16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca

Jianhua Gong[1], Weiduo Si[1], Robert J. Forster[2], Ruilin Huang[3], Hai Yu[1], Yulong Yin[3], Chengbo Yang[3] & Yanming Han[4]

[1]Food Research Program, Agriculture and Agri-Food Canada, Guelph, ON, Canada; [2]Lethbridge Research Center, Agriculture & Agri-Food Canada, Lethbridge, AB, Canada; [3]Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, Hunan, China; and [4]Maple Leaf Foods Agresearch, Guelph, ON, Canada

Correspondence: Jianhua Gong, Food Research Program, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, ON, Canada N1G 5C9. Tel.: +1 519 780 8027; fax: +1 519 829 2600; e-mail: gongj@agr.gc.ca

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Abstract

Mucosa-associated microbiota from different regions of the gastrointestinal (GI) tract of adult broilers was studied by analysis of 16S rRNA gene sequences. The microbiota mainly comprised Gram-positive bacteria along the GI tract. Fifty-one operational taxonomic units (OTUs) (from 98 clones) were detected in the ceca, as compared with 13 OTUs (from 49 clones) in the crops, 11 OTUs (from 51 clones) in the gizzard, 14 OTUs (from 52 clones) in the duodenum, 12 OTUs (from 50 clones) in the jejunum and nine OTUs (from 50 clones) in the ileum. Ceca were dominantly occupied by clostridia-related sequences (40%) with other abundant sequences being related to *Faecalibacterium prausnitzii* (14%), *Escherichia coli* (11%), lactobacilli (7%) and *Ruminococcus* (6%). Lactobacilli were predominant in the upper GI tract and had the highest diversity in the crop. Both *Lactobacillus aviarius* and *Lactobacillus salivarius* were the predominant species among lactobacilli. Candidatus division *Arthromitus* was also abundant in the jejunum and ileum.

Introduction

Different gastrointestinal (GI) tract regions of chickens play different roles in feed digestion, nutrient absorption and intestinal health, all of which are important to the health and well-being of the animals. Whereas the crop, gizzard and duodenum of chickens have major functions in feed digestion, the jejunum and ileum are the principal sites of nutrient absorption (Freeman, 1976; Riesenfeld *et al.*, 1980; Sklan, 1980; Barrow, 1992). In the ceca, extensive bacterial fermentation occurs, resulting in further nutrient absorption, detoxification of harmful substances and prevention of pathogen colonization (Moran, 1982; Barrow, 1992; Csordas, 1995). It is generally accepted that the intestinal microbiota contributes to intestinal function and thus has significant impact on the growth and health of chickens.

The chicken intestinal microbiota has largely been studied by culture-based methods (reviewed by Barrow, 1992). Because these methods are inapplicable to noncultivable bacteria and are selective for readily cultivated bacteria (Ricke & Pillai, 1999; Theron & Cloete, 2000; Gong *et al.*, 2002a), our previous understanding of the intestinal microbiota may be inaccurate and certainly incomplete. To overcome these limitations, molecular approaches were recently used to characterize the intestinal microbiota, including both cultivable and noncultivable bacteria. Although some drawbacks were recognized with the molecular methods (Farrelly *et al.*, 1995; Suzuki & Giovannoni, 1996; Qiu *et al.*, 2001), culture-independent studies that mainly focused on cecal and ileal microbiota (Apajalahti *et al.*, 2001; Gong *et al.*, 2002a, b; Knarreborg *et al.*, 2002; Lan *et al.*, 2002; Zhu *et al.*, 2002; Lu *et al.*, 2003; Amit-Romach *et al.*, 2004) have further advanced our understanding of chicken intestinal microbial ecology, including the diversity, phylogeny, distribution and success of the bacteria as well as the effect of bird age, diet and administration of dietary antibiotics on the composition of microbiota in these two GI tract regions. For example, culture-independent analyses revealed a highly diverse bacterial community in the ceca, mainly comprising Gram-positive bacteria (Gong *et al.*, 2002a; Zhu *et al.*, 2002; Lu *et al.*, 2003). The diversity and complexity of the community structure of cecal bacteria were much higher than had been reported previously by culture-based studies. Moreover, a large number of 16S

rRNA genes isolated from cecal microbiota were also found to be related to uncultured bacteria identified in human feces and in the intestine of other animals (including pigs, ruminants and mice). Although this observation illustrated the great diversity of cecal microbiota, it also suggested a similarity of the intestinal ecosystem across animal species (Gong *et al.*, 2002a; Lu *et al.*, 2003). In addition to studies on cecal microbiota, culture-independent analyses also revealed that ilea had a less complicated bacterial community structure dominated by lactobacilli (Gong *et al.*, 2002b; Lu *et al.*, 2003), thus supporting the earlier observations by culture-based methods.

We need to further our understanding of chicken intestinal microbial ecology in order to provide a solid scientific basis for the effective use and development of probiotics. Effective probiotics are thought to be associated with, and interact with, the mucosal layer of the intestines. We have therefore investigated the community structures and phylogenetic relationship of bacteria associated with the mucosa of different intestinal regions of broiler chickens with a particular interest in the gizzard, duodenum and jejunum. This is, to our knowledge, the first report that provides information on mucosa-associated microbiota throughout the GI tracts of broiler chickens using culture-independent techniques through analysis of 16S rRNA gene sequences.

