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Antibiotic Manipulation of Intestinal Microbiota To Identify Microbes Associated with *Campylobacter jejuni* Exclusion in Poultry▽†

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The ability of various subsets of poultry intestinal microbiota to protect turkeys from colonization by *Campylobacter jejuni* was investigated. Community subsets were generated *in vivo* by inoculation of day-old poults with the cecal contents of a *Campylobacter*-free adult turkey, followed by treatment with one antimicrobial, either virginiamycin, enrofloxacin, neomycin, or vancomycin. The *C. jejuni* loads of the enrofloxacin-, neomycin-, and vancomycin-derived communities were decreased by 1 log, 2 logs, and 4 logs, respectively. Examination of the constituents of the derived communities via the array-based method oligonucleotide fingerprinting of rRNA genes detected a subtype of *Megamonas hypermegale* specific to the *C. jejuni*-suppressive treatments.

Campylobacter jejuni, a spiral, flagellated epsilonproteobacterial commensal of poultry, is the predominant cause of bacterial food-borne illness in the United States, resulting in approximately 2 million cases per year. A role for endogenous poultry intestinal microbiota in competitive exclusion (CE) of *Campylobacter* was first investigated in 1982 (38). Since then, numerous studies have attempted to identify microbes associated with *Campylobacter* CE. Suspensions of intestinal bacteria, isolated from *Campylobacter*-free adult poultry and passaged under strict anaerobic conditions, were found to protect chicks from colonization by the pathogen (31). Bacteria derived from the scrapings of broiler intestinal mucosa were proven more effective than the earlier fecal culture, a result not surprising, as *Campylobacter* is known to preferentially colonize cecal crypts (4, 39). The CE function of the bacterial suspensions decreased with time in storage, however (39, 40). Evidence also indicates that CE may depend on the presence of strictly anaerobic bacteria (31). As an oxygen gradient likely occurs from the host epithelium into the luminal contents, a CE role for both mucosal and luminal microbes in concert is likely.

Attempts have been made to identify specific microbes antagonistic to *Campylobacter*, and initial attempts isolated mucin-dwelling organisms with *in vitro* antagonistic effects against the pathogen (35, 36). Recent experiments have identified numerous bacterial groups producing anti-*Campylobacter* bacteriocins (29, 41, 42, 44, 45). Direct treatment of market-weight birds with the therapeutic bacteriocin *Enterococcus faecium* E 50-52 is effective for removal of *Campylobacter* spp. immediately prior to slaughter (44).

Despite progress toward a solution to contamination of poultry products by *Campylobacter* species, incomplete or intermittent CE protection, combined with a lack of studies addressing long-term CE efficacy, indicates that the *Campylobacter* colonization problem is far from solved (35). In addition, risk factors for campylobacteriosis other than direct consumption of contaminated poultry include consumption of fresh vegetables and bottled water (14). *Campylobacter* has been found in poultry manure used to fertilize crops as well as in runoff from these farms (22, 24, 50). We believe that novel approaches for studying microbial ecology in the gut are necessary for development of intervention strategies, including competitive exclusion.

The work described here takes a functional approach to identify microbes associated with protection of the intestine from *Campylobacter jejuni* colonization, an approach we are calling antibiotic dissection. The cecal contents from a *Campylobacter*-free adult turkey were inoculated into day-old poults and the microbial communities in these poults modified by treatment with therapeutic levels of antibiotics. The resulting modified microbiota were then tested for the ability to outcompete a *C. jejuni* challenge, and a microbe potentially associated with *C. jejuni* exclusion was identified.

