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ORIGINAL ARTICLE

Rumen-like methanogens identified from the crop of the folivorous South American bird, the hoatzin (*Opisthocomus hoazin*)

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The hoatzin is the only known avian species with foregut fermentation. It is a primarily folivorous feeder and has a distended crop and lower/distal esophagus, which has evolved for the microbial fermentation of ingested feed. Crop samples collected from 10 individual animals from the Apure River area, Apure State, Venezuela were examined for the presence and density of methanogens using 16S rRNA gene clone libraries and real-time PCR prepared from pooled and individual PCR products. A total of 197 clones were examined, revealing 24 different methanogen 16S rRNA sequences, or phylotypes. Of the 24 unique phylotypes, 16 (171 of 197 clones) formed five unique clades within the genus *Methanobrevibacter* with the largest group of clones (118 clones) 98.7% similar to *Methanobrevibacter ruminantium*. The remaining eight phylotypes (26 clones) formed four unique clades that had only 94.0–96.7% identity to *Methanosphaera stadtmanae*. Based upon 98% sequence identity, we identified 17 of the 24 methanogen phylotypes from the hoatzin as possible new species and strains, with three phylotypes representing possible new genera (<94.5% sequence identity). Although none of the hoatzin methanogen phylotypes had 100% sequence identity to any other archaeal sequences in the GenBank database, the hoatzin crop methanogen sequences formed sister groups with known rumen methanogens. Mean population densities (numbers per gram wet weight) of methanogenic archaea, rumen bacteria and ciliate protozoa, estimated using real-time PCR, were 5.80×10^9, 7.93×10^{12} and 3.31×10^5, respectively. The crop microbial data presented here provide an excellent example of convergent evolution of foregut fermentation in the hoatzin, similar to that of ruminants.
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Introduction

There are approximately 9000 species of birds within the Class Aves, but only one species, the hoatzin (*Opisthocomus hoazin*), has evolved a foregut fermentation system that is similar to that of ruminant animals. The hoatzin is a neotropical, obligate folivorous feeder, weighing approximately 700–750 g, that inhabits the low-land riverine swamps and gallery forests of South America, from Guyana and Brazil, north to Venezuela, and west to Ecuador and Bolivia. It has an unfeathered blue face with maroon eyes and a spiky crest on its head, and the sole extant species within the family Opisthocomidae. It is often considered one of the most primitive of birds as the hoatzin chick has two claws on the first and second digits of their wings to help it grip branches and clamber about awkwardly. The taxonomic position of this enigmatic bird has been greatly debated and still cannot be confidently placed (Hackett *et al.*, 2008).

In other avian species, food is broken up in the gizzard, but in the hoatzin, its crop and lower/distal esophagus have functionally evolved into large, well-developed, fermentation structures, comprising about 70% of the digestive tract and weighing up to 17.7% of the animal's total mass. As a result, the muscular crop has displaced the flight muscles and keel of the sternum, making the hoatzin a poor flyer (Grajal *et al.*, 1989). The increased size of the foregut allows a long retention of the food and microbial digestion of the folivorous diet. As a result, mean retention times for the hoatzin are among the longest recorded for a bird (Grajal *et al.*, 1989), and digestion efficiencies are very similar to those of ruminant animals (Grajal and Parra, 1995). Short-chain fatty acids, a by-product of microbial fermentation, are also

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present in the crop in high concentrations similar to that of ruminants.

Like ruminants, the hoatzin has evolved a complex gut microbial community that includes bacteria, methanogenic archaea and protozoa to coordinate plant biomass breakdown, with methane gas produced as a by-product. Because of the evolutionary distance between the Aves and the Artyodactyla, the hoatzin is an excellent example of convergent evolution of foregut fermentation, and provides an excellent opportunity to study if the analogous function of the crop and the rumen leads to similar microbial diversity. A recent study by Godoy-Vitorino *et al.* (2008) characterized the crop's bacterial population in six wild adult hoatzin birds and reported that 94% of the 580 16S rRNA phylotypes recovered were novel species.

Information on methanogens from the hoatzin is limited with one conference abstract reporting just two partial 16S rRNA sequences (<625 bp), one belonging to the genus *Methanobrevibacter* and the other to the genus *Methanosphaera* (Garcia-Amado *et al.*, 2007). However, methanogens have been reported from other avian species. *Methanobrevibacter woesei* was isolated from enrichments of goose feces (Miller *et al.*, 1986; Miller and Lin, 2002) and was also the predominant methanogen in chicken ceca (Saengkerdsub *et al.*, 2007). *Methanogenium*-like strains also appear to have been reported from chicken and turkey feces, but no sequence data were reported (König, 1986; Miller *et al.*, 1986).

The objectives of the present study were to construct a 16S rRNA gene library to elucidate the composition of the methanogen population in the crop of the hoatzin; to test the hypothesis that *Methanobrevibacter* phylotypes are likely to be the most dominant component in the hoatzin 16S rRNA gene clone library; to determine if the methanogens present in the hoatzin crop are more closely related to methanogens from other avian species, or more similar to methanogens from ruminants and to use real-time PCR to determine the density of methanogens, bacteria, and protozoa in the crops of individual animals.

Materials and methods

Source of samples

A permit (Licencia de Caza con fines cientificos) to capture hoatzins was obtained from the Viceministerio de Ordenación y Administración Ambiental Oficina Administrativa De Permisiones, in Venezuela, before commencement of the research. Ten wild adult hoatzins were killed in the Apure River region, Apure State, Venezuela (6° 55.6′ N, 68° 31.5′ W) by a professional shooter. After slaughter, the contents of each crop were immediately measured and fixed with two times the sample volume with 100% ethanol, and stored at room temperature in a 100 ml storage vial for transport. Information on age and sex of individuals is unavailable.

DNA extraction, clone library construction and sequencing

DNA was extracted using a cetyltrimethylammonium bromide method described elsewhere (Wright *et al.*, 1997), and PCR-amplified with methanogen-specific primers Met86F and Met1340R (Wright and Pimm, 2003), using an iCycler (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia). The following conditions were used: 4 min hot start at 94 °C, followed by denaturation for 35 s at 94 °C, annealing for 35 s at 58 °C and 2 min of extension at 72 °C. On the 35th and final cycle, extension time was increased to 10 min.

A 16S rRNA gene library was constructed from the pooled PCR product from each animal using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). All positive clones were selected and suspended into 100 μl of Milli-Q water. Approximately, 10 μl of this suspension was lysed at 80 °C for 4 min and added to a PCR mastermix containing the primers Met86F and Met1340R. PCR amplification continued according to the conditions described above and the resulting PCR products were digested with the enzyme *Hae*III according to the manufacturer's instructions. Digested products were run on a 4% molecular screening agarose (Roche Diagnostics, Australia Pty Ltd, Castle Hill, NSW, Australia) and bands were visualized using the Bio-Rad Gel Doc 2000 gel documentation system.

The resulting restriction fragment length polymorphisms were initially grouped together according to their riboprint patterns and up to seven representatives from each unique pattern were sequenced in both directions using four internal 16S primers (Met448F, Met102F, Met448R and Met1027R) (Wright and Pimm, 2003). Sequencing was performed using the AB genetic analyzer 3130 XL platform (Applied Biosystems, Melbourne, VIC, Australia) with big dye terminator and *Taq*FS matrix. Sequences were proofread and confirmed in both directions and all ambiguities were removed. The online chimeric detection program Bellerophon (Huber *et al.*, 2004) was used to identify if any chimeric sequences were present in the library.

Distance data were generated from the clone library using the Kimura (1980) two-parameter model and analyzed using the computer program DOTUR (Schloss and Handelsman, 2005) to group clone sequences into operational taxonomic units (OTU), based on a 98% sequence identity cutoff. Clones were designated 'HZ' to indicate hoatzin, followed by the clone number.

