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Identification and Quantification of Methanogenic Archaea in Adult Chicken Ceca▽

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By using molecular methods for the identification and quantification of methanogenic archaea in adult chicken ceca, 16S rRNA genes of 11 different phylotypes, 10 of which were 99% similar to *Methanobrevibacter woesei*, were found. Methanogen populations, as assessed by cultivation, and the 16S rRNA copy number were between 6.38 and 8.23 cells/g (wet weight) and 5.50 and 7.19 $\log_{10}$/g (wet weight), respectively.

Methanogens, members of the domain *Archaea*, have been isolated from various animals (13, 14). For avian species, only one report regarding the isolation of methanogens from chicken, goose, and turkey feces exists (13). Based on cell wall composition, however, the strains isolated from chicken and turkey feces appeared to belong to the genus *Methanogenium* (11). Analysis of the 16S rRNA genes of the methanogens from chicken and turkey feces have not been reported. In the present study, methanogens were identified by using 16S rRNA gene clone libraries. In addition, methanogens in these cecal contents were quantified by a most probable number (MPN) method and by real-time PCR.

Ceca were obtained from 56- to 72-week-old female leghorn chickens maintained on a layer ration described previously (3). Twenty-five chickens were divided into five groups and were sacrificed to remove their cecal contents. Ceca from each group were pooled in an anaerobic glove box and were designated samples 1 through 5. Samples for PCR analysis were isolated directly from the pooled ceca after introduction into an anaerobic hood (Coy Laboratory Products, Grass Lake, MI). Tenfold dilutions were inoculated into five serum tubes containing Balch 1 medium supplemented with rumen fluid and additional NH_4Cl (1, 12). Each tube was flushed with 80% H_2–20% CO_2 under 200 kPa of pressure. The tubes were incubated while stationary at 37°C and mixed once per day manually. After 20 days, the level of methane in the headspace gas was determined with a gas chromatograph (model 8610C; SRI, Torrance, CA). Tubes with methane concentrations greater than 100 ppm (μg/ml) were counted positive for the determination of methanogens by the MPN method. The freeware MPN calculator (VB6 version; Michael Curiale [http://www.i2workout.com/mcuriale/mpn/index.html]) was used to calculate the MPN. In this study, fresh bovine rumen fluid was used as a positive control. It was collected from a cannulated Holstein-Friesian cow maintained on a 50% alfalfa hay–50% flaked-corn diet. The cecal samples were stored at −80°C until DNA extraction. Microbial genomic DNA was isolated by the method of Wright et al. (25) with some modifications. The DNA solution was stored at −20°C.

Methanogenic 16S rRNA genes from five cecal samples were amplified using the methanogen-specific forward and reverse primers Met86F and Met1340R (26). The PCR conditions and cloning protocol followed the protocol described by Wright et al. (27). The PCR products from 420 clones were digested with HaeIII (Promega, Madison, WI). Clones representing all HaeIII restriction fragment length polymorphism patterns were bidirectionally sequenced with ABI Prism BigDye primer cycle sequencing kits (Applied Biosystems, Foster City, CA).

The 16S rRNA sequences from this study were used to query GenBank. To place these sequences within a phylogeny of representative methanogenic archaea, some 16S rRNA gene sequences from GenBank were included in the analysis. The alignment was generated with ClustalW (23). The neighbor joining tree was constructed using Phylogenetic Analysis Using Parsimony and Other Methods software (PAUP* 4.0b) (21) employing a distance matrix calculated with the Jukes-Cantor correction model. The tree was subjected to 1,000 replicates of bootstrapping, and the percentages of replicates supporting a given node are indicated in Fig. 1.

Calibration standards for the quantitative PCR assays were developed with a 10-fold dilution series of plasmid containing sequence CH101. The plasmid copy number was calculated from the plasmid molecular weight, and the plasmid concentration was measured with Picogreen (Molecular Probes, Eugene, OR) using a Spectrafluor Plus microtiter plate reader (Tecan, Inc., Research Triangle Park, NC). The quantitative PCRs using primers MBT857F and MBT1196R and TaqMan probe MBT929F were described previously by Yu et al. (28). The assays were performed in triplicate with two PCRs. Results are presented as means ± standard deviations.

