4	Pw-e9	Unidentified rumen bacterium RF15 (99.4)	AB189877
P 1	a	7	Pa1-e3	β-*Proteobacteria Roseateles depolymerans* (99.4)	AB189878
	b	3	Pb1-e1	γ-*Proteobacteria*	AB189879
				Terrahaemophilus aromaticivora (98.8)	
P 2	a	4	Pa2-e1	β-*Proteobacteria Ralstonia* sp. APF11 (99.4)	AB189880
		2	Pa2-e3	α-*Proteobacteria Sphingomonas* sp. BF2 (97.8)	AB189881
	b	3	Pb2-e3	γ-*Proteobacteria*	AB189886
				Terrahaemophilus aromaticivora (98.8)	
P 3	a	9	Pa3-e2	β-*Proteobacteria Roseateles depolymerans* (99.4)	AB189882
		11	Pa3-e5	β-*Proteobacteria*	AB189883
				Uncultured bacterium clone csbio160330 (99.4)	
		12	Pa3-e6	β-*Proteobacteria Ralstonia* sp. APF11 (99.6)	AB189884
		8	Pa3-e8	γ-*Proteobacteria Ruminobacter amylophilus* (95.2)	AB189885
	b	8	Pb3-e2	α-*Proteobacteria* Uncultured bacterium (98.8)	AB189887
		7	Pb3-e4	α-*Proteobacteria Amaricoccus tamworthensis* (97.7)	AB189888
		8	Pb3-e6	β-*Proteobacteria* Uncultured bacterium (99.4)	AB189889
P 4	a	3	Pa4-e4	α-*Proteobacteria Sphingomonas* sp. BF14 (99.4)	AB189890
		10	Pa4-e5	β-*Proteobacteria Ralstonia* sp. APF11 (98.8)	AB189891
		1	Pa4-e7	β-*Proteobacteria*	AB189892
				Uncultured bacterium csbio160330 (99.4)	
	b	3	Pb4-e5	β-*Proteobacteria* Uncultured bacterium (99.4)	AB189914

Source, see Table 2.

lated to *M. smithii* are the endosymbiotic methanogens that carried by nearly every rumen ciliate cell independent of the genera. The sequences related to the genus *Thermoplasma* were newly detected from a cell of *I. intestinalis* and three cells of *O. purkynjei*. The *Thermoplasmales* listed in this figure (AB02044, AB036326) have been isolated from termite gut as minor symbiotic methanogens (Shinzato et al., 1999), although *Methanobacteriales* were the predominant symbionts for termites. *Methanomicrobiales* such as *Methanocorpusclum* spp. have been detected as endosymbiotic methanogens of *Metopus* spp., *Trimyema* spp., and *Cyclidium* spp., typical fresh-water anaerobic protozoa (Embley and Finlay, 1993; Finlay et al., 1993). *Methanomicrobiales* was detected from two cells of *I. intestinalis* and three cells of *O. purkynjei*. This suggests the presence of some minor methanogenic archaea specific to the rumen ciliates, although the *Methanobrevibacter* spp., most likely *M. smithii*-like *Methanobacteriales*, may be the predominant methanogenic archaea associated with rumen ciliates.

Bacterial sequences

Bacterial sequences detected from *P. multivesiculatum* cells before incubation are listed in Table 3. They resembled common ruminal bacteria, such as *Ruminococcus albus* and *Streptococcus bovis*. Bacterial sequences detected upon a 48 h incubation under a mixture of antibiotics were substantially changed to