Materials and methods

Animals and sampling

Broiler chickens (Ross × Ross) were reared under controlled conditions similar to commercial production at the animal research facilities of Maple Leaf Foods (Burford, ON, Canada). Management and experimental procedures were in accordance with the guidelines of the Canadian Council on Animal Care (1993). In the hatchery, chicks were vaccinated to protect from Marek's disease and infectious bronchitis. The birds were fed typical all-vegetable broiler starter, grower and finisher diets (manufactured by Shur-Gain, Maple Leaf Foods), composed of wheat, corn and soybean meal, plant oil, together with supplements of amino acids, major and trace minerals, and vitamins. Zinc bacitracin ($55\,mg\,kg^{-1}$) was included in the diets as a growth-promotant. The birds were healthy and no disease outbreaks were noticed during the experimental period. When they were 5-weeks-old, five birds were selected randomly and humanely killed by cervical dislocation. Samples were collected from six different intestinal regions, including crop, gizzard, duodenum (first half of the intestine from gizzard to Meckel's diverticulum), jejunum (second half of the intestine from gizzard to Meckel's diverticulum), ileum (Meckel's diverticulum to ileal–cecal conjunction) and ceca (both). All the samples were kept on ice and processed immediately after dissection. All collected samples from each intestinal region of the five chickens were combined for preparation of bacterial samples that were subsequently used for DNA extraction.

Intestinal wall-associated bacterial samples, which we refer to as mucosa-associated bacteria, were prepared as described previously (Gong *et al.*, 2002a). Briefly, each intestinal region was opened longitudinally. After removal of the digesta, they were briefly washed three times in saline to remove unattached or loosely attached bacteria from the walls. Bacterial cells were then released from the walls by two washes in saline containing 0.1% Tween 80 with vigorous hand shaking for 30 s per wash followed by centrifugation (27 000 ***g***, 20 min) at 4 °C to pellet the cells. This procedure was previously shown to release about 95% of the wall-associated bacterial cells without detectable lysis of bacterial cells (Li *et al.*, 2003). Samples for DNA extraction were frozen in liquid nitrogen and stored at − 70 °C.

Cell lysis and DNA extraction

Bacterial cells prepared from the five chickens were lysed and genomic DNA was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Canada) according to the manufacturer's instructions. This kit was evaluated previously against the bead-beating method for cell lysis and DNA extraction and demonstrated to be appropriate for ecological studies of intestinal microbiota (Li *et al.*, 2003).

Random cloning and sequencing of 16S rRNA genes

16S rRNA genes were amplified by PCR from the genomic DNA samples of mucosa-associated bacteria using eubacterial primers F8 (5′-AGAGTTTGATCCTGGCTCAG-3′) and R1492 (5′-GGTTACCTTGTTACGACTT-3′) (Eden *et al.*, 1991). PCR mixtures were the same as described previously (Whitford *et al.*, 1998). The thermocycle program was 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C for 25 cycles followed by 10 min at 72 °C. Two PCR amplicon libraries were combined and then cloned into a vector, pCR®4-TOPO®, using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions, and partially sequenced with an ABI PRISM™ 377 Automated DNA Sequencer.

Data analyses

Putative chimeric sequences were identified using the program Check_Chimera (Maidak *et al.*, 1999) and the Mallard program (http://www.cf.ac.uk/biosi/research/biosoft) implementation of the Pintail algorithm (Ashelford *et al.* 2005). Partial 16S rRNA gene sequences corresponding to *Escherichia coli* 16S rRNA gene bases 400–1050 were compared directly with the GenBank, EMBI and DBJI nonredundant nucleotide databases using BLASTN. The

presumptive relationships of rRNA gene sequences to uncultured bacteria located in GenBank were further analysed through RDP II Sequence Match. The cloned sequences were aligned to a database of almost 28 000 sequences using the ARB software package (Ludwig *et al.*, 2004) and the 'ssu_jan04' database. The aligned sequences were exported to the Seaview and Phylo_win packages (Galtier *et al.*, 1996) where trees were reconstructed using neighbor-joining, the Kimura-2 correction and pair-wise gap removal. Reliability of internal branches was assessed using 1000 bootstraps (values above 60% are shown). The aligned sequences were also used for analysis in DOTUR (Schloss & Handelsman, 2005) to determine operational taxonomic units (OTUs), conduct rarefaction analysis and determine Chao1 richness estimates. An OTU was defined as comprising sequences with less than a 2% difference as detected in DOTUR by the furthest-neighbor method.

Sequence accession numbers

The partial sequences of 39 cloned 16S rRNA genes showing $< 97\%$ similarity to existing database sequences were deposited in GenBank under accession numbers AY654947–AY654985. Among them, 24 were from the ceca, seven from the duodenum, three from the ileum, two each from the gizzard and jejunum, and one from the crop.

Results

Bacteria associated with the mucosa of ceca

Partial sequences of 98 random 16S rRNA gene clones from bacteria associated with the cecal mucosa of five birds were analysed. The presumptive relationships of these sequences were obtained from database comparison. The cloned sequences had 47 closest database sequences with degrees of similarity from 92% to 100% (Table 1). These clones were also highly diverse, with many sequences related to the sequences identified in human feces, in the pig or chicken GI tracts, or in the bovine rumen. There were ten groups comprising three or more closely related sequences. Clostridia were the most abundant (39 of 98 clones), representing 40% of the clones. Twenty-three of these clones were assigned to *Clostridium orbiscendens*. *Faecalibacterium prausnitzii* and *E. coli* were the second largest groups (representing 14% and 11% of the clones, respectively) followed by lactobacilli (7%) and *Ruminococcus* (6%). In addition, 24 of the 98 clones had $< 97\%$ similarity to existing database sequences, and which may thus represent novel species previously unidentified in the chicken GI tract.