In our study, 11 phylotypes were observed among the 420

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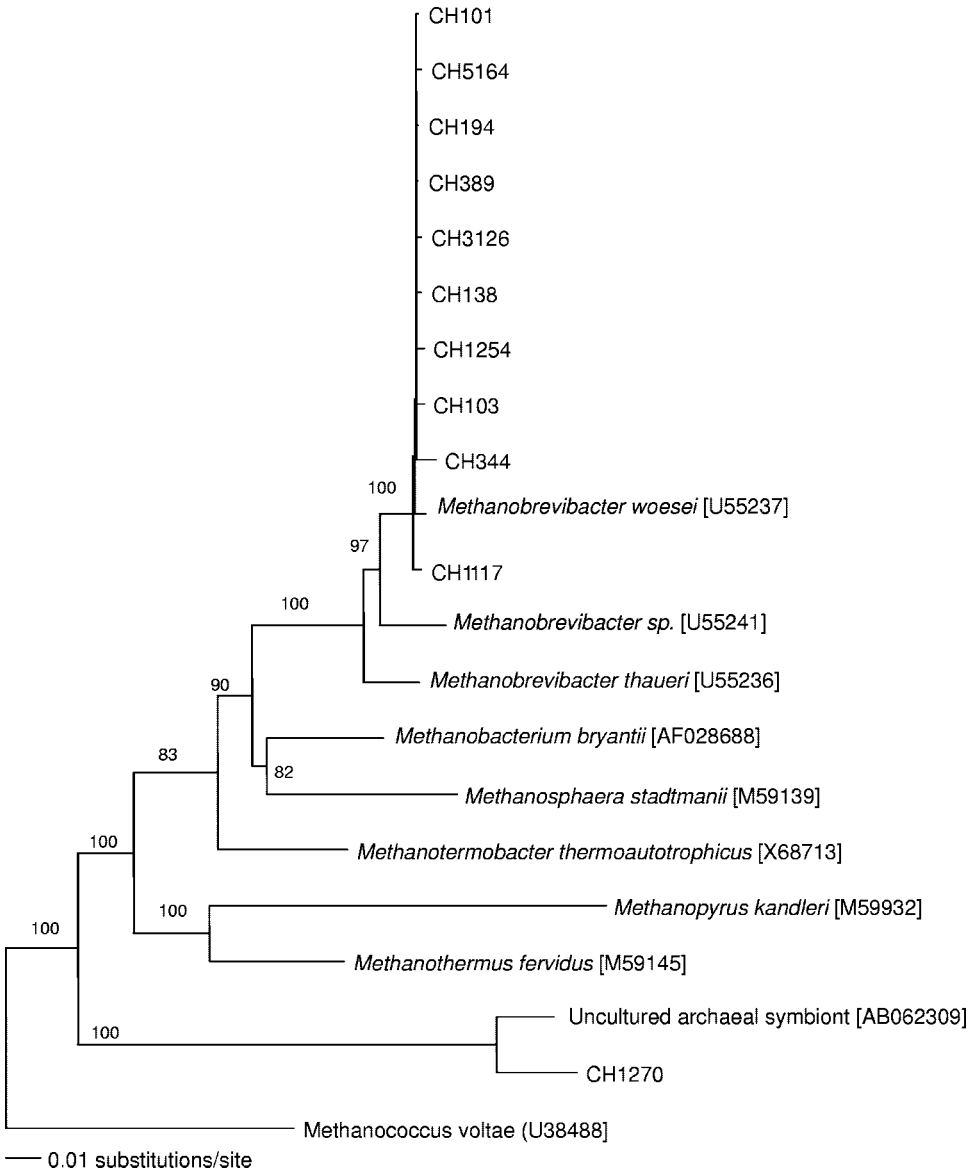

