

Abstract

Identification and quantification of methanogenic archaea in adult chicken ceca by molecular methods detected eleven different 16S rRNA genes, ten of which were 99% similar to *Methanobrevibacter woesei*. Methanogen populations, as assessed by cultivation, and the 16S rRNA copy number were between 6.38 to 8.23 and 5.50 to 7.19 log₁₀ per gram wet weight, respectively.

Short-Form Paper

Methanogens, members of the domain *Archaea*, have been isolated from various animals (13-14). For avian animals, only one report exists regarding the isolation of methanogens from chicken, goose, and turkey feces (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 these two methanogens has not been reported. In the current study, methanogens were identified by using 16S rRNA gene clone libraries. In addition, methanogens in these cecal contents were quantified by a MPN method, and by real-time PCR.

Ceca were obtained from female Leghorn 56 to 72-week chickens maintained on a layer ration described previously (3). Twenty-five chickens were divided into five groups and were sacrificed to remove cecal contents. Ceca from each group were pooled in an anaerobic glove box and were designated as samples 1 through 5. Samples for PCR analysis were isolated directly from the pooled ceca after introduction into the hood. Ten-fold dilutions were inoculated into five serum tubes containing BRN medium (1, 12). Each tube was flushed with 80% H₂/ 20% CO₂ under 200 kPa. The tubes were incubated standing at 37 °C and mixed once per day manually. After 20 days, methane was

1 determined in the headspace gas by GC (SRI, model 8610C, Torrance, CA). Tubes with
2 methane concentrations greater than 100 ppm ($\mu\text{g/ml}$) were counted positive for the
3 determination of methanogens by MPN. The freeware MPN calculator (VB6 version;
4 Michael Curiale [members.ync.net/mcuriale/mpn/index.html]) was used to calculate the
5 MPN. In this study, fresh bovine rumen fluid was used as a positive control. It was
6 collected from a cannulated Holstein-Friesian cow maintained on a 50 % alfalfa hay, 50
7 % flaked corn diet. The cecal samples were stored at $-80\text{ }^{\circ}\text{C}$ until DNA extraction.
8 Microbial genomic DNA was isolated by the method of Wright et al. (25) with some
9 modifications. The DNA solution was stored at $-20\text{ }^{\circ}\text{C}$.

10 Methanogenic 16S rRNA genes from five cecal samples were amplified using
11 methanogen-specific forward and reverse primers Met86F and Met1340R (26). The PCR
12 conditions and cloning protocol followed the protocol of Wright et al. (27). The PCR
13 products from 420 clones were digested with *Hae*III (Promega, Madison, WI). Clones
14 representing all *Hae*III restriction fragment length polymorphism patterns were
15 bidirectionally sequenced with ABI Prism® BigDye® Primer Cycle Sequencing kits
16 (Applied Biosystems, Foster City, CA).

17 The 16S rRNA sequences from this study were used to query Genbank. To place
18 these sequences within a phylogeny of representative methanogenic archaea, some 16S
19 rRNA gene sequences from Genbank were included in the analysis. The alignment was
20 generated with ClustalW (23). The neighbor joining tree was constructed in Phylogenetic
21 Analysis Using Parsimony and Other Methods (PAUP* 4.0b) (21) employing a distance
22 matrix calculated with the Jukes-Cantor correction model. The tree was subjected to 1000
23 replicates of bootstrapping, the percentage of replicates supporting a given node are

1 indicated on Fig. 1. The sequences obtained in this study are available in GenBank under
2 accession numbers DQ445715 to DQ445725.

3 Calibration standards for the quantitative PCR assays were developed with a 10-
4 fold dilution series of plasmid containing sequence CH101. Plasmid copy number was
5 calculated from plasmid molecular weight, and plasmid concentration was measured with
6 Picogreen (Molecular Probes, Eugene, OR) using a Spectrafluor Plus (Research Triangle
7 Park, NC). The quantitative PCR reactions using primers MBT857F, MBT1196R, and
8 TaqMan probe MBT929F are described in Yu et al. (28). The assays were triplicates in
9 two PCR reactions. Results are presented as the mean \pm standard deviations.