The phylogenetic relationship of the reference and cloned 16S rRNA gene sequences is shown in Fig. 1. The majority clustered with Gram-positive taxa. The sequences related to clostridia had different branch locations in the phylogenetic tree, reflecting the taxonomic diversity of clostridial groups (Collins *et al.*, 1994). The clusters were mainly related to *Clostridium leptum*, *Clostridium orbiscindens* and *Clostridium* cluster XIV (AJ002591). The rarefaction curve of the cecal sample is shown below in Fig. 4. Whereas the 98 cloned sequences were placed into 51 OTUs at the 2% difference level by DOTUR, the Chao1 richness estimate produced 121 OTUs.

Bacteria associated with the mucosa of the upper GI tract

Partial sequences of 252 random 16S rRNA gene clones from bacteria associated with the mucosa of the upper GI tract (including the crop, gizzard, duodenum, jejunum and ileum) of the five birds were analysed. Among them, 49 clones were from the crop, 51 from the gizzard, 52 from the duodenum, and 50 from the jejunum and ileum. The cloned sequences had 26 closest database sequences with degrees of similarity between 92% and 100% (Table 2). Lactobacilli were predominant in the upper GI tract, with 184 clones representing ten closest database sequences. *Lactobacillus aviaries* (98 clones) comprised the largest group followed by *Lactobacillus salivarius* (56 clones). In addition to the large group of lactobacilli, 32 cloned sequences were related to candidatus division *Arthromitus* (X80834) and 16 sequences to clostridia. There were also a small number of cloned sequences related to sequences identified previously in human faeces, and in the pig or chicken GI tracts. In contrast to the cecal sequences, clones from the upper GI tract had a much lower percentage of sequences (15 of 253) showing similarity of $< 97\%$ to existing database sequences.

Predominant groups of the cloned sequences were located in the different regions of upper GI tract. Sequences related to *L. aviaries* were abundant in all the regions of the upper GI tract except for the crop. They represented 43% of the cloned sequences in the gizzard, 29% in the duodenum, 24% in the jejunum and 26% in the ileum. Sequences related to *L. salivarius* were also abundant in most regions of the upper GI tract, including the crop, duodenum, jejunum and ileum. They represented 63% of the cloned sequences in the crop, 19% in the duodenum, 22% in the jejunum and 12% in the ileum. Sequences related to candidatus division *Arthromitus* were abundant in the jejunum and ileum, representing 34% and 28% of the cloned sequences in these regions, respectively. They were not detected in the crop and gizzard of the birds.

The phylogenetic relationship among cloned sequences from mucosa-associated bacteria in each region of the upper GI tract was analysed. Figure 2 shows the phylogenetic analysis of the 152 cloned sequences from mucosa-associated bacteria in the small intestine, including the duodenum, jejunum and ileum. The microbiota in the duodenum exhibited the highest diversity in the small intestine, with 14 OTUs (from 52 clones) at 2% difference level. The Chao1

Table 1. Molecular species detected on the mucosa of ceca*

Closest group	Closest species/clone	GenBank accession no.	Similarity (%)	Number of clones
Acetanaerobacterium	*A. elongatum*	AY487928	93–94	2
Bilophila	*B. wadsworthia*	L35148	98–100	3
Bacteroides	*B. capillosus*	AY136666	92–97	17
Clostridium	*C. algidixylanolyticum*	AF092549	96	1
	C. celerecrescens	AY604564	92–94	2
	C. clostridioforme	AY169422	97	1
	C. orbiscindens	Y18187	96–97	3
		AY730664	95	1
		AY730665	94–95	2
	C. lituseburense	M59107	99	1
	C. leptum	AJ305238	94	1
	C. saccharolyticum	Y18185	94	1
	Clostridium sp.	AF304435	87	1
		Y10028	96	1
	Clostridiales bacterium	DQ168652	93	1
Escherichia	*E. coli*	AF403733	99	1
		AE016770	98–100	7
		AP002566	99	2
		AB035922	99	1
Eubacterium	*E. desmolans*	L34618	94	3
	E. halii	L34621	95	1
	E. tortuosum	L34683	93	2
	Eubacterium sp.	U81762	96	1
Faecalibacterium	*F. prausnitzii*	AJ270469	97	1
		AY169429	92–96	5
Firmicutes	*Firmicutes* sp.	AF432140	94	1
Lactobacillus	*L. salivarius*.	AF420311	99	1
	Lactobacillales bacterium	AY581272	94–95	4
Peptococcus	*Peptococcus niger*	X55797	93	1
Pseudobutyrivibrio	*P. ruminis*	AY178843	94	1
Megamonas	*M. hypermegale*	AJ420107	95–97	4
Ruminococcus	*R. schinkii*	X94964	93	1
	R. torques	L76604	95	1
	Ruminococcus sp.	AJ315979	96–97	2
Sporobacter	*S. termitidis*	Z49863	93	1
Subdoligranulum	*S. variabile*	AJ518869	96–97	2
Uncultured bacteria†	cknm300-B2-4	AF376204	99–100	3
	cknm326-B4-13	AF376230	98	1
	p-2529-18B5	AF371569	94	1
	p-1940-962-3	AF371813	97	1
	p-4154-6Wa5	AF371714	92	1
	cc142	DQ057372	98	1
	CFT19A7	DQ455828	97	1
	CFT19B1	DQ455833	99	5
	CFT19G10	DQ455889	100	1
	M1_1e10	DQ014926	94	1
	LH65	AY916208	96	1
Total number				98

*Determined by GenBank BLASTN queries with additional analysis through RDP Sequence Match. Bacterial names were assigned to rRNA gene sequences with closest BLASTN matches to named organisms located in GenBank.