FIG. 1. Phylogeny of partial 16S rRNA sequences from chicken ceca placed within the context of several methanogenic species within the *Archaea*. Sequences harvested from GenBank are followed by accession numbers in brackets. The scale bar represents the nucleotide substitution rate. The *Methanococcus voltae* (U38488) sequence served as the outgroup.

clones. Of the total number of clones, 406 belonged to phylotype CH101, accounting for 92.86 to 100% of the total clone libraries in five samples, while the other phylotypes consisted of only 1 or 2 clones each (Table 1). Despite our finding of 11 different sequences in chicken ceca, sequence identity data show that all of the sequences, except sequence CH1270, were 98.97 to 99.45% similar to the 16S rRNA sequence of *Methanobrevibacter woesei* GS (U55237), a methanogen isolated from goose feces (13). Sequence CH1270 had 97.62% sequence identity to an uncultured archaeon, clone ConP1-11F (GenBank accession number AY911630.1). However, phylotype CH1270 was not identifiable to the species level.

Primers Met86F and Met1340R were designed from the conserved region of the 16S rRNA genes from 82 methanogens and can amplify 26 diverse strains of methanogens (26); therefore, these primers were used in this experiment. Factors such as the number of mismatches, the location of the mismatches, and the primer location in relation to secondary structures influence primer specificity (19). There was one mismatched base pair between the 16S rRNA genes of *M. woesei* (U55237) and primer Met1340R. However, primers Met86F and 1340R exactly match the 16S rRNA genes of *Methanogenium cariaci* (M59130) and *Methanogenium organophilum* (M59131). According to Skillman et al. (19), these primers have more specificity to *Methanogenium cariaci* and *Methanogenium organophilum* than to *Methanobrevibacter woesei* GS. However, *Methanogenium*-related sequences were not found in this study. Even though our approach is more accurate than the cultural methods, it still has a bias in amplification and formation of chimeric molecules (24). In addition, these primers may

TABLE 1. The 420 clones of 16S rRNA genes obtained in this study

16S rRNA phylotype	No. of clones[a]	Size (bp)	GenBank accession no.	Nearest taxon	% Sequence identity
CH101	406 (96.67)	1,266	DQ445715	*Methanobrevibacter woesei* GS (U55237)	99.21
CH103	2 (0.48)	1,264	DQ445725	*Methanobrevibacter woesei* GS (U55237)	99.21
CH138	2 (0.48)	1,268	DQ445716	*Methanobrevibacter woesei* GS (U55237)	98.97
CH194	1 (0.24)	1,266	DQ445722	*Methanobrevibacter woesei* GS (U55237)	99.21
CH1117	2 (0.48)	1,266	DQ445717	*Methanobrevibacter woesei* GS (U55237)	98.97
CH344	1 (0.24)	1,263	DQ445720	*Methanobrevibacter woesei* GS (U55237)	98.97
CH389	1 (0.24)	1,263	DQ445719	*Methanobrevibacter woesei* GS (U55237)	99.45
CH3126	1 (0.24)	1,263	DQ445724	*Methanobrevibacter woesei* GS (U55237)	99.29
CH5164	1 (0.24)	1,262	DQ445721	*Methanobrevibacter woesei* GS (U55237)	99.13
CH1254	2 (0.48)	1,264	DQ445718	*Methanobrevibacter woesei* GS (U55237)	99.05
CH1270	1 (0.24)	1,256	DQ445723	Uncultured archaeon clone ConP1-11F (AY911630.1)	97.62

[a] Percentages of methanogens are given in parentheses for all 420 clones examined in this study.

not recover sequences from some archaea existing in gastrointestinal tracts. *Thermoplasmales*- and *Crenarchaeota*-associated sequences have been observed in the gastrointestinal tracts of various animals (5, 8, 10, 17–19, 22).

Clearly, phylogenetic analysis supported the conclusion that the predominant methanogenic species found in chicken ceca is *Methanobrevibacter woesei*, while Miller et al. (13) isolated *Methanogenium* spp. from chicken and turkey feces. It is not surprising that all sequences, with the exception of sequence CH1270, were very closely related to *M. woesei* GS, as shown in Fig. 1. This is because previous studies have indicated that the primary methanogens in animal intestinal tracts belong to the genus *Methanobrevibacter* (9). *Methanogenium* spp., however, originate from aquatic environments (16). In contrast to that in ruminant animals, methanogen diversity in nonruminants appears to be minimal. In this study, 11 phylotypes were observed in chicken ceca while 65 phylotypes were identified in sheep rumen (27). Eckburg et al. (4) found that all 1,524 archaeal sequences in human intestinal tracts belonged to *Methanobrevibacter smithii*.

The numbers of methanogens quantified in the bovine rumen fluid sample and the four cecal samples are shown in Table 2. Based on an MPN enumeration, the methanogen population in bovine rumen fluid was found to be 7.15 $\log_{10}$ cells/ml. The number of methanogens in the rumen fluid was similar to that found in previous studies (15, 20). The number of methanogens in chicken ceca closely resembled that in both horse and pig ceca and ranged from 4 to 6 $\log_{10}$ cells/g (wet weight) and 6.78 16S rRNA copies/g (wet weight) (2, 15).

MBT primers (28), designed specifically for the order *Methanobacteriales*, were used to quantify the methanogen population. The mean$\log_{10}$ 16S rRNA copy numbers per gram (wet weight) of cecum and the corresponding standard deviations are shown in Table 2. We found that the 16S rRNA copy number per gram (wet weight) in the samples was between 5.50 and 7.19 $\log_{10}$. The results of this experiment revealed that the 16S rRNA copy number in four samples, particularly samples 4 and 5, were similar to the numbers estimated by the MPN method. The $\log_{10}$ 16S rRNA copy numbers per gram (wet weight) in samples 2 and 3 were lower than the lower numbers of the 95% confidence limits enumerated by the MPN method used in this study. Our results show that methanogens in chicken ceca potentially have 1 16S rRNA copy per cell. In general, all methanogens have only one or two 16S rRNA genes (6). However, a recent study showed that the genome sequence of *Methanosphaera stadtmanae* contains four copies of 16S rRNA genes (7).

In conclusion, the results of culture-independent approaches and MPN enumeration show that the methanogen community is less diverse and that *Methanobrevibacter woesei* is the predominant methanogen in chicken ceca. The population levels

TABLE 2. Numbers of methanogenic archaea in bovine rumen fluid and chicken cecal contents

Sample[a]	MPN results[b]			No. of 16S rRNA copies/g (wet wt) determined by real-time PCR[c]	Reference
	No. of cells/g (wet wt) or no. of cells/ml of bovine rumen fluid	95% Confidence limit			
		Lower	Upper		
Bovine rumen fluid	5–8	NK[d]	NK	NK	15
Bovine rumen fluid	6–8	NK	NK	NK	20
Bovine rumen fluid	7.15	6.72	7.58	ND[e]	This study
Chicken cecal sample 2 (6 birds)	6.45 (7.08)	6.00	6.87	5.50 ± 0.11	This study
Chicken cecal sample 3 (6 birds)	8.23 (8.88)	7.82	8.67	7.19 ± 0.09	This study
Chicken cecal sample 4 (6 birds)	6.73 (7.36)	6.23	7.23	6.76 ± 0.08	This study
Chicken cecal sample 5 (6 birds)	6.38 (7.04)	5.96	6.81	6.78 ± 0.12	This study

[a] The numbers of methanogenic archaea in all cecal samples, except sample 1, were quantified by using the MPN method and real-time PCR.
[b] Values in parentheses are $\log_{10}$ MPNs per gram (dry weight).
[c] Values are mean $\log_{10}$ 16S rRNA copy number per gram (wet weight) ± standard deviation.
[d] NK, not known.
[e] ND, not determined.

of methanogenic archaea inhabiting this ecosystem are similar to those in other domestic animals studied.

Nucleotide sequence accession numbers. The sequences obtained in this study are available in GenBank under accession numbers DQ445715 to DQ445725.

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