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Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen

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Abstract

We reported the first attempt to describe mucosa-associated bacterial populations in the chicken ceca by molecular analysis of 16S rRNA genes. Bacteria in the mucosa were highly diverse but mainly Gram-positive with low G+C. *Fusobacterium prausnitzii* and butyrate-producing bacteria comprised the largest groups among 116 cloned sequences. Twenty five percent of the clones had less than 95% homology to database sequences. Many sequences were related to those of uncultured bacteria identified in human feces or the bovine rumen. Terminal restriction fragment length polymorphism (T-RFLP) analysis revealed some differences between bacterial populations present in the mucosa and lumen of ceca. Greater resolution of bacterial population was obtained using a culture-independent approach rather than a culture-based approach.

Keywords: Chicken; Cecal bacterium; 16S rRNA; Phylogeny; Diversity

1. Introduction

The normal gut microflora plays an important role in the health and well-being of host animals. In poultry, the absence of healthy microflora in the ceca has been considered to be a major factor in the susceptibility of chicks to bacterial infection [1]. To prevent chicks from bacterial infection, poultry producers in North America currently rely on the prophylactic use of antibiotics. There is public concern about the development and spread of antibiotic resistance in bacteria, which has led to greater interest in the use of probiotics in commercial practice to control bacterial infection and reduce the reliance on antibiotics. Clearly, a better understanding of microbial ecology of the chicken gut is required for the development of probiotics and their most effective use.

Chicken gut microflora have been studied previously by culture-based methods (reviewed in [1]). Because of the selectivity of the culture approach for readily cultivated bacteria, it may introduce a biased view of microbial diversity. Increasingly, molecular approaches are being used to examine the diversity of gut microflora independent of culturing [2–4]. While molecular approaches based on PCR may introduce bias of a different kind [5–7], they provide powerful tools to investigate the phylogenetic diversity of microbes in gut samples [2–4].

This report is the first study to our knowledge, to use molecular analysis of 16S rRNA genes to examine the phylogenetic diversity of bacterial communities in mucosa of chicken ceca, to compare bacterial populations in the mucosa and lumen, and to compare the results of culture-dependent and -independent methods to analyze mucosal bacteria.

2. Materials and methods

2.1. Chicken maintenance and sample collection

Broiler chickens (Ross/Ross) were reared under controlled management similar to commercial practice. Management and experimental procedures were according the

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welfare guideline of the Animal Care Committee, University of Guelph (AUP 98R161). The birds were fed with corn-soy broiler diets [8] containing 18–22% crude protein and 3073–3195 kcal ME per kg and no antibiotics. Gut samples were collected from the ceca of 10 6-week-old broiler chickens. All gut samples were kept on ice and processed immediately after dissection. Bacterial samples from digesta of ceca were prepared essentially by the method of Apajalahti et al. [9]. To prepare cecal wall-associated bacterial samples, ceca were opened longitudinally and briefly washed three times in saline to remove unattached or loosely attached bacteria from the wall. Bacterial cells were then released from the cecal wall by two washes in saline containing 0.1% Tween 80 with vigorous hand shaking for 30 s per wash followed by centrifugation ($27\,000 \times g$, 20 min) at 4°C to pellet the cells. This fraction of bacterial cells was referred to as mucosal bacteria in our investigation. The procedure described above was shown to release about 95% bacterial cells from the cecal wall, which was as efficient as scraping mucosa to prepare the bacterial samples. Samples for DNA extraction were frozen in liquid nitrogen and stored at −70°C, while samples for bacterial growth and storage were collected anaerobically and stored in 15% glycerol at −70°C.

2.2. Bacterial growth

Bacteria were grown at 41°C on brain–heart-infusion agar (BHI, Difco) supplemented with hemin (5 mg l^{-1}), yeast extract (5 g l^{-1}), and L-cysteine (0.5 g l^{-1}) in an anaerobic atmosphere (80% N_2, 10% CO_2, and 10% H_2).

2.3. Cell lysis and DNA extraction

Bacterial samples were subjected to five freeze–thaw cycles, alternating between liquid nitrogen and 65°C for 5 min in the presence of β-mercaptoethanol (5 μl ml^{-1}), followed by bead-beating as described previously [2] to lyse cells. DNA was extracted from cell lysates using the method of phenol/chloroform extraction and ethanol precipitation as described [2].