Phylogenetic reconstruction

Seventy additional 16S rRNA gene sequences representing all the methanogen orders and other

major lineages within the Euryarchaeota were included in the phylogenetic analysis, and three members of the Crenarchaeota (*Pyrolobus fumarius*, *Sulfolobus acidocaldarius* and *Thermosphaera aggregans*) were used as the outgroup. All sequences were globally aligned using the Dedicated Comparative Sequence Editor program (de Rijk and de Wachter, 1993) and further refined manually based on the secondary structure model. PHYLIP (version 3.62C; Felsenstein, 2004) was used to construct a neighbor-joining tree (Saito and Nei, 1987), which was bootstrap resampled 1000 times.

Real-time PCR analysis
Each crop sample was analyzed using real-time PCR to estimate the number of methanogens (using mcrA genes), bacteria (using 16S rRNA genes) and ciliate protozoa (using 18S rRNA genes). The external standards used for the real-time PCR amplifications have been previously validated for bacteria (Denman and McSweeney, 2006), ciliate protozoa (Sylvester *et al.*, 2004) and methanogenic archaea (Denman *et al.*, 2007), and have been discussed elsewhere (Sundset *et al.*, 2009). Real-time PCR amplifications were carried out with the Bio-Rad iCycler in a 25 µl volume containing the following reagents: 1.0 µl template DNA (10 ng), 400 nM (final concentration) of each primer, 12.5 µl iQ SYBR Green supermix (Bio-Rad) and 9.5 µl ddH_2O. Real-time PCR amplification was initiated by a hot start at 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s. A final melting curve analysis was carried out by continuously monitoring fluorescence between 60 and 95 °C with 0.5 °C increments every 10 s. Three dilutions of DNA were amplified and the C_t of the most efficient PCR was recorded.

Nucleotide sequence accession numbers
The sequences from this study have been deposited in the GenBank database under the accession numbers EU547210–EU547233.

Results

Sequence examination and phylogenetic analysis of clones
A total of 197 clones were examined, revealing 24 unique sequences, or phylotypes (Table 1). Of these, 12 phylotypes represented by 166 clones had 96.7–98.7% sequence identity to *Methanobrevibacter ruminantium*, eight phylotypes represented by 26 clones had 94.0–96.7% sequence identity to *Methanosphaera stadtmanae* whereas four phylotypes, represented by only five clones, had 94.3–96.3% sequence identity to *Methanobrevibacter olleyae*. No chimeras were identified.

Pair-wise distance data (not shown) of these 24 phylotypes revealed that the average genetic divergence over all possible pairs of sequences was 4.8% with the greatest genetic distance being 11.1% between clones HZ-37 and HZ-39. Using a similarity criterion of 98%, DOTUR analysis (Schloss and Handelsman, 2005) indicated the 24 phylotypes formed nine OTUs (Figure 1). Three of the nine OTUs (OTU 2, OTU 6 and OTU 9) were represented by a single phylotype, whereas the largest OTU (OTU 5) was composed of seven phylotypes (HZ-01,

Table 1 16S rRNA clones from pooled PCR samples from the crop of 10 hoatzin from Venezuela