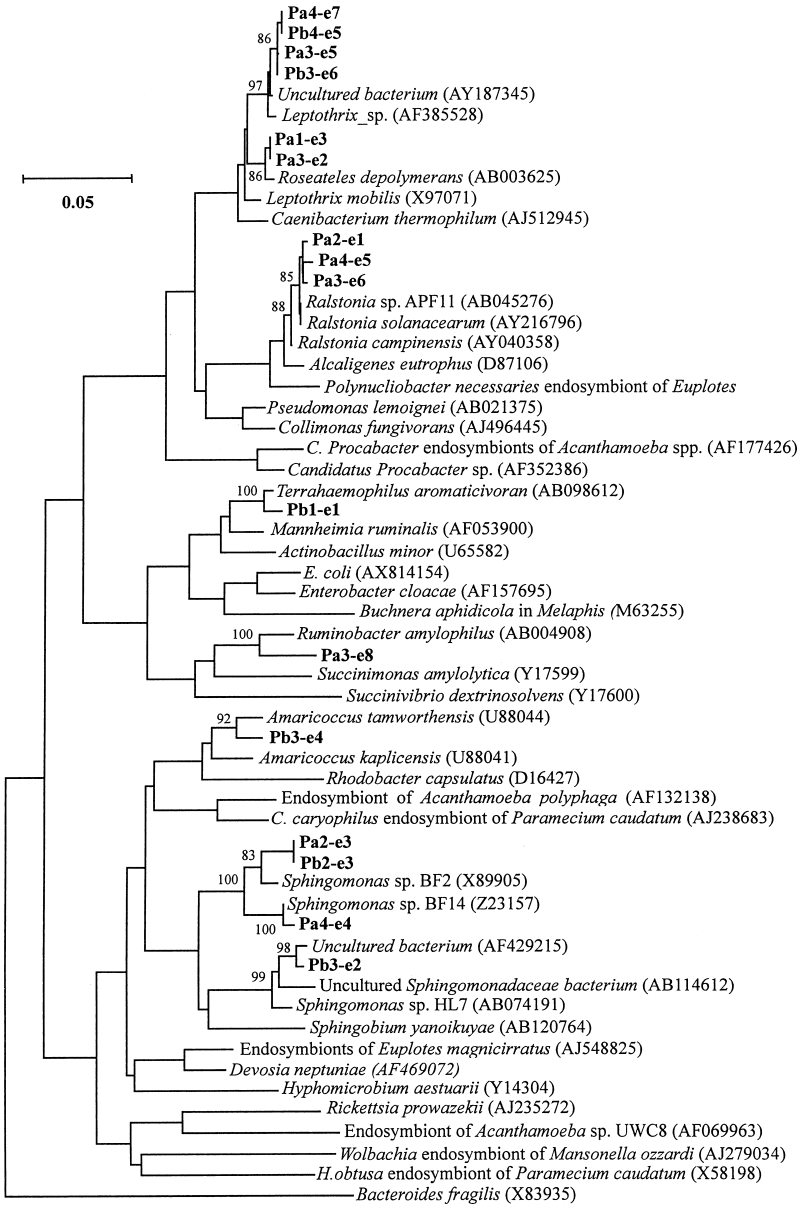

Fig. 2. Phylogenetic placement of proteobacterial 16S rDNA sequences from the single cell of *Polyplastron multivesiculatum* cultured with antibiotic treatment (a) and (b).

The database sequences have the GenBank accession numbers in brackets. The *Bacteroides fragilis* sequence is used as the outgroup for rooting the tree. Since the maximum-parsimony method, maximum-likelihood method, and neighbor-joining method showed the same placement, the neighbor-joining tree is presented. The numbers around the nodes are the confidence levels (%) generated from 1,000 bootstrap trials. The scale bar is in fixed nucleotide substitutions per sequence position. Bootstrap values above 80% are given.

reveal more proteobacteria and less common ruminal bacteria (Table 3). The inclusion of chloramphenicol further reduced the detection of bacterial sequences common in the rumen (Table 3).

These were α-proteobacteria, such as *Sphingomonas* spp. and *Amarricoccus* spp., β-proteobacteria, such as *Polynucleobacter* spp.-related *Ralstonia* spp., *Leptothrix* spp., and *Ralstonia* spp., and γ-proteobacteria, such as *Terrahaemophilus* spp. (Fig. 2). Interestingly, *Polynucleobacter* spp.-related *Ralstonia* were detected as a ciliate-associated β-proteobacteria. *Polynucleobacter* sp. was identified as the endosymbiotic proteobacteria of the fresh water ciliate *Euplotes aediculatus* (Springer et al., 1996). As Gortz and

Brigge (1998) indicated, most of the intracellular bacteria in protozoa are proteobacteria. Both Pa1-e3 and Pa3-e2 had a similar sequence to *R. depolymerans*. Both Pb3-e6 and Pb4-e5 had a similar sequence to an uncultured β-proteobacterium. Pb3-e1, Pa4-e3, and Pb4-e2 had a similar sequence to uncultured rumen bacteria 3C0d-16, which seems to be a low GC gram-positive bacterium such as *Firmicutes*. Unlike methanogens, the distribution of bacterial sequences was not uniform for the *P. multivesiculatum* cells tested, although two of the cells, Pa1 and Pa3 for the antibiotic mixture (a) and Pb3 and Pb4 for the antibiotic mixture (b), carried the same bacteria, U29676 and AB034014, respectively. Therefore, it is still inconclusive that these are the endosymbiotic bacteria for rumen ciliates.

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