10 In our study, eleven phlotypes were observed from the total 420 clones. From the
11 total clones, 406 clones belonged to phylotype CH101, accounting for 92.86 to 100 % of
12 the total clone libraries in five samples, while the other phlotypes consisted of only 1 or
13 2 clones (Table 1). Despite finding eleven different sequences in chicken ceca, sequence
14 identity data show that all of the sequences, except sequence CH1270, were 98.97 to
15 99.45 % similar to the 16S rRNA sequence of *M. woesei* GS (U55237), a methanogen
16 isolated from goose feces (13). Sequence CH1270 had a 97.62 % sequence identity to an
17 uncultured archaeon clone ConP1-11F (AY911630.1). However, phylotype CH1270 was
18 not identifiable to the species level.

19 Primers Met86F and Met1340R were designed from the conserved region of the
20 16S rRNA genes from 82 methanogens and can amplify 26 diverse strains of
21 methanogens (26), therefore, these primers were used in this experiment. Factors, such as
22 the number of mismatches, location of the mismatches and primer location in relation to
23 secondary structures, influence primer specificity (19). There was one mismatched base

1 pair between 16S rRNA gene of *M. woesei* (U55237) and primer Met1340R. However,
2 primers Met86F and 1340R exactly match with the 16S rRNA genes of *Methanogenium*
3 *cariaci* (M59130) and *Methanogenium organophilum* (M59131). According to Skillman
4 et al. (19), these primers have more specificity to *Methanogenium cariaci* and
5 *Methanogenium organophilum* than *M. woesei* GS. However, *Methanogenium*-related
6 sequences were not found in this study. Even though this approach is more accurate than
7 the cultural methods, it still has a bias in amplification and formation of chimeric
8 molecules (24). In addition, these primers may not recover sequences from some archaea
9 existing in gastrointestinal tracts. *Thermoplasmatales*- and *Crenarchaeota*-associated
10 sequences have been observed in gastrointestinal tracts of various animals (5, 8, 10, 17-
11 19, 22).

12 Clearly, phylogenetic analysis supported the conclusion that the predominant
13 methanogenic species found in the chicken ceca is *M. woesei* while Miller et al. (13)
14 isolated *Methanogenium* spp. from chicken and turkey feces. It is not surprising that all
15 sequences were very closely related to *M. woesei* GS with the exception of sequence
16 CH1270 as shown in Fig. 1. This is because previous studies have indicated that the
17 primary methanogens in animal intestinal tracts belong to the genus *Methanobrevibacter*
18 (9). *Methanogenium* spp., however, originate from aquatic environments (16). In
19 contrast to ruminant animals, methanogen diversity in non-ruminants appears to be
20 minimal. In this study, 11 phylotypes were observed in chicken ceca while 65 phylotypes
21 were identified in sheep rumen (27). Eckburg et al. (4) found that all 1524 archaeal
22 sequences in human intestinal tracts belonged to *M. smithii*.

1 The number of the methanogens quantified in the bovine rumen fluid sample and
2 the four cecal samples are shown in Table 2. Based on a MPN enumeration, the
3 methanogen population in bovine rumen fluid was found to be 7.15 log₁₀ cells/ml. The
4 number of methanogens in the rumen fluid was similar to those found in previous studies
5 (15, 20). The number of methanogens in chicken ceca closely resembled that in both
6 horse and pig ceca and ranged from log₁₀ of 4 to 6 and 6.78 per gram wet weight,
7 respectively (2, 15).

8 MBT primers (28), designed specifically for the order *Methanobacteriales*, were
9 used to quantify the methanogen population. The means of log₁₀ 16S rRNA copy number
10 per gram wet weight cecum with the corresponding standard deviations are shown in
11 Table 2. We found that the 16S rRNA copy number per gram wet weight in the samples
12 was between log₁₀ 5.50 to 7.19. The results of this experiment revealed that the copy
13 number of 16S rRNA in four samples, particularly samples 4 and 5, were similar to the
14 numbers estimated by MPN. The log₁₀ 16S rRNA copy number per gram wet weight in
15 samples 2 and 3 was less than the lower numbers of 95 % confidence limits enumerated
16 by the MPN method used in this study. Our results show that methanogens in chicken
17 ceca potentially have one 16S rRNA copy per cell. In general, all methanogens have only
18 one or two 16S rRNA genes (6). However, a recent study showed that the genome
19 sequence of *Methanosphaera stadtmanae* contains 4 copies of 16S rRNA genes (7).

20 In conclusion, by using culture independent approaches and MPN enumeration,
21 the results show that the methanogen community was less diverse and *M. woesei* was the
22 predominant methanogen in chicken ceca. The population levels of methanogenic

1 archaea inhabiting this ecosystem were also similar to those in the other domestic
2 animals.

3 We thank Meyer J. Wolin, Terry Miller, and Egidio Currenti of Wadsworth
4 Center for providing *M. woesei* GS and technical assistance. We are grateful to Ann
5 Marie Prazak of United States Department of Agriculture, Southern Plains Agricultural
6 Research Center for supplying bovine rumen fluid. We appreciate the critical comments
7 of Timothy Kral from the Department of Biological Sciences, the University of Arkansas.
8 This research was supported by Hatch grant H8311 administered by the Texas
9 Agricultural Experiment Station, USDA-NRI grant number 2002-02614 and U.S. Poultry
10 and Egg Association grant #485.

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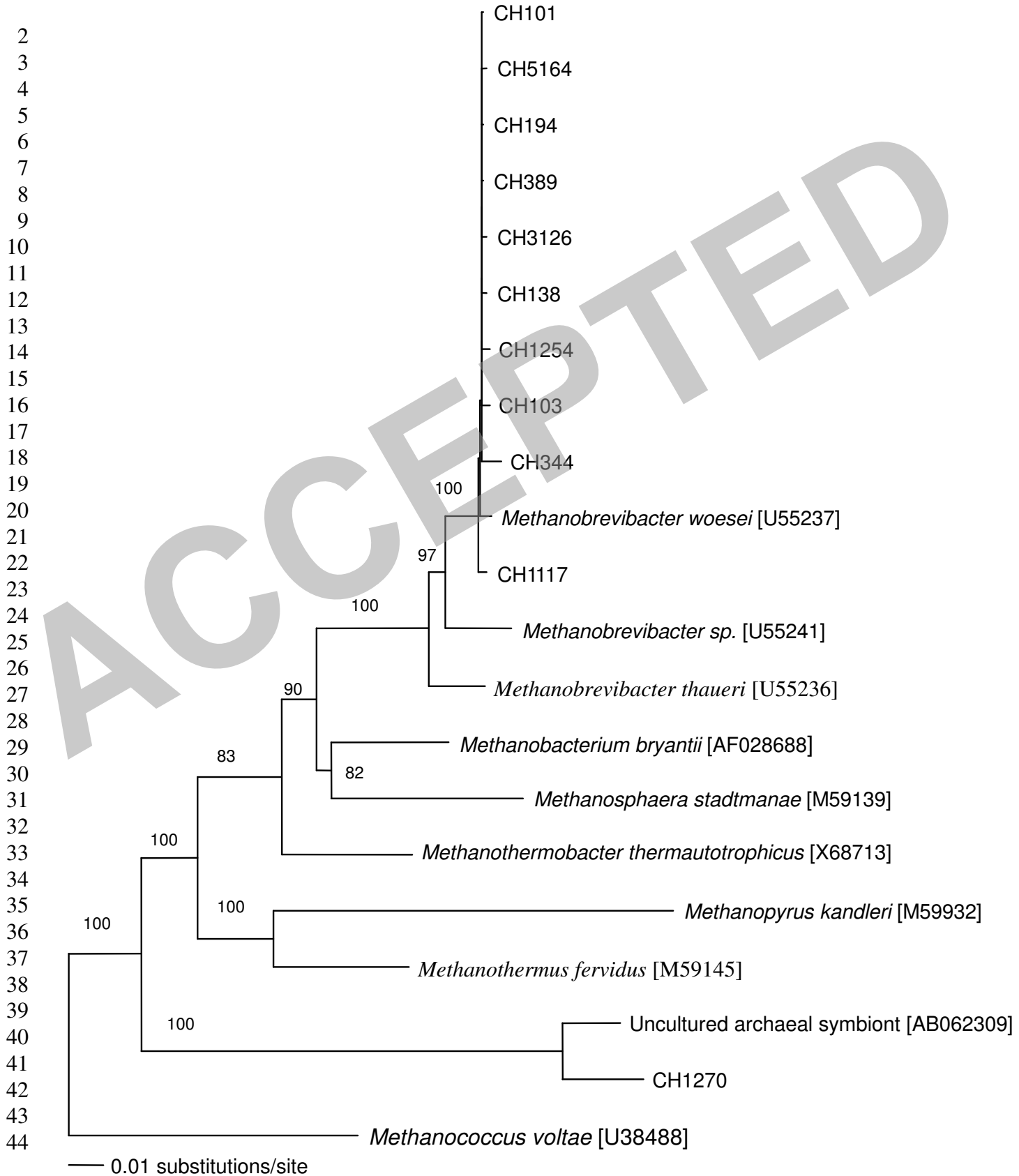
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1 **Figure 1.** Phylogeny of partial 16S rRNA sequences from chicken ceca placed within the
2 context of several methanogenic species within the *Archaea*. Sequences harvested from
3 Genbank are followed by accession numbers in brackets. The scale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

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1 **Table 1.** The 420 clones of 16S rRNA genes obtained in this study.
 2
 3

16S rRNA phylotype	No. of clones	Size (bp)	GenBank accession no.	Nearest taxon	% Sequence identity
CH101	406 (96.67) ^a	1266	DQ445715	<i>Methanobrevibacter woesei</i> GS (U55237)	99.21
CH103	2 (0.48)	1264	DQ445725	<i>Methanobrevibacter woesei</i> GS (U55237)	99.21
CH138	2 (0.48)	1268	DQ445716	<i>Methanobrevibacter woesei</i> GS (U55237)	98.97
CH194	1 (0.24)	1266	DQ445722	<i>Methanobrevibacter woesei</i> GS (U55237)	99.21
CH1117	2 (0.48)	1266	DQ445717	<i>Methanobrevibacter woesei</i> GS (U55237)	98.97
CH344	1 (0.24)	1263	DQ445720	<i>Methanobrevibacter woesei</i> GS (U55237)	98.97
CH389	1 (0.24)	1263	DQ445719	<i>Methanobrevibacter woesei</i> GS (U55237)	99.45
CH3126	1 (0.24)	1263	DQ445724	<i>Methanobrevibacter woesei</i> GS (U55237)	99.29
CH5164	1 (0.24)	1262	DQ445721	<i>Methanobrevibacter woesei</i> GS (U55237)	99.13
CH1254	2 (0.48)	1264	DQ445718	<i>Methanobrevibacter woesei</i> GS (U55237)	99.05
CH1270	1 (0.24)	1256	DQ445723	Uncultured archaeon clone ConP1-11F (AY911630.1)	97.62

4
 5 ^a Percentage of methanogens in parenthesis for all 420 clones examined in this study.

1 **Table 2.** The log₁₀ numbers of methanogenic archaea in bovine rumen fluid and chicken
 2 cecal contents.
 3

Sample	cells/ g wet wt. (or ml. bovine rumen fluid)	MPN		16S rRNA copy number/ g wet wt.	Reference
		95 % Confidence limits			
		Lower	Upper		
Bovine rumen fluid	5 to 8	NK ^a	NK	NK	15
Bovine rumen fluid	6 to 8	NK	NK	NK	20
Bovine rumen fluid	7.15	6.72	7.58	ND ^b	This study
Chicken cecal sample 2 ^c (6 birds)	6.45 (7.08) ^d	6.00	6.87	5.50 ± 0.11 ^e	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

4
 5 ^a NK, not known.

6 ^b ND, not determined.

7 ^c All cecal samples, except sample 1, were quantified the numbers of methanogenic
 8 archaea by using the MPN method and real-time PCR.

9 ^d Log₁₀ MPN per gram dry weights are given in parentheses.

10 ^e The mean of log₁₀ 16S rRNA copy number per gram wet weight and standard
 11 deviations.

12
 13