16S rRNA	*No. clones*[a]	*Outgroup*	*Accession no.*	*Size (bp)*	*Nearest valid taxon*	*% Seq. identity*
HZ-04	2	1	EU547211	1265	*Msp. stadtmanae*	95.7
HZ-22	17	1	EU547219	1265	*Msp. stadtmanae*	96.7
HZ-39	1	1	EU547229	1265	*Msp. stadtmanae*	96.5
HZ-51	1	2	EU547233	1260	*Msp. stadtmanae*	94.0
HZ-21	1	3	EU547218	1260	*Msp. stadtmanae*	95.5
HZ-32	1	3	EU547224	1260	*Msp. stadtmanae*	94.2
HZ-13	2	4	EU547216	1260	*Msp. stadtmanae*	95.0
HZ-36	1	4	EU547227	1260	*Msp. stadtmanae*	95.4
HZ-01	118	5	EU547210	1260	*Mbr. ruminantium*	98.7
HZ-26	2	5	EU547220	1260	*Mbr. ruminantium*	98.3
HZ-27	1	5	EU547221	1260	*Mbr. ruminantium*	98.7
HZ-31	1	5	EU547223	1260	*Mbr. ruminantium*	98.6
HZ-34	1	5	EU547225	1260	*Mbr. ruminantium*	98.3
HZ-35	2	5	EU547226	1260	*Mbr. ruminantium*	98.4
HZ-45	2	5	EU547232	1260	*Mbr. ruminantium*	98.6
HZ-37	1	6	EU547228	1260	*Mbr. ruminantium*	96.7
HZ-09	1	7	EU547213	1260	*Mbr. ruminantium*	97.5
HZ-15	35	7	EU547217	1260	*Mbr. ruminantium*	97.6
HZ-42	1	7	EU547230	1260	*Mbr. ruminantium*	97.6
HZ-43	1	7	EU547231	1260	*Mbr. ruminantium*	97.4
HZ-10	1	8	EU547214	1265	*Mbr. olleyae*	96.1
HZ-11	1	8	EU547215	1265	*Mbr. olleyae*	96.3
HZ-28	1	8	EU547222	1265	*Mbr. olleyae*	96.1
HZ-08	2	9	EU547212	1265	*Mbr. olleyae*	94.3

[a]A total of 197 total clones.

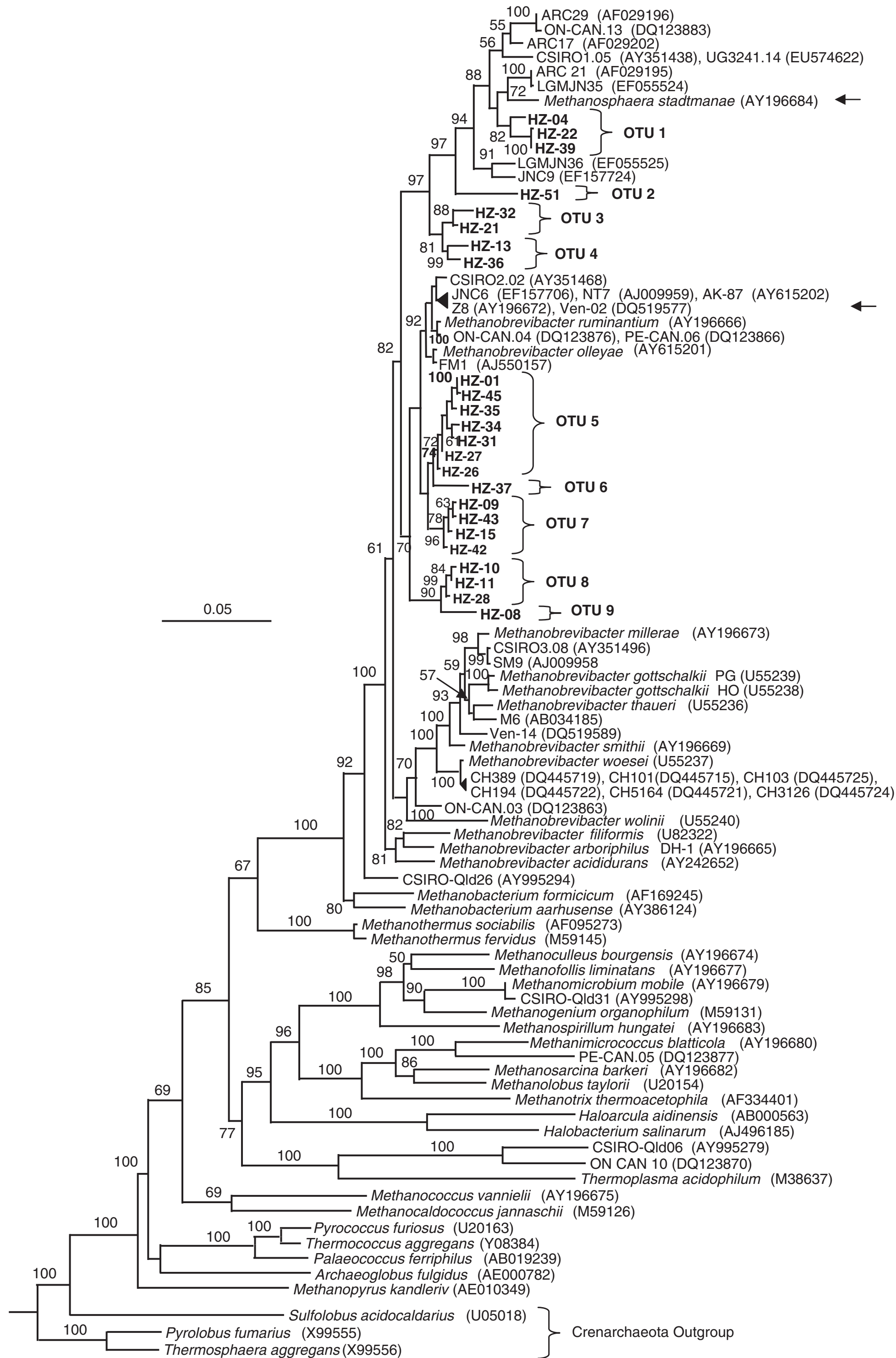

Figure 1 A distance matrix tree of the archaea derived from 16S rRNA evolutionary distances produced by the Kimura two-parameter (Kimura, 1980) correction model and constructed using the neighbor-joining method (Saito and Nei, 1987). Bootstrap supports are indicated as a percentage at the base of each bifurcation. Bootstrap values less than 50% are not shown. Evolutionary distance is represented by the horizontal component separating species in the figure. The scale bar corresponds to five changes per 100 positions. Arrows indicate the phylogenetic placement of the two partial methanogen 16S rRNA gene sequences (<625 bp) from a published abstract (see Garcia-Amado *et al*., 2007).

HZ-26, HZ-27, HZ-31, HZ-34, HZ-35 and HZ-45), which included the most prevalent phylotype, HZ-01 (118 clones).

Phylogenetic analysis

Bootstrap data of the neighbor-joining tree (Figure 1) supported the Euryarchaeota as a monophyletic

Table 2 Densities (cell numbers per gram wet weight) of methanogens, bacteria and protozoa in the crop of the hoatzin

Sample	*Methanogens*	*Bacteria*	*Protozoa*
Hoatzin crop 1	2.22×10^{8}	3.35×10^{12}	4.67×10^{3}
Hoatzin crop 2	3.68×10^{8}	1.20×10^{13}	3.04×10^{6}
Hoatzin crop 3	9.85×10^{8}	5.92×10^{12}	8.65×10^{4}
Hoatzin crop 4	2.91×10^{10}	8.21×10^{12}	3.97×10^{3}
Hoatzin crop 5	4.39×10^{9}	1.47×10^{13}	9.09×10^{2}
Hoatzin crop 6	1.69×10^{9}	2.05×10^{13}	2.51×10^{4}
Hoatzin crop 7	4.58×10^{8}	6.33×10^{12}	1.19×10^{4}
Hoatzin crop 8	2.28×10^{9}	8.70×10^{11}	1.30×10^{3}
Hoatzin crop 9	2.85×10^{9}	3.67×10^{12}	1.31×10^{5}
Hoatzin crop 10	1.57×10^{10}	3.89×10^{12}	6.49×10^{3}
Mean (s.e.m.)	5.80×10^{9} (2.97×10^{9})	7.93×10^{12} (2.03×10^{12})	3.31×10^{5} (3.01×10^{5})

group (100%), as well as the orders Halobacteriales (100%), Methanococcales (69%), Methanomicrobiales (100%), Thermoplasmatales (100%), Methanosarcinales (100%) and Methanobacteriales (67%). All 24 crop methanogen phylotypes grouped within the Methanobacteriales. Of these, five phylotypes (HZ-13, HZ-21, HZ-32, HZ-36 and HZ-51) branched basal to a clade consisting of *Methanosphaera stadtmanae*, three crop methanogens (HZ-04, HZ-22 and HZ-39) and several environmental clones from the rumen environment. At the 98% identity level, these eight phylotypes formed four OTUs (OTU 1, OTU 2, OTU 3 and OTU 4). The remaining 16 crop methanogen phylotypes (that is, five OTUs) all branched basal to a clade consisting of two validly recognized methanogen species, *Methanobrevibacter ruminantium* and *Methanobrevibacter olleyae*, and more environmental clones from the rumen environment.

Crop microbial population densities

Crop cell densities (per gram wet weight) of methanogens, bacteria and protozoa for each animal are presented in Table 2, and ranged from 2.22×10^{8} to 2.91×10^{10} for methanogens, 8.70×10^{11} to 2.05×10^{13} for bacteria and 9.09×10^{2} to 3.04×10^{6} for the ciliate protozoa. Mean density of methanogens relative to the mean density of bacteria was approximately 0.07%.

Discussion

Nearly 20 years after Grajal *et al*. (1989) first reported foregut fermentation in a nonmammalian animal, information on the microbial consortia resident in the hoatzin's crop is now being elucidated using molecular approaches (Godoy-Vitorino *et al*., 2008). In the present study, 197 clones were examined and 24 different phylotypes were grouped into nine OTUs based on a similarity criterion of 98% (Table 1). This level of identity was used as many validly described methanogen species would not be identified using the 97% similarity criterion. For example, based on a 97% similarity cutoff, these four validly described methanogen species, *Methanobrevibacter smithii*, *Methanobrevibacter millerae*, *Methanobrevibacter thaueri* and *Methanobrevibacter gottschalkii*, would be considered a single OTU, thereby underestimating methanogen speciation. On the contrary, the nine OTUs are likely to represent nine new species and 1–3 new genera (<94.5% identity; OTU 2, OTU 3 and OTU 9), once cultivated isolates are established and characterized.

None of the 24 phylotypes had 100% sequence identity to any of the existing sequences in the GenBank database. In comparison, Godoy-Vitorino *et al*. (2008) examined the bacterial diversity of the hoatzin's crop and grouped 1235 16S rRNA sequences into 580 different phylotypes, and reported that 94% of the 580 phylotypes were unclassified at the species level and presumed to be new species. All 24 methanogen phylotypes from the present study branched early before the mammalian gut methanogens *Methanobrevibacter ruminantium*, *Methanobrevibacter olleyae* and *Methanosphaera stadtmanae*. We believe that the early branching of the novel crop methanogen sequences may represent an ancestral parallel lineage of gut methanogens because the hoatzin dates back to the Eocene, some 50 million years before the arrival of ruminants (Cracraft, 1971). Ruminants just entered South America, via the Isthmus of Panama, during the middle Pliocene (Prothero and Foss, 2007), only 2.5–3.0 million years ago after the ocean level subsided.

As much as 86.8% of clones (171 of 197 clones) belonged to the genus *Methanobrevibacter* (Figure 1). This finding is consistent with other studies where *Methanobrevibacter* strains accounted for the vast majority of methanogens in chicken ceca (Saengkerdsub *et al*., 2007), the lower termite *Reticulitermes speratus* (Shinzato *et al*., 1999) and the rumen (Miller and Wolin, 1986; Sharp *et al*., 1998; Tokura *et al*., 1999; Whitford *et al*., 2001; Irbis and Ushida, 2004; Skillman *et al*., 2004, 2006; Wright *et al*., 2004, 2008). Moreover, this finding supports our hypothesis that the genus *Methanobrevibacter* is the most dominant constituent of foregut fermentation systems. The remaining 13.2% of clones (26 of 197) had 94.3–96.3% identity to

Methanosphaera stadtmanae, which has been identified in the rumen by others (Whitford *et al*., 2001; Wright *et al*., 2004, 2006, 2007; Skillman *et al*., 2006). Despite Garcia-Amado *et al*. (2007) reporting only two partial methanogen 16S rRNA sequences (<625 bp), one with 100% identity to *Methanobrevibacter* sp. Ven-02 and the other with 100% identity to *Methanosphaera stadtmanae*, these two sequences were not found in the present study.

No clones were affiliated with *Methanobrevibacter smithii*, *Methanobrevibacter millerae* or *Methanobrevibacter gottschalkii*, three methanogens commonly detected in the rumen of domestic ruminants. Moreover, no clones were identified from the Methanosarcinales, the Methanomicrobiales or from the clade consisting of distantly related uncultivated archaea. The absence of sequences from these other methanogens, as well as the distantly related archaeal clade, is consistent with another published study from Venezuela, where only *Methanobrevibacter* and *Methanobacterium*-like 16S rRNA gene sequences were recovered from the ovine rumen (Wright *et al*., 2008).

Methanogens present in the hoatzin crop were more closely related to methanogens from ruminants, rather than methanogens from other avian species. Saengkerdsub *et al*. (2007) examined the methanogenic archaea in adult chicken ceca, using the same methanogen-specific forward and reverse primers (Met86F and Met1340R), and grouped 420 clones into 11 different phylotypes, 10 of which were 99% similar to *Methanobrevibacter woesei*. In one other study (Miller *et al*., 1986), methanogen strains isolated from turkey and chicken feces appeared to belong to the genus *Methanogenium* (König, 1986). However, in the present study, none of the hoatzin crop methanogens was closely related to *Methanobrevibacter woesei* or *Methanogenium* spp. or grouped within the same clade containing either methanogen.

The mean density of hoatzin crop methanogens (5.80×10^9 cells per gram wet weight) from the present study was up to 20 000 times greater than methanogen densities found in chicken cecal samples, which were based on both most probable number enumeration and 16S rRNA copy numbers (Saengkerdsub *et al*., 2007). However, the mean density of methanogens in the hoatzin crop was in agreement with real-time PCR densities of methanogens from cattle from Canada using 16S rRNA (7.93×10^4 to 1.62×10^9; Hook *et al*., 2009), and cattle from Australia using the mcrA genes (9.8×10^8, Evans *et al*., 2009; 1.34×10^9, Denman *et al*., 2007). The mean density of hoatzin crop bacteria (7.93×10^{12}) from the present study was in agreement with previously published studies from the hoatzin, having viable cell counts ranging from 1.1×10^9 to 4.97×10^{12} (Grajal *et al*., 1989; Dominguez-Bello *et al*., 1993; Garcia-Amado *et al*., 2007), and with a study on another avian species, the green-rumped parrotlet, having 1.1×10^5 to 7.3×10^{12} colony-forming units in the crop (Pacheco *et al*., 2004). In addition, the mean density (3.31×10^5) of ciliate protozoa was also in agreement with previously published microscopic protozoal cell counts of 10^4 per gram (Dominguez-Bello *et al*., 1993).

In conclusion, methanogens resident in the crop of the hoatzin are novel and phylogenetically distinct from rumen methanogens, despite being more similar in identity and density to methanogens from ruminants, compared to methanogens from the ceca of other avian species. Once cultivars are established, hoatzin crop methanogens may provide useful insights into the evolution of the rumen methanogens.

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