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Phylogenetic analysis of *Methanobrevibacter* isolated from feces of humans and other animals

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Abstract Comparative 16S rRNA gene sequence and genomic DNA reassociation analyses were used to assess the phylogenetic relationships of *Methanobrevibacter* fecal isolates. The 16S rRNA gene sequences of *Methanobrevibacter smithii* strain PS and the human fecal isolates B181 and ALI were essentially identical, and their genomic DNA reassociated at values greater than 94%. The analysis of 16S rRNA sequences of the horse, pig, cow, rat, and goose fecal isolates confirm that they are members of the genus *Methanobrevibacter*. They had a high degree of sequence similarity (97–98%) with the 16S rRNA gene of *M. smithii*, indicating that they share a common line of descent. The 16S rRNA genes of the horse and pig isolates had 99.3% sequence similarity. Sequence analysis of the 16S rRNA gene of the sheep fecal isolate showed that it formed a separate line of descent in the genus *Methanobrevibacter*. Genomic DNA reassociation studies indicate that the horse, pig, cow, and goose fecal isolates represent at least three new species. The horse and pig isolates were the only animal isolates that had > 70% genomic DNA reassociation and represent strains of a single species. The cow, goose, and sheep isolates had little or no genomic DNA reassociation with *M. smithii* or with each other. The relationship of the rat isolate to the other animal isolates was not determined. An evaluation of the relationship of 16S rRNA gene sequence similarity and genomic DNA reassociation of *Methanobrevibacter* and other methanogenic archaea indicated that genomic DNA reassociation studies are necessary to establish that two methanogenic organisms belong to the same species.

Key words Archaea · Methanogen · *Methanobrevibacter* · 16S rRNA gene · Genomic DNA reassociation

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Introduction

Methanogens that reduce CO_2 with H_2 to form methane are common inhabitants of gastrointestinal tract ecosystems. *Methanobrevibacter* is a major intestinal genus. Five of the currently recognized species of the genus have been isolated from gut ecosystems. *Methanobrevibacter ruminatium* (formerly *Methanobacterium ruminantium*) is the type species and was the first methanogen isolated from the bovine rumen (Smith and Hungate 1958). *Methanobrevibacter smithii* is the numerically dominant methanogen species in the large bowel of humans that harbor a methanogenic flora (Miller and Wolin 1982, 1983; Miller et al. 1982; Weaver et al. 1986). The morphology, cell wall structure, immunology, DNA base ratios, genomic DNA reassociation, and other physiological features are all consistent with the identification of the human fecal isolates as *M. smithii* (Miller and Wolin 1982, 1986; König 1986; Miller et al. 1986). The hindgut of the subterranean termite *Reticulitermes flavipes* harbors three newly described species: *Methanobrevibacter cuticularis*, *Methanobrevibacter curvatus*, and *Methanobrevibacter filiformis* (Leadbetter and Breznak 1996; Leadbetter et al. 1998).

The morphology, physiological characteristics, DNA base ratios, and immunology of methanogens isolated from feces of horse, pig, cow, rat, goose, and sheep show that they are members of the genus *Methanobrevibacter* (Miller et al. 1986; Conway de Macario et al. 1987). Also, all of the isolates have cell sacculi composed of pseudomurein (König 1986). However, none of these features demonstrate the phylogenetic relationship of the animal isolates to the present species of the genus or to each other.

This study was initiated to determine the phylogenetic relationships of fecal methanogens from horse, pig, cow, rat, goose, and sheep to those in the bovine rumen and human large intestine ecosystems. We used 16S rRNA gene sequence analysis and genomic DNA reassociation to assess the relationships.

Materials and methods

Bacterial strains and growth conditions

The methanogens used for 16S rRNA gene sequence determination included *M. smithii* type strain PS (DSM 861); human fecal isolates *M. smithii* strain ALI (DSM 2375) and strain B181; the animal fecal isolates HO (horse), SH (sheep), GS (goose), CW (cow), PG (pig), and RT (rat). Strains HO, SH, GS, CW, PG, and 13181 have been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany, DSM). In addition, two other species of *Methanobrevibacter* [*M. arboriphilicus* type strain DH1 (DSM 1125) and *M. ruminantium* type strain M1 (DSM 1093)] were included in the genomic DNA reassociation study.

All methanogens were cultured using the serum bottle modification of the Hungate technique (Miller and Wolin 1974) in medium 1 (Balch et al. 1979) supplemented with 10% (v/v) rumen fluid and an additional 1% NH_4Cl (Miller et al. 1982) under H_2 CO_2 (80 : 20, v/v; 202 kPa). All cultures were incubated with rotation or on a reciprocal shaker at 37° C.

Cells used for isolation of 16S rRNA gene sequences were grown in 5 ml medium in serum-bottle-finished tubes (18 × 150 mm; Miller and Wolin 1974). The cultures were regassed and repressurized with H_2 CO_2 at least once daily for 2–3 days. Cells were harvested by centrifugation and stored as a cell paste at –76° C. Anaerobic solid-liquid biphasic culture techniques as described by Miller et al. (1986) were used to obtain cells for genomic DNA reassociation studies.

Nucleic acid extraction and PCR amplification of 16S rRNA genes

The methanogen cells were broken by mechanical disruption on a reciprocating shaker (Mini-Beadbeater; Biospec Products, Bartlesville, Okla., USA) with zirconium beads by a slight modification of the procedure of Lin and Stahl (1995). In brief, cells from 5 ml culture were combined with approximately 3 g beads, 600 µl TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)], and 600 µl phenol equilibrated with TE buffer (pH 8.0) and were disrupted on the shaker for 2 min at 4° C. The nucleic acids were extracted at least three times with equal volumes of phenol and phenol/chloroform. RNase A was added to the nucleic acid preparation to a final concentration of 50 µg/ml, and the sample was incubated at 37° C for 30 min to digest RNA. The DNA was precipitated by the addition of ammonium acetate (2 M final concentration) and isopropanol (one volume) at –20° C overnight. The DNA pellet was recovered by centrifugation. The DNA pellet was washed with 70% ethanol, resuspended in water, and stored at –20° C.

Ribosomal DNA was amplified with a Perkin-Elmer ThermoCycler 9600 (Perkin-Elmer, Norwalk, Conn., USA) as follows: denaturation at 94° C for 1 min, annealing at 50° C for 30 s, and extension at 72° C for 1 min for the first cycle. In the remaining 29 cycles, template denaturation time was reduced to 30 s. All reagents used for PCR were purchased from Perkin-Elmer, and the reaction mixture contained 2.5 m*M* $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 mM deoxynucleotide triphosphates, 2.5 units of *Taq* polymerase, 10 pmol each of oligonucleotide primers, and 250 ng of DNA. The primers were:

MbrF: TCTGTTTGATCCTGGCAGA
MbrR1: AAGGAGGTGATCCAGCC
MbrUR: ACGGGCGGTGTGTGCA

Amplified DNA was visualized by electrophoresis on a 1% agarose gel containing ethidium bromide in 0.04 M Tris-acetate with 1 mM EDTA (Sambrook et al. 1989). Amplified 16S rRNA genes were purified for sequencing with Microcon 100 spin columns (Amicon, Beverly, Mass., USA) according to the manufacturer's instructions.

Sequencing of amplified rRNA genes

Sequences of the rRNA genes were determined with an Applied Biosystems (Foster City, Calif., USA) model 373A automatic DNA sequencer using the *Taq* DyeDeoxy terminator cycle sequencing method as recommended by the manufacturer. Part of the 16S rRNA gene from the cow isolate CW was sequenced manually using standard dideoxynucleotide chain termination sequencing with [α-^{35}S]dATP and a Sequenase PCR Product Sequencing Kit (United States Biochemical, Cleveland, Ohio, USA) following the manufacturer's instructions. Universal 16S rRNA gene-specific sequencing primers (Lane 1991) and the PCR amplification primers were used as primers in the sequencing reactions. Sequences for *M. smithii* strain PS, the two human fecal isolates, and the other animal fecal isolates were deposited in GenBank under accession nos. U55233–U55241.