†No closest BLASTN matches to a known bacterium were identified in GenBank. RDP Sequence Match analysis then assigned eight such rRNA gene sequences (with closet matches to AF376204 or DQ455833) to *Faecalibacterium prausnitzii*, five rRNA gene sequences (closely matching DQ455889, DQ014926, AY916208, AF371569 or AF371813) to *Clostridium*, two rRNA gene sequences (closely matching DQ455828 or AF376230) to Lactobacillales bacterium, and two rRNA gene sequences (closely matching DQ057372 or AF371714) to *Ruminococcus*. The clones in GenBank were generated from chicken, pig or human intestine.

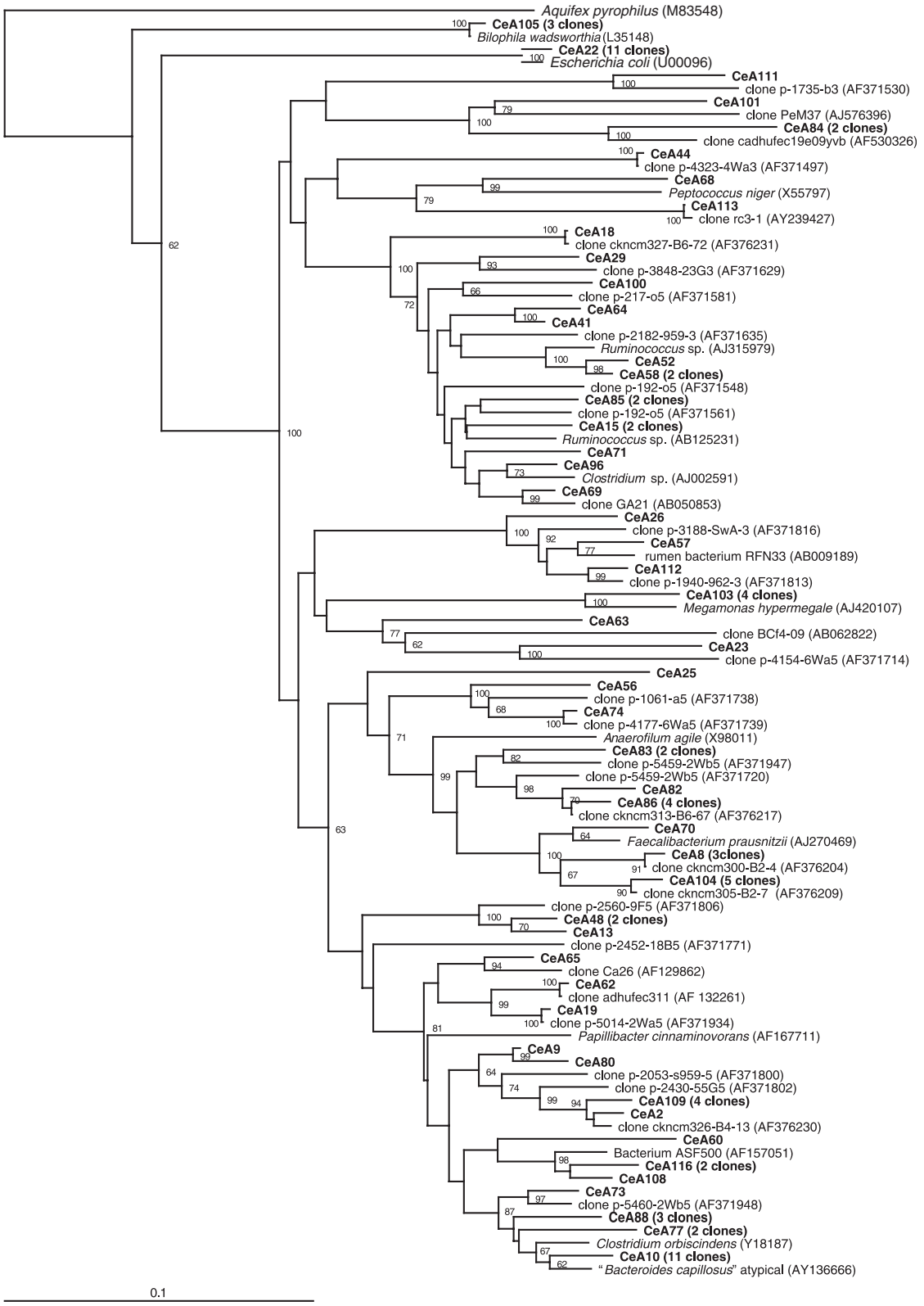

Fig. 1. Unrooted phylogenetic tree of mucosa-associated bacteria in the chicken ceca constructed by a neighbor-joining method. The scale bar represents a sequence divergence of 10%. Clones represented by CeA were generated from this study, with names of CCCM submitted to GenBank. CeA100, 101, 15, 23, 25, 26, 29, 41, 52, 53, 55, 56, 58, 59, 6, 60, 63, 64, 68, 71, 79, 81, 83 and 85 had $< 97\%$ similarity to the existing database sequences of 16S rRNA genes, and were assigned GenBank accession numbers AY654947–AY654969.

Table 2. Molecular species detected on the mucosa of the upper gastrointestinal tract*

Closest group	Closest species	GenBank accession no.	Similarity (%)	Number of Clones
Candidatus *Arthromitus*†	TBD	X80834	97–100	32
Clostridium	*C. difficile*	AF072474	95–96	13
	C. orbiscindens	Y18187	96	1
	Clostridium sp.	AF157052	93	1
		DQ445858	95	1
Escherichia	*E. coli*	AE016770	100	1
		AF527825	99	1
		AE000406	99	1
		AP002566	99	1
Eubacterium	*E. tenue*	M59118	95–96	7
		U81762	96	1
Enterococcus	*E. faecium*	AY172570	99	1
Faecalibacterium	*F. prausnitzii*	AJ413954	96	3
		X85022	94	1
Lactobacillus	*L. aviarius*	AB001836	92–100	98
	L. crispatus	AF257097	96–99	4
	L. gasseri	AF243165	99	1
		AF519171	97–99	16
	L. salivarius	AY137589	97–100	19
		AF420311	96–100	37
	L. reuteri	X76328	99	3
	L. acidophilus johnsonni	M99704	98–99	2
	Lactobacillus sp.	AY005048	99	2
		AY098490	94	2
Ruminococcus	*R. torques*	L76604	95	1
Veillonella	*V. atypica*	X84007	94–95	2
Total number				252

*Determined by GenBank BLASTN queries with additional analysis through RDP Sequence Match. Bacterial names were assigned to rRNA gene sequences with closest BLASTN matches to named organisms located in GenBank.

†TBD, to be determined; Members of this group of bacteria can be divided into different species on the basis of host specificity and 16S rRNA sequences. Candidatus *Arthromitus* represents a subline within the *Clostridium* subphylum (Snel et al., 1995).

richness estimate was 28 OTUs. The 52 cloned sequences formed five major clusters represented by clones DuA17 (*L. aviaries*), DuA50 (*L. salivarius*), DuA13 (*Lactobacillus* species), DuA10 (unidentified species) and DuA6 (*Lactobacillus reuteri*), respectively. The microbiota in the jejunum had the second highest diversity with 12 OTUs detected in the sample (50 clones), although the Chao1 richness estimate was 40 OTUs. The majority of cloned sequences formed three major clusters represented by clones JuA11 (*L. aviaries*), JuA14 (*L. salivarius*) and JuA10 (candidatus division *Arthromitus*), respectively. Nine OTUs were detected in the ileal sample (50 clones) with 12 OTUs of the Chao1 richness estimate. There were four major clusters with each represented by clones IleA10 (*L. aviaries*), IleA5 (*L. salivarius*), IleA22 (candidatus division *Arthromitus*) and IleA14 (unidentified species).

Figure 3 shows the phylogenetic analysis of the 100 cloned sequences from the crops and gizzards. Both samples were exclusively composed of lactobacilli. The crops had two large clusters with 32 and 12 clones, which were represented by clones CpA4 (*L. salivarius*) and CpA11 (*L. johnsonii*), respectively. The remaining five clones represented five different molecular species related to *Lactobacillus*. The 51 cloned sequences from the gizzard were placed at three different positions in the tree with 50 sequences represented by clones GzA1 (*L. aviaries*) and GzA10 (*Lactobacillus* species). The rarefaction curves, representing the number of OTUs discovered as the number of sequences sampled increases, are shown for the crops and gizzard samples in Fig. 4. There were 13 and 11 OTUs detected in the crops and gizzard, respectively, with a Chao1 richness estimate of 58 OTUs for the crops and 13 OTUs for the gizzard. The crops had the greatest diversity of lactobacilli in the chicken GI tracts.

Discussion

The mucosa-associated microbiota from each of six different intestinal regions, including the crop, gizzard, duodenum, jejunum, ileum and ceca, of five chickens was collected. The samples from the same intestinal region were subsequently combined for DNA preparation. Because the samples were from the same chickens, the

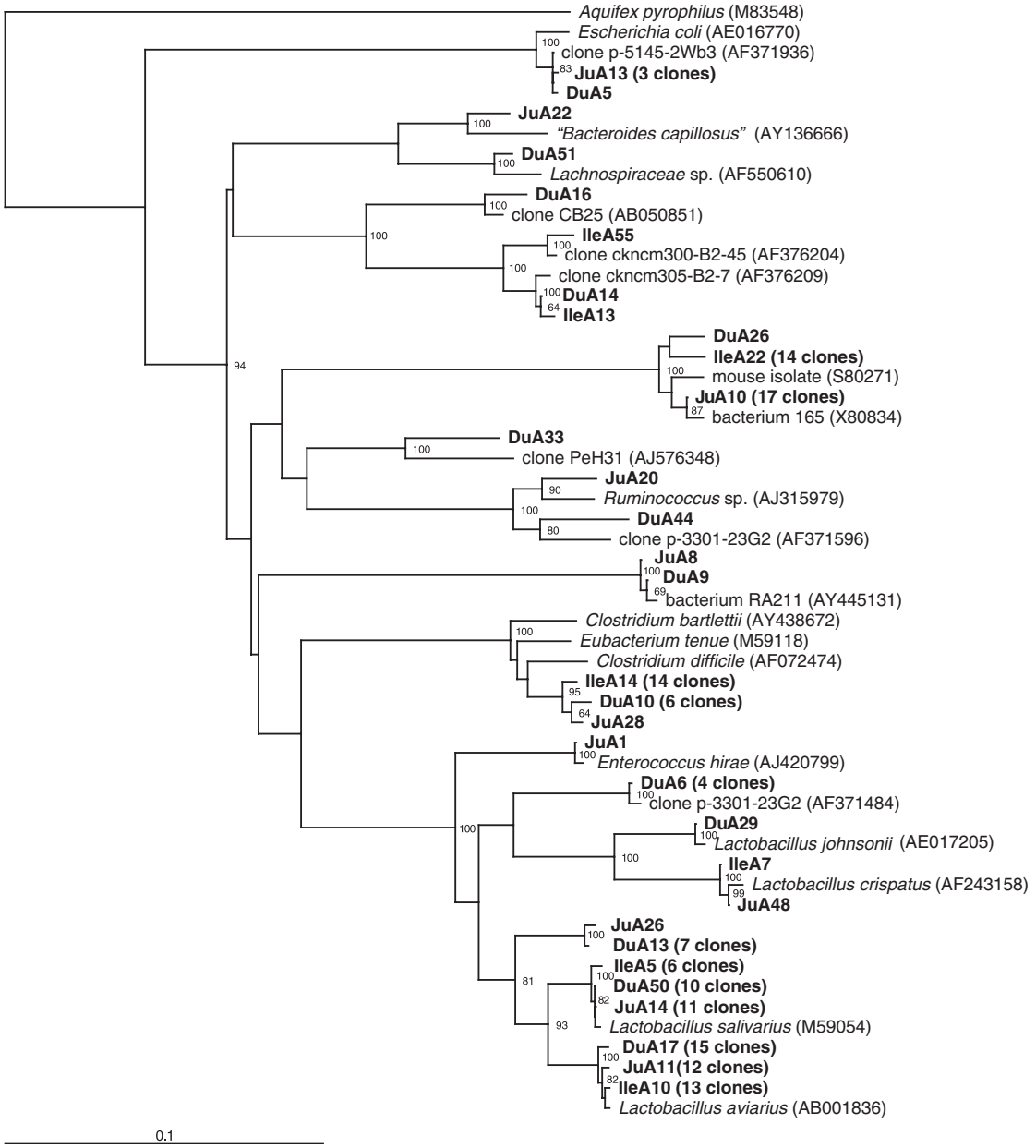

Fig. 2. Unrooted phylogenetic tree of mucosa-associated bacteria in the small intestine constructed by a neighbor-joining method. The scale bar represents a sequence divergence of 10%. Clones represented by DuA, JuA and IleA were generated from this study. Clones of DuA were generated from the duodenum, JuA from the jejunum and IleA from the ileum. DuA13, 24, 33, 38, 44, 52 and 9 had $< 97\%$ similarity to the existing database sequences of 16S rRNA genes, and were assigned GenBank accession numbers AY654971–AY654979 and under names CDDN. JuA8 and 26 had $< 97\%$ similarity to the existing database sequences of 16S rRNA genes, and were assigned GenBank accession numbers AY654983 and AY654982 and under names CJJN8 and CJJN26. IleA14, 34 and 6 had $< 97\%$ similarity to the existing database sequences of 16S rRNA genes, and were assigned GenBank accession numbers from AY654979 to AY654981 and under names CILM.

bacterial communities represented the mucosa-associated microbiota in the different intestinal regions of these birds as a whole, regardless of differences in microbiota among individual chickens.

During our BLASTN analysis, some 16S rRNA gene clones had the closet matches to a database sequence (AY136666) in GenBank. This sequence (AY136666) was originally identified as being from *Bacteroides capillosus* when submitted. In fact, it is more closely related to sequences of the Gram-positive *Clostridium orbiscindens* than from species of the Gram-negative *Bacteroides*. The 16S rRNA gene clones generated from the present study with the closet matches to

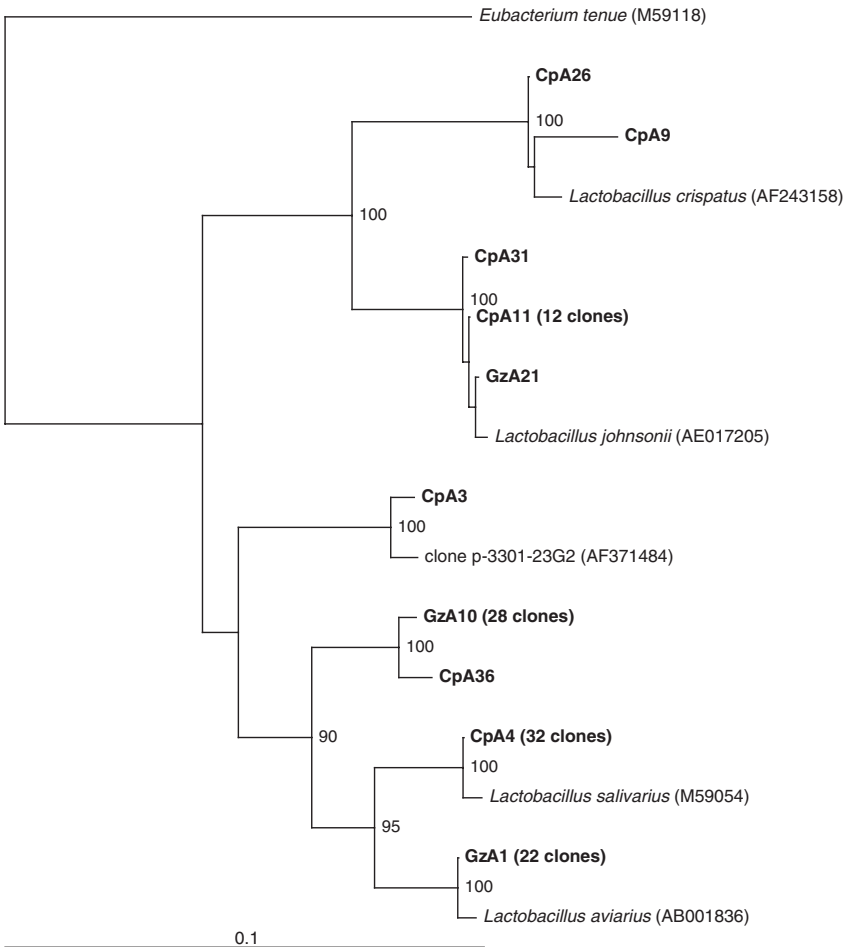

Fig. 3. Unrooted phylogenetic tree of bacteria on the wall of crops and gizzards constructed by a neighbor-joining method. The scale bar represents a sequence divergence of 10%. Clones represented by CpA and GzA were generated from this study. CpA36 had $< 97\%$ similarity to the existing database sequences of 16S rRNA genes. This clone has GenBank accession number AY654970 with the name CCRP36.

AY136666 were therefore regarded as *C. orbiscindens* in the microbiota composition analysis. Nevertheless, further investigations of *B. capillosus* strains should be carried out to determine their correct phylogenetic placement.

The chicken intestinal microbiota has previously been investigated using culture-based methods (Lev & Briggs, 1956; Smith, 1965a, b; Barnes *et al.*, 1972; Salanitro *et al.*, 1974, 1978; Barnes, 1979). These studies have shown that Gram-positive bacteria were usually cultured, the ceca had a high density and numerous types of bacteria, and the small intestine had a simpler bacterial community dominated by lactobacilli. This general description has been supported by recent studies on ileal and cecal microbiota with culture-independent DNA techniques, although the molecular analysis has revealed remarkable microbial diversity beyond that of the early reports of culture-based studies (Gong *et al.*, 2002a, b; Zhu *et al.*, 2002; Lu *et al.*, 2003). The present study has provided further evidence on bacterial communities along the GI tracts of broiler chickens, which also appears to support previously reported trends regarding intestinal region-specific microbiota and its increasing complexity in distal regions.

Our previous studies on the diversity of mucosa-associated microbiota from broiler chickens assigned 115 and 51 rRNA gene clones from the ceca and ilea, respectively, to 49 and 15 database sequences (Gong *et al.*, 2002a, b). While *Faecalibacterium prausnitzii* and butyrate-producing bacteria (mainly *F. prausnitzii*, *Clostridium* and *Ruminococcus*) comprised the largest groups in the ceca (representing 29% and 25% of cecal clones, respectively), lactobacilli and *Enterococcus cecorum* were predominant in the ilea (representing 45% and 29% of ileal clones, respectively). The present study revealed a similar degree of diversity in the mucosa-associated microbiota (Tables 1 and 2). Lactobacilli were also predominant in the small intestine, gizzard and corp. However, the community structures of cecal and ileal microbiota were different. The major changes observed in this study include a significant increase in the population of *C. orbiscendens* and *E. coli* (up to 25% and 11% of the cloned sequences, respectively) in the ceca and the lack of *Enterococcus cecorum* detected in the mucosa associated with the small intestine (Table 2). In the present study, the chickens were from the commercial production line of Maple Leaf Foods. The broiler diets fed to these chickens were

medicated, unlike those used in our previous studies. In addition, the major dietary components and supplements as well as their levels used in this trial differed from previous studies, although all the diets including broiler starter, grower and finisher were typical all-vegetable based. These differences may in part explain the different observations on microbiota compositions from our different studies.

Similar to other studies on microbiota from cecal lumen using DNA techniques (Zhu *et al.*, 2002; Lu *et al.*, 2003), the present study revealed a highly diverse population of microbiota with some members being related to bacteria identified in human and other animal GI tracts including pigs, ruminants and mice. As with other reports, the present study also found that low-G+C Gram-positive bacteria were dominant in the ceca with clostridia being the most abundant group. These observations were, in particular, similar to the report by Lu *et al.* (2003) on broiler chickens fed a vegetarian corn-soy diet devoid of feed additives, in which 65% of the clones in their 16S rRNA gene libraries were *Clostridiaceae*-related with other abundant sequences being related to *Faecalibacterium* (14%), *Lactobacillus* (8%) and *Bacteroides* (5%). Zhu *et al.* (2002) also described similar results on clostridia in the ceca from chickens fed a corn-soy diet that contained animal proteins and an anticoccidial compound. However, they reported that 40% of their library sequences were related to *Sporomusa* or enteric bacteria related to the *Gammaproteobacteria*, such as *E. coli*, which was not detected in the studies reported by Lu *et al.* (2003) and by our group (Gong *et al.*, 2002a).