2.4. Random cloning of 16S rRNA genes

16S rRNA genes were amplified by PCR from genomic DNA of mucosal bacteria using eubacterial primers F8 (5′-AGAGTTTGATCCTGGCTCAG-3′) and R1492 (5′-GGTTACCTTGTTACGACTT-3′). PCR reaction mixtures were the same as described previously [2]. The amplification 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. PCR products were cloned into the 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.

2.5. Sequence and phylogenetic analysis

Partial 16S rDNA sequences corresponding to *Escherichia coli* 16S rRNA bases 400–1050 were compared directly with the GenBank, EMBL, and DBJI non-redundant nucleotide databases using BLAST. Sequence alignment and phylogenetic analysis were conducted as described previously [2]. Briefly, cloned 16S rDNA sequences and closely related reference sequences were manually aligned using the program SeqPup (Don Gilbert, Biocomputign Office, Biology Department, Indiana University, Bloomington, IN, USA). Phylogenetic trees were generated using a neighbor-joining method [10], in the PHYLO_WIN package [11], with pair-wise gap removal and Jukes–Cantor correction [12]. In order to validate the tree, statistical bootstrapping [13] was carried out with data resampled 1000 times. Sequences were also compared by generating similarity matrices. Putative chimeric sequences were identified using the program Check Chimera [14].

2.6. T-RFLP (terminal restriction fragment length polymorphism) analysis

Two pairs of eubacterial primers, F8 and R1492, F8 and R926 (5′-CCGTCAATTCCTTTRAGTTT-3′) [15] were used for T-RFLP. F8 was labeled with 6-FAM (6-carboxyfluorescein, Applied Biosystems) while the reverse primers were labeled with NED (Applied Biosystems). PCR conditions were the same as those for random cloning. Aliquots of amplified rDNA products were separately digested with *Alu*I, *Hha*I, and *Msp*I (New England Biolabs) according to the manufacturer's instructions. The lengths of T-RFs were determined by comparison with Rox-labeled internal standards using an ABI PRISM™ 377 Automated DNA sequencer and GeneScan® Analysis Software (Applied Biosystems).

3. Results

3.1. Diversity and phylogenetic analysis

Microscope-counts of bacterial cells were 10^{10}–10^{11} cells g^{-1} cecal digesta and about 10^{11} cells in total recovered from the cecal mucosa of one bird. More than 90% of bacterial cells stained Gram-positive.

Partial sequences of 116 random 16S rDNA clones from bacteria present in the cecal mucosa were analyzed. In addition to *E. coli*, at least 48 other molecular species were obtained among cloned sequences. The presumptive relationships of these sequences were obtained from database comparison. As shown in Table 1, 75% of cloned sequences exhibited a 95% or higher identity to database sequences, and were assigned to the closest genus. There were 29 cloned sequences (25%) with less than 95% of

Table 1
Bacteria found in the mucosa of chicken ceca

Cloned rDNA sequence	Homology to database sequence[a]	
	≥95%	<95%
Bacilli	1	2
Butyrate-producing bacteria	27	2
Clostridia	5	1
Enterococcus cecorum	7	0
E. coli	1	0
Eubacteria	2	2
F. prausnitzii	7	0
Holdemania filiformis	1	1
Lactobacilli	4	0
Ruminococci	11	4
Streptococcus alactolyticus	2	0
Uncultured bacteria from human feces[b]	14	9
Uncultured rumen bacteria[b]	5	6
Uncultured bacteria from mouse gut[b]	0	1
Others	0	1
Total number of clones	87	29[c]

[a]Based on a BLAST analysis of partial 16S rDNA sequences corresponding to *E. coli* 16S rRNA bases 400–1050.
[b]Sequences showing a similarity in the databases to those identified in human feces, bovine rumen, or mouse gut.
[c]GenBank accession numbers: AF429354–AF429382.

relatedness to database sequences. We also analyzed full-length 16S rRNA gene sequences from the seven predominating cloned sequences to confirm the homology determined by partial sequence analysis. Only two clones exhibited 1% of variation in the sequence homology, suggesting that partial sequences used in this study can represent full-length sequences for the analysis.

Phylogenetic analysis revealed that the cloned sequences were mainly those of low G+C, Gram-positive bacteria (Fig. 1). This group was highly diverse, with many sequences being related to sequences identified in human feces [16] or in the bovine rumen [2]. There were 11 groups comprising four or more closely related sequences. The largest group comprised sequences (34) more or less related to *Fusobacterium prausnitzii*. Of the 116 clones, more than 25% were related to butyrate-producing bacteria.

3.2. T-RFLP analysis of bacterial populations present in the mucosa and lumen

T-RFLP analysis was used to compare bacterial populations in the mucosa and lumen. Fig. 2 shows the T-RFLP profiles generated by *Hha*I and *Msp*I digestions. While *Msp*I and *Alu*I were unable to differentiate the two bacterial populations significantly, *Hha*I generated several major T-RFs with a preference in the mucosa or lumen. For instance, one T-RF (197 bp) was mainly located in the mucosa, which was hardly detected in the lumen (Fig. 2A).

3.3. Comparison of culture-dependent and -independent mucosal bacteria

Two random 16S rDNA clone pools from the mucosal samples were compared. One was the pool (culture-independent) used for the phylogenetic analysis, the other was generated from the mucosal bacteria grown on BHI agar for 2 days. As shown in Table 2, the BHI-cultured sample had a less diverse bacterial population and a less complicated community structure than the uncultured sample.

4. Discussion

Previous studies using culture-based methods have defined cultivable microflora in the chicken gut (reviewed in [1]). The present study was to investigate the diversity and phylogenetic relationships of mucosa-associated cecal bacteria, both cultivable and non-cultivable, by molecular analysis of 16S rRNA genes. 16S rDNA analysis has shown greater diversity of the bacterial population in the chicken ceca than had previously been achieved by the culture approach. Twenty nine out of 116 cloned sequences showed less than 95% homology to database sequences, which may represent new species [17] previously unidentified in the chicken gut. Some sequences were related to uncultured bacteria reported from human feces [16] or from the bovine rumen [2]. While the ecological and physiological role of these bacteria remains to be determined, it is likely that previous estimates by cultures may have overestimated certain groups of bacteria, such as lactobacilli and clostridia, in the cecal microflora. Gut-surface-associated bacterial population has long been of a research interest because of their importance in pathogen control, immune modulation, and their effects upon nutrient absorption by their hosts. Our observations reported here may, therefore, have significant implications for the health and nutrition of the host, and are particularly relevant for the development of probiotics and their effective use.

T-RFLP analysis has become a useful tool for the studies of microbial ecology, as a generated T-RFLP profile can serve as a 'community fingerprint' to characterize a particular microbial community [15]. In this study, the use of *Hha*I was able to differentiate bacterial populations present in the mucosa and lumen, as indicated by the seven major polymorphic bands which were located differently in the mucosa or lumen (Fig. 2A). It is unclear at present that which bacterial groups contribute to the polymorphism. However, our sequence analysis of full-length 16S rRNA genes from the seven predominating cloned sequences revealed that one group butyrate-producing bacteria would generate a 5′-T-RF upon digestion with *Hha*I which has a similar size to one of the seven polymorphic bands (197 bp). In addition, our recent cloning and sequence analysis suggested that the butyrate-producing bacteria were present in the mucosa, but not in the

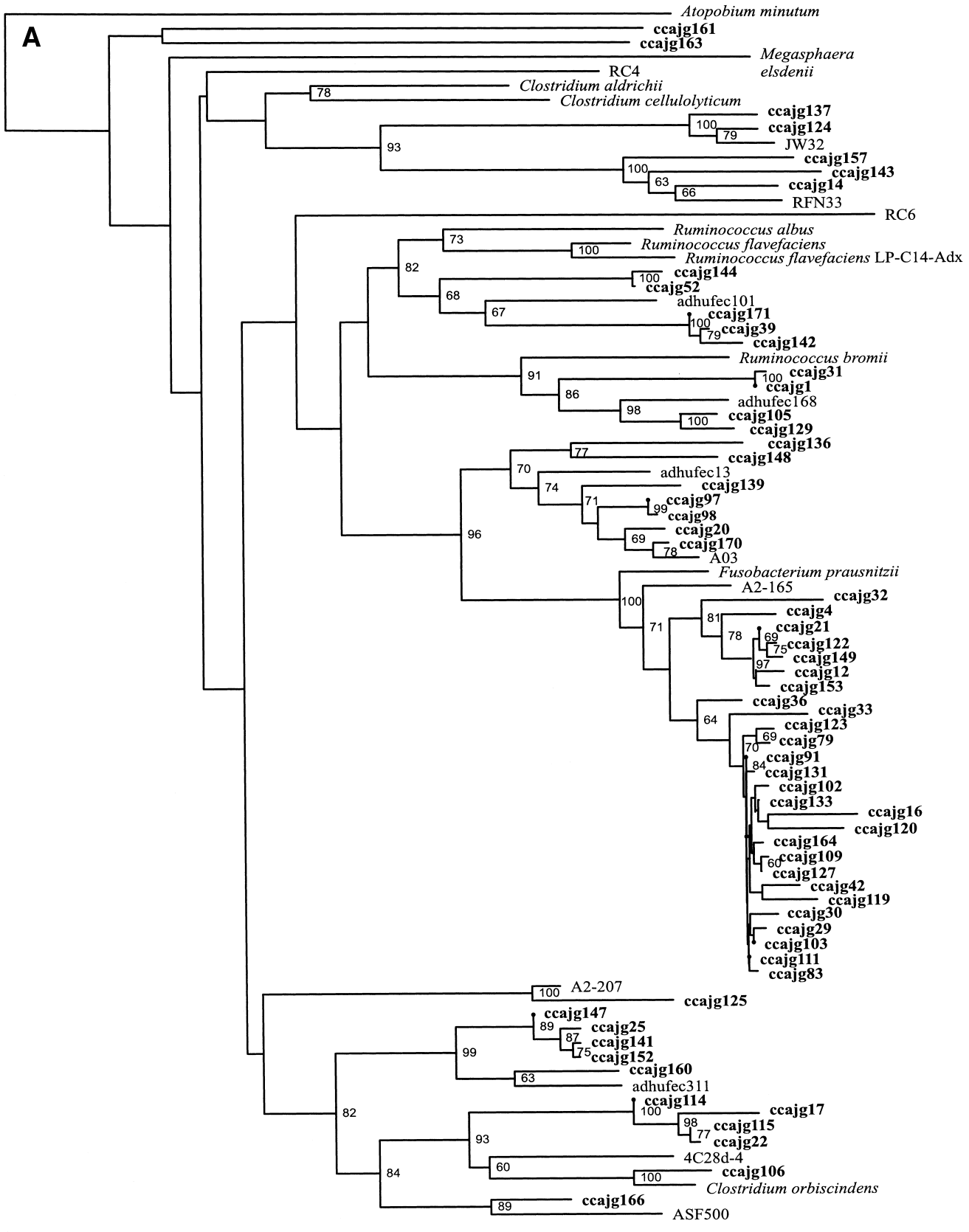

Fig. 1. A,B: Unrooted phylogenetic tree of mucosal bacteria in the chicken ceca constructed using a neighbor-joining method. Our cloned sequences are shown in bold numbers. Bootstrap values for 1000 trees are shown at branch points. Only values of 60% or above are shown. The bar represents a sequence divergence of 0.1%.

Fig. 1. (*continued*)

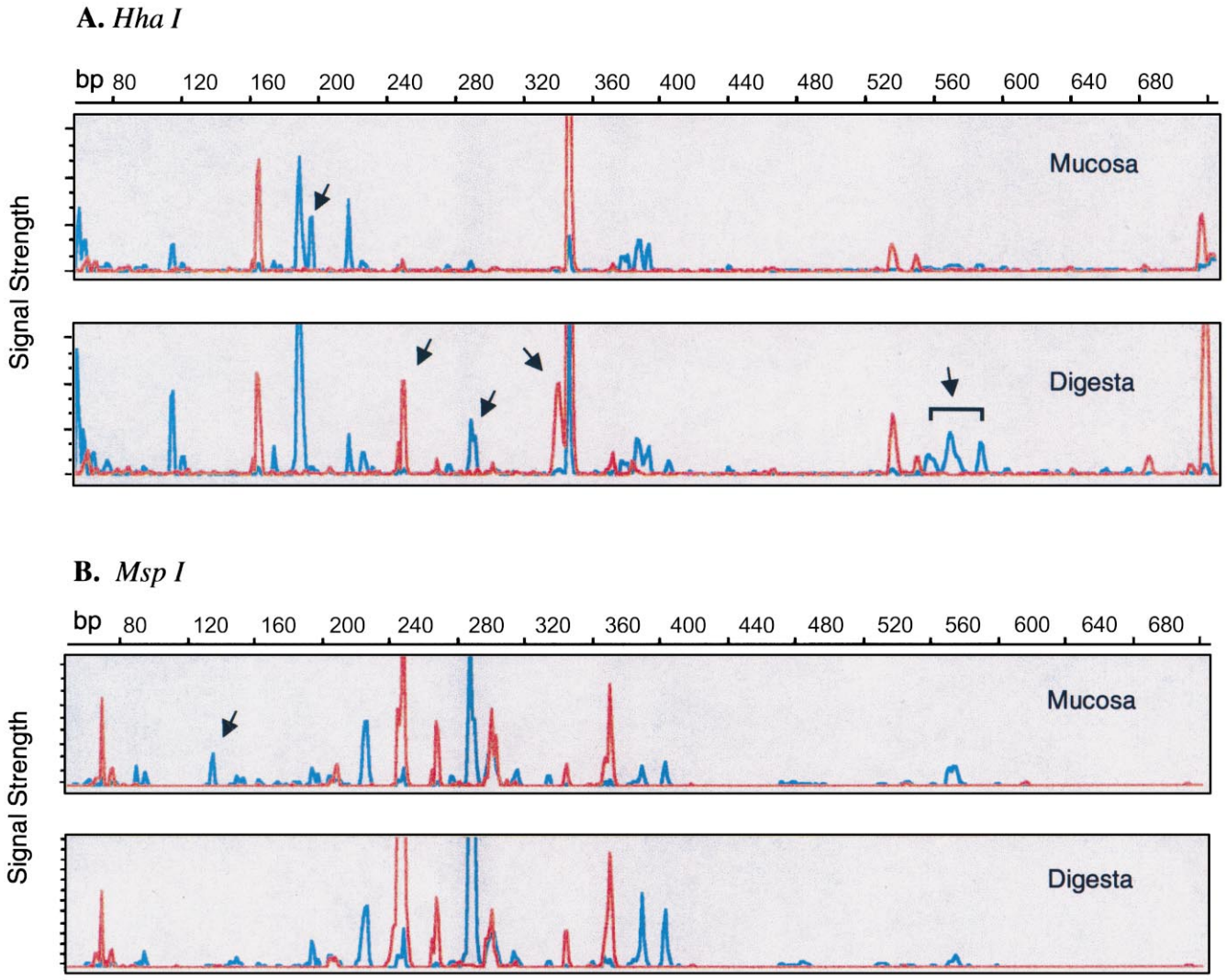

Fig. 2. TRFLP analysis of 16S rDNA amplified by primers F8 and R926 from bacteria present in the mucosa and lumen of chicken ceca. PCR products were digested with *Hha*I (A) or *Msp*I (B). F8 and R926 were labeled with 6-FAM (blue) and NED (orange), respectively. The major T-RF bands with a different location are indicated by arrows.

Table 2
Culture-dependent and -independent bacteria in the mucosa of chicken ceca[a]

	Culture-independent		Culture-dependent[b]	
Cloned sequence	No. of clones	Percent	Number of clones	Percent
Butyrate-producing bacteria	29	25	0	0
Clostridia	6	5	4	7
E. cecorum	7	6	26	44
E. coli	1	1	21	36
F. prausnitzii	7	6	0	0
Lactobacilli	4	4	4	7
Ruminococci	15	13	1	2
Sequences identified in human feces[c]	23	20	2	3
Sequences identified in bovine rumen[c]	11	10	0	0
Sequences identified in mouse gut[c]	1	1	0	0
Others	12	10	1	2
Total	116	101	59	101

[a]Based on a BLAST analysis. Cloned sequences were assigned to the closest database sequences.
[b]Culture-dependent bacteria were grown on BHI agar and analyzed by random cloning and sequence analysis of 16S rRNA genes. Culture-independent bacteria were analyzed by direct DNA extraction from cecal samples, random cloning and sequence analysis of 16S rRNA genes.
[c]Sequences showing a similarity in the databases to those identified in human feces, bovine rumen, or mouse gut.

lumen of ceca (data not shown). These data imply that the major T-RF (197 bp) may represent one group butyrate-producing bacteria.

In our comparison of culture-dependent and -independent bacterial populations from chicken ceca, the bacteria population revealed by the culture-dependent approach exhibited a lower diversity and a less complicated community structure. This may reflect the limitation of the culture method since some bacteria may not be cultivable. Furthermore, any medium used is more or less selective for certain groups of cultivable bacteria. In our comparison, we have presented groups of sequences as percentages of the total number of cloned sequences. This is an approximation since the exact proportion was not necessarily conserved in the PCR step. Alternative molecular methods, such as Real Time PCR, dot blot or in situ hybridization will be needed to confirm these results.

One significant observation in the present study was the predominance of butyrate-producing bacteria in the chicken ceca. These bacteria were more or less closely related to *F. prausnitzii*, or related to unidentified butyrate-producing bacteria isolated from human feces [16]. Butyrate plays an important role in animal health by influencing the regulation, metabolism and development of colonic epithelial cells [18,19]. The ecological and physiological significance of this numerically important group of bacteria in the chicken ceca remains to be elucidated.

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