Phylogenetic analysis

The 16S rRNA gene sequences of *M. arboriphilicus, M. ruminantium, M. cuticularis,* and *M. curvatus* were obtained from the Ribosomal Data Base Project (Maidak et al. 1997) and GenBank. A partial 16S rRNA sequence of *M. smithii* strain PS was kindly provided by D. A. Stahl (Northwestern University, Evanston, Ill., USA) as well. Sequences were manually aligned with ambiguous regions and alignment gaps omitted from the analysis. A total of 1,187 nucleotide positions was used in the comparative analysis. Secondary structural constraints were taken into consideration in sequence alignment. Sequence similarities were determined as previously described by Montgomery et al. (1988). Evolutionary distance analyses were performed on a SUN SPARC station using the programs contained in the PHYLIP package (Felsenstein 1993) that were implemented through the Genetic Data Environment (GDE) sequence editor. Pairwise evolutionary distances were estimated by using DNADIST with Jukes and Cantor correction (Jukes and Cantor 1969). A phylogenetic tree was calculated by using the Fitch-Margoliash method with the program FITCH. The stability of the relationship was assessed by the bootstrap method with the programs SEQBOOT, DNADIST, FITCH, and CONSENSE.

Genomic DNA reassociation study

Genomic DNA was isolated from 1–2 g wet cells by a modification of the procedure of Meyer and Schleifer (1974). Cells were suspended in 5 ml saline-EDTA buffer (pH 7.0) and disrupted in a French pressure cell at 67,533 kPa. Unbroken cells were removed from the lysate by centrifugation, resuspended in 5 ml buffer, and disrupted again by passage through the French pressure cell. The procedure was repeated to yield a combined lysate of 20 ml. The lysate was incubated with 1 mg Pronase K (Sigma, St. Louis, Mo., USA) for 30 min at 60° C. The procedure of Meyer and Schleifer (1974) using hexadecyltrimethylammonium bromide (Sigma) was then followed with the volumes of reagents adjusted accordingly. The DNA preparation was dialyzed overnight at 4° C against 100 vol. of 0.1 × standard saline-citrate buffer (Marmur 1961). The T_m of each purified DNA preparation was determined by thermal denaturation as described by Marmur and Doty (1962). *Escherichia coli* (ATCC 11775) DNA was included as a reference in each determination.

DNA reassociation was analyzed by the direct-binding nitrocellulose membrane method (Johnson 1994). Probe DNA was labeled with 5′-^{32}P-deoxycytidine using a commercial nick translation kit (Amersham, Cleveland, Ohio, USA). The hybridization temperature used was 25° C below the T_m of the labeled DNA. The radioactivity of probe DNA annealed to homologous and heterologous DNA bound to filters was determined in Aquasol (New England Nuclear, Boston, Mass., USA) with a scintillation counter. The sequence similarity was calculated from the counts bound onto the heterologous DNA membrane filter divided by the counts bound onto the homologous DNA membrane filter. Results were multi-

plied by 100 to give the percent of reassociation. Membrane-bound, unlabeled DNA from *E. coli* (ATCC 11775) was included in all analyses as a control of nonspecific binding of the labeled probe DNA.

Results

16S rRNA gene sequencing

More than 90% of the 16S rRNA gene sequence was recovered for most strains, using the complete 16S rRNA from *Methanobacterium thermoautrophicum* strain Marburg (1,481 base pairs) as a reference. For strains CW and RT, 86% of 16S rRNA sequences were recovered.

The sequence of the 16S rRNA gene of *M. smithii* strain PS had a single base mismatch with the genes of the human fecal isolates B181 and ALI. Sequences from isolates B181 and ALI were identical to each other and differed from *M. smithii* strain PS by substitution of a G for an A at position 69 of the 16S rRNA (*M. thermoautrophicum* strain Marburg numbering), resulting in a change from a TA base pair to a TG pair. The partial *M. smithii* strain PS 16S rRNA sequence, obtained using reverse transcriptase and provided by D. Stahl (Northwestern University, Evanston, Ill., USA), was identical to the corresponding sequence of *M. smithii* strain PS obtained in this study.

The 16S rRNA gene sequences from the horse, pig, cow, rat, and goose fecal isolates had a high degree of sequence similarity (97–98%) with the 16S gene of *M. smithii* (Table 1). The 16S rRNA genes of the isolates from horse and pig shared 99.3% sequence similarity. The 16S rRNA gene sequence of the sheep fecal isolate SH had a low degree of sequence similarity (94% or less) to the other animal fecal isolates or to *M. smithii*, *M. arboriphilicus*, or *M. ruminantium* (Table 1).

A dendrogram depicting the phylogenetic relationships of the *Methanobrevibacter* type species and the human and other animal isolates with representative Methanobacteriaceae is shown in Fig. 1. All of the fecal isolates

Table 1 16S rRNA sequence similarity (*below the diagonal*) and genomic DNA reassociation (*above the diagonal*) values for *Methanobrevibacter*. The *left value* for reciprocal hybridization values corresponds to use of the row-organism's DNA as probe (*PS Methanobrevibacter smithii* type strain; *B181* and *ALI Metanobrevibacter* human fecal isolates; *HO*, *PG*, *CW*, *RT*, *GS*, and *SH Methanobrevibacter* animal fecal isolates; *DH1 Methanobrevibacter arboriphilicus* type strain; *M1 Methanobrevibacter ruminantium* type strain)

	PS[a]	B181[a]	ALI[a]	HO	PG	CW	RT[a]	GS	SH	DH1[a]	M1[a]
PS		110/78	112/88	36/27	31/28	24/15	48/37	41/39	30/18	14/21	23/23
B181	100.0		91/113				32/43			8/34	21/28
ALI	100.0	100.0					36/43			9/34	38/32
HO	97.3	97.3	97.3		73/108	33/38		29/39	23/32	11/19	10/22
PG	97.5	97.5	97.5	99.3		37/28		33/28	40/25	14/13	14/28
CW	98.0	98.0	98.0	98.1	98.5			18/22	31/25	6/10	13/28
RT	97.5	97.5	97.5	96.1	96.3	96.6				12/28	31/9
GS	97.2	97.2	97.2	95.7	95.7	96.5	97.4		27/19	18/29	14/26
SH	93.9	93.9	93.9	92.8	92.9	92.8	92.7	93.2		9/15	11/21
DH1	94.9	94.9	94.9	93.8	94.1	94.6	94.1	94.7	92.8		14/11
M1	94.7	94.7	94.7	93.4	93.8	93.9	93.7	94.5	93.3	94.7	

[a] DNA reassociation values were previously published in a review by Miller and Wolin (1986)

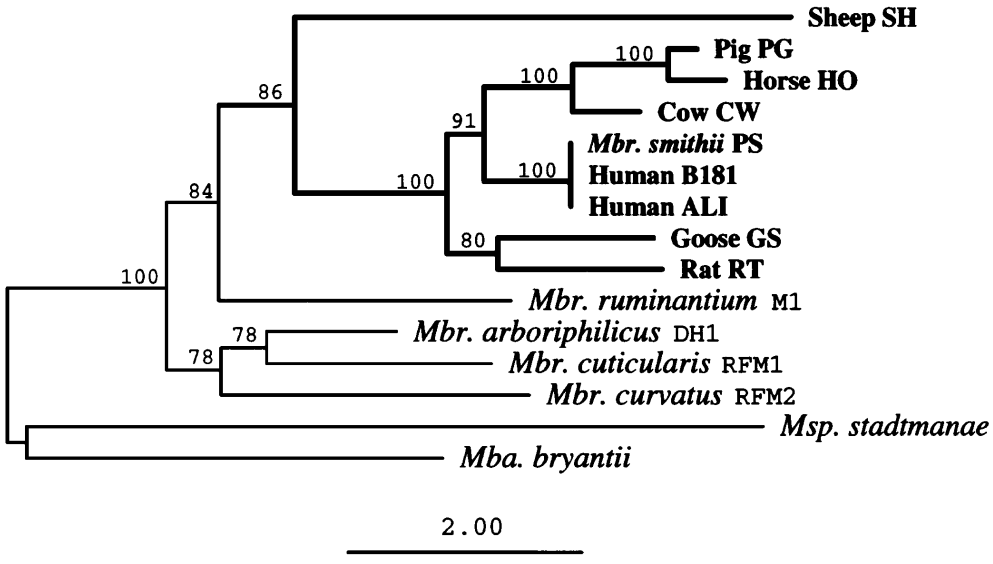

Fig. 1 Phylogenetic tree based on 16S rRNA gene comparative sequence analysis (*Mbr. Methanobrevibacter*, *Msp. Methanosphaera*, *Mba. Methanobacterium*; *bar* 2 estimated nucleotide changes per 100 positions)

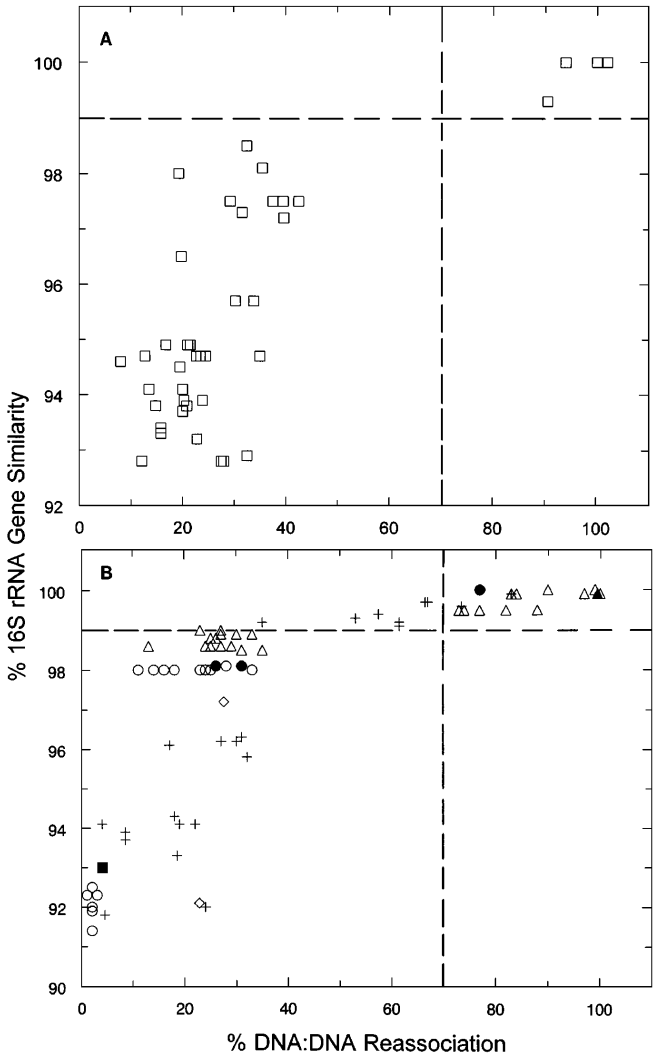

Fig. 2 A, B Comparison of 16S rRNA gene sequence similarity and genomic DNA reassociation values. The *dashed lines* indicate 70% genomic DNA reassociation and 99% 16S rRNA gene sequence similarity values. **A** *Methanobrevibacter* species and animal fecal isolates listed in Table 1. The average of reciprocal DNA reassociation values is plotted in the figure. **B** Other methanogenic archaea. DNA-DNA hybridization values are taken from the following references: △ *Methanobacterium* (Touzel et al. 1992), ▲ *Methanobacterium* (Kotelnikova et al. 1993), ○ *Methanosarcina* (Sowers et al. 1984), ● *Methanosarcina* (Maestrojuan et al. 1992), ◇ *Methanococcoides* (Franzmann et al. 1992), ■ *Methanogenium* (Xun et al. 1989), and + *Methanococcus* (Keswani et al. 1996). The 16S rRNA sequence similarity values for the data sets Δ through ■ were calculated with Jukes-Cantor correction as described in Materials and methods from comparison of sequences deposited in public domain data bases. The values for the + data set are the uncorrected 16S rRNA gene sequence similarity values provided by W. B. Whitman (University of Georgia, Athens, Ga., USA)

grouped together within the genus *Methanobrevibacter*, with a bootstrap value of 100%. However, sequence analysis revealed that sheep isolate SH formed a separate line of descent, representing a new species at a level comparable to the presently recognized species of *Methanobrevibacter*. The phylogenetic grouping of the fecal isolates with *M. smithii* was supported by bootstrap values of 86% (for all animal fecal isolates used in this study) and 100% (for all isolates excluding the sheep fecal isolate).

Genomic DNA reassociation

The genomic DNA sequences of the human fecal isolates B181 and ALI were virtually identical to *M. smithii* strain PS as assessed by DNA reassociation studies (Table 1). The genomic DNAs of isolate B181, isolate ALI, and strain PS reassociated at values greater than 94%.

The rat fecal isolate RT showed approximately 40% genomic DNA reassociation with *M. smithii* and the human fecal isolates (Table 1). It did not share any genomic similarity with either *M. ruminantium* or *M. arboriphilicus*. Its genomic similarity to the other animal isolates was not determined. The horse, pig, cow, goose, and sheep isolates shared little sequence similarity with the genomic DNA of *M. smithii* (Table 1). Their DNA reassociation values with *M. smithii* ranged from 20% for the cow isolate (CW) to 40% for the goose isolate (GS). The horse (HO) and pig (PG) isolates were the only non-human animal isolates that had a high degree of genomic DNA similarity, as shown by > 70% reassociation values.

Comparison of 16S rRNA gene sequence similarity and DNA reassociation values

The relationship between 16S rRNA gene sequence similarity and DNA reassociation values of the fecal isolates, *M. smithii*, *M. arboriphilicus*, and *M. ruminantium* is shown in Fig. 2 A. When 16S rRNA gene sequences were related at similarities greater than 99%, the genomic DNA reassociation values were greater than 70%. When 16S rRNA gene sequences were less than 99% related, the genomic DNA reassociation values were less than 50%. None of the animal fecal isolates had sufficient genomic similarity to reassociate at values between 50 and 70%. Using the public domain data bases, we also calculated the 16S rRNA sequence similarity values for methanogens with published genomic DNA reassociation values (references in Fig. 2 B). Figure 2B shows the relationship between 16S rRNA gene sequence similarity and genomic DNA similarity as reflected by reassociation values for other genera of methanogenic archaea. In most cases, > 99% 16S rRNA gene sequence similarity also reflected > 70% genomic DNA reassociation between two methanogens. Notable exceptions were *Methanococcus* pairs having greater than 99% rRNA gene sequence similarity and genomic DNA reassociation values of 53–67% or, in one case, only 35% genomic DNA reassociation (Fig. 2 B).

Discussion

Methanobrevibacter taxonomy

The phylogeny of the genus *Methanobrevibacter* was established almost 20 years ago on the basis of the similarities of the 16S rRNA oligonucleotide catalogs (Balch et al. 1979). Phenotypic differentiation of species of *Methanobrevibacter* is unsatisfactory because of the lack of distinguishing morphological, biochemical, and physiological characteristics. There are phenotypic differences between strains, e.g., bile sensitivity, formate utilization, requirements for acetate, coenzyme M, 2-methylbutyrate, and probably amino acids (Miller et al. 1986; Miller 1989; Leadbetter and Breznak 1996). These markers are useful for relating strain characteristics to strain requirements and activities in their native habitats. However, the limited number of markers and the lack of information about their distribution among strains of *Methanobrevibacter* species mandate the use of more powerful molecular tools for establishing phylogenetic relationships.

Refinements in methodology have facilitated the determination of the 16S rRNA gene sequences of methanogens. Our sequencing study of the *Methanobrevibacter* species confirms the characteristics of species identified by the analyses of oligonucleotide catalogs by Woese and colleagues (Balch et al. 1979). Application of sequence analysis to the animal fecal isolates that we studied shows that they are members of the genus and that the sheep fecal isolate is a new species.

We found that the isolate from sheep feces has only 93–94% 16S rRNA gene sequence similarity with other species of the genus and all of the other fecal isolates. It represents a separate line of descent within the genus. The original speciation of *Methanobrevibacter* based on 16S rRNA oligonucleotide catalogs corresponds to less than 95% 16S rRNA sequence similarity between species (Woese 1987). One newly described species (*M. cuticularis*) has 96.9% 16S rRNA gene sequence similarity to *M. arboriphilicus*, and another (*M. curvatus*) has 95.3% similarity to *M. arboriphilicus* (Leadbetter and Breznak 1996). The sheep fecal isolate that we studied shows low or no genomic DNA reassociation with *M. smithii* PS and the *Methanobrevibacter* isolates from horse, pig, cow, and goose feces. Based on the 16S rRNA gene sequence and genomic DNA reassociation studies, it represents a new taxon at the species level.

Using the same criteria, the isolates from horse, pig, cow, rat, and goose feces are clearly distinct from *M. arboriphilicus* and *M. ruminantium*. The 99.3% 16S rRNA gene sequence similarity for the isolates from horse and pig and their high degree of DNA reassociation indicate species identity between these two isolates from two different animals. The 97–98% 16S rRNA gene sequence similarity between these two strains, the isolates from the cow, rat, and goose, and *M. smithii* suggests a common line of descent within the genus. However, the genomic DNA reassociation studies show little genetic similarity between these animal fecal isolates and *M. smithii*. We propose that they represent new species of the genus. Based on DNA reassociation, the goose fecal isolate is a different species from the isolates from horse, pig, and cow. Similarly, the horse and pig isolates represent a species different from the cow isolate. Thus, the isolates from horse, pig, cow, and goose represent three distinct species. Studies of genomic DNA relationships between the rat strain and other animal fecal strains are necessary to decide whether the rat strain represents a distinct species.

Our study shows that the human fecal isolates ALI and B181 are virtually identical to *M. smithii* PS, with respect to both their 16S rRNA gene sequences and their genomic DNA relatedness. They differ from one another by a single base substitution in the 16S rRNA gene sequences. *M. smithii* PS was isolated from municipal anaerobic sewage contents, and it is likely that *M. smithii* PS originated from human feces. Immunological and other analyses of strains from a large number of fecal samples from different humans indicate that it is widely prevalent in humans (Miller and Wolin 1982; Miller et al. 1982; Weaver et al. 1986). Thus far it has not been isolated from the feces of other animals or from the forestomach contents of ruminants.

Relationship between 16S rRNA gene sequence similarity and genomic DNA reassociation values

Wayne et al. (1987) have proposed that phenotypically related bacterial strains (members of the domain Bacteria) with approximately 70% or greater genomic DNA relatedness constitute a single bacterial species. In contrast, those having < 70% but > 20% similarity are considered to be different species within a genus (Johnson 1984).

The relationship between genomic DNA reassociation and 16S rRNA gene sequence similarity within the domain Bacteria has been summarized by Stackebrandt and Goebel (1994). On the basis of literature values, they found that when 16S rRNA gene sequence similarity values below 97% it is unlikely that the genomic DNA of two organisms will reassociate to more than 60%. Paradoxically, they have found that two organisms can have 16S rRNA similarity values greater than 97.5% and yet have either low genomic DNA reassociation values or very high values of reassociation. Stackebrandt and Goebel have proposed that when 16S rRNA gene similarities are over 97%, genomic DNA reassociation studies are required to assess the relation of two organisms as a single or separate species.

We evaluated the relationship between genomic DNA similarity and 16S rRNA gene similarity for *Methanobrevibacter* (Fig. 2 A). We found that > 99% 16S rRNA gene sequence similarity corresponded to > 70% genomic DNA similarity as measured by DNA reassociation. The *Methanobrevibacter* that had less than 99% 16S rRNA gene sequence similarity had less than 50% DNA reassociation values. A similar relationship seems to apply to other methanogenic archaea as well (Fig. 2 B). The results de-

picted in Fig. 2 with the methanogenic archaea are consistent with the earlier analysis of the Bacteria by Stackebrandt and Goebel (1994): < 97% rRNA gene sequence similarity predicts low genomic DNA hybridization. However, it should be emphasized that only a limited number of studies of methanogenic archaea have been reported in the literature (see references in Fig. 2B) in comparison to the number of studies with Bacteria (see Stackebrandt and Goebel 1994). Since some *Methanococcus* species had > 99% rRNA gene sequence similarity but less than 70% genomic DNA reassociation, and in view of the paradox observed by Stackebrandt and Goebel in the Bacteria, it is premature to conclude that > 99% 16S rRNA gene sequence similarity always predicts high genomic DNA reassociation values between two methanogenic archaea, i.e. that the two organisms are the same species. Thus, the available information on methanogenic archaea reinforces the concept that genomic DNA reassociation is the gold standard for differentiating two organisms at the species level (Stackebrandt and Goebel 1994).

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