One significant advance made by this study is the characterization of mucosa-associated microbiota in the gizzard, duodenum and jejunum in addition to the microbiota in the ileum and crop. Although we further confirmed previous observations on lactobacilli, including their dominance in the ileum and diversity in the crop revealed by both culture-dependent (Fuller, 1973, 1975, 1977) and -independent methods (Guan *et al.*, 2003), we found that the gizzard was exclusively and the duodenum and jejunum were predominately occupied by lactobacilli. In addition to lactobacilli, sequences related to candidatus division *Arthromitus* were abundant in the jejunum and ileum in the present study. This group of bacteria is commonly associated with a healthy GI tract in animals (Snel *et al.*, 1995) and has previously been reported in the chicken ileum (Lu *et al.*, 2003). The duodenum (proximal small intestine) is characterized by rapid flow of the highly fluid digesta, which results in relatively low numbers of bacteria (10^3–10^5 cells g^{-1} digesta). Traditionally, the ileum (the distal small intestine) is considered to harbor a more diverse and numerically greater (10^8–10^9 cells g^{-1} digesta) population of bacteria. In the present study, however, we found that the microbiota associated with the mucosa of the duodenum was more diverse than that of the ileal mucosa (14 vs. nine OTUs). In fact, it was the most diverse community in the upper GI tract.

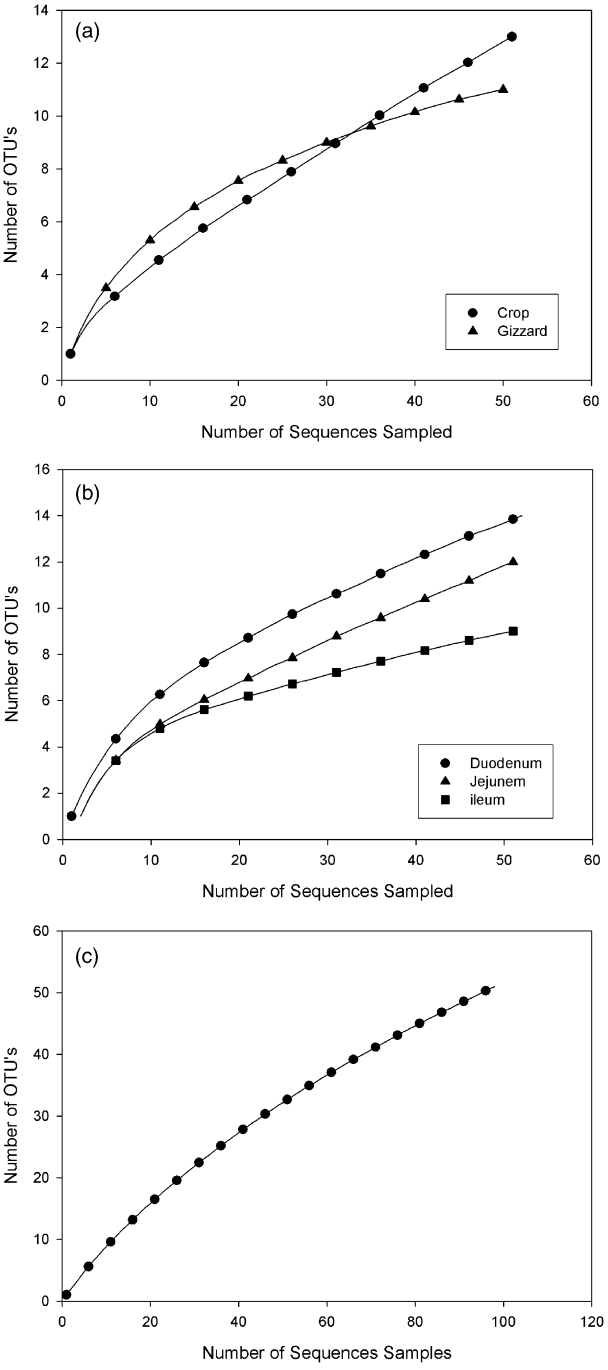

Fig. 4. Rarefaction curves of observed operational taxonomic units (OTUs) recovered from bacterial samples collected from mucosa of different intestinal regions: (a) crops and gizzard; (b) duodenum, jejunum and ileum; and (c) ceca.

The composition of chicken intestinal microbiota can be significantly influenced by diet (Knarreborg *et al.*, 2002; Lu *et al.*, 2003) and other factors, such as the age of birds

(Knarreborg *et al.*, 2002; van der Wielen *et al.*, 2002; Lu *et al.*, 2003; Zhu & Joerger, 2003; Amit-Romach *et al.*, 2004) and antibiotic administration (Knarreborg *et al.*, 2002; Smirnov *et al.*, 2005), which in turn will determine its microbial diversity and community structures. In this study, we analysed limited numbers of 16S rRNA gene clones from different intestinal regions of five chickens. Whereas the sequenced clone libraries from the gizzard, duodenum and ileum covered 85%, 50%, and 75% of the OTUs predicted by the Chao1 analyses (Fig. 4), respectively, the crop, jejunum and cecum samples were poorly covered (22–42%) and may have revealed only predominant groups of bacteria in these communities. To investigate microbial diversity in the chicken GI tracts fully, a larger number of 16S rRNA gene clones generated from chickens fed different diets or raised under different conditions should be collected by PCR amplification using different pairs of universal PCR primers, as reported by Zhu *et al.* (2002) and Leser *et al.* (2002).

The GI tract-surface-associated microbiota has long been studied because of its importance in pathogen control, immune modulation and its effects upon nutrient absorption by their hosts. In this study, the community of bacteria associated with the mucosa of thew chicken GI tract (from crops to ceca) and their phylogenetic relationships were determined. The predominant bacterial groups in each region of the GI tract were also identified. These data may have significant implications for the health and nutrition of chickens, and are particularly relevant to the development of probiotics and their most effective use for poultry.

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