MATERIALS AND METHODS

Antibiotic dissection. Beltsville White turkey poults were hatched from the *Campylobacter*-free USDA flock housed on the National Animal Disease Center campus, with four poults per treatment. On day 1 after hatch, an adult was culled from the Beltsville White flock. The adult turkey was anesthetized to surgical depth by intramuscular injection with telazol (6 mg/kg of body weight), ketamine (8 mg/kg), and xylazine (4 mg/kg), followed by decapitation. Ceca were removed, and cecal contents were mixed 1:1 (vol/vol) with 1× phosphate-buffered saline (PBS). Poults were randomly assigned into six treatment groups, the uninoculated control (UC), inoculated control (IC), virginiamycin (VIR)-treated, enrofloxacin (ENR)-treated, neomycin (NEO)-treated, and vancomycin (VNC)-treated groups (Table 1). Antibiotics were chosen for both relevance to the poultry industry and variety of target molecule, such that the 50S and 30S ribosomal subunits, as well as RNA polymerase (RNAP) and bacterial cell walls, were targeted (Table 1). In addition, the chosen antibiotics are known to target various combinations of aerobes, anaerobes, and Gram-positive and -negative

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TABLE 1. Treatment groups of poults inoculated with *Campylobacter*- free adult turkey intestinal contents and the antibiotics used to derive the six treatment groups[a]

Treatment group	Adult inoculum	Antibiotic target molecule	Sensitivity			
			Anaerobes	Aerobes	Gram-positive bacteria	Gram-negative bacteria
UC	−					
IC	+					
VIR	+	50S	S	R	S	R
ENR	+	RNAP	R	S	S	S
NEO	+	30S	R	S	R	S
VNC	+	Cell wall	S	R	S	R

[a] UC, uninoculated control; IC, inoculated control; VIR, virginiamycin; ENR, enrofloxacin; NEO, neomycin; VNC, vancomycin. Antibiotic targets and sensitivities are derived from references 8, 19, and 43. S, sensitive; R, resistant.

bacteria, further suggesting that antibiotic treatment would generate varied microbial communities (Table 1). Roughly 0.3 to 0.5 ml diluted adult cecal contents per poult was administered to five of the groups by oral gavage directly into the crop. A remaining control group (UC) was sham inoculated with 1× PBS. Poults were housed in BSL-2 isolators and given UV-irradiated feed and filter sterilized water *ad libitum*. The UC and IC treatment groups received sterile water and food only. In an effort to achieve therapeutic treatment levels, the VIR treatment group received sterile food containing 0.3 mg/g virginiamycin, the ENR group received sterile water containing 0.05 mg/ml enrofloxacin, the NEO group received sterile water containing 0.3 mg/ml neomycin sulfate, and the VNC group received sterile water containing 0.8 mg/ml vancomycin. The therapeutic dose of enrofloxacin was determined from Baytril product inserts. Therapeutic doses of virginiamycin and neomycin were determined from primary literature describing treatment of poultry for necrotic enteritis (18) and colibacillosis (30), respectively. Vancomycin was used at the same dose as avoparcin used to treat necrotic enteritis (33). Avoparcin was not used, as it was never approved for use in the United States, having been linked to vancomycin cross-resistance (51). Individuals within each isolator were assumed to have consumed equivalent antibiotic amounts. To maintain isolation, the birds in these experiments were not weighed; however, weights taken from a separate group of poults suggest these poults were ≤80 g. Antibiotic treatment was maintained for 7 days posthatch. Days 8 through 14 posthatch constituted a resting period allowing antibiotics to be cleared from the intestinal tract. On day 15, poults were challenged via oral gavage with 5×10^3 CFU of chloramphenicol-resistant *Campylobacter jejuni* 11168 (28). *C. jejuni* 11168 was kindly donated by Qijing Zhang from the Department of Veterinary Microbiology & Preventive Medicine, Iowa State University. On day 22 posthatch, poults were killed as described above. One cecum from each poult was frozen on dry ice for storage until DNA could be isolated, and serial dilutions were made in 1× PBS from the contents of the other cecum. All animal experiments were conducted with approval by, and under the guidance of, the National Animal Disease Center institutional animal care and use committee.

***Campylobacter* culture.** Serial dilutions of cecal contents were plated on TSB agar supplemented with 10% defibrinated horse blood, 30 μg/ml cephalothin, and 5 μg/ml chloramphenicol. Plates were microaerobically incubated (5% O_2, 10% CO_2, and 85% N_2) at 42°C for 48 h and *C. jejuni* colonies counted.

DNA extraction. DNA was isolated from 200 mg cross sections of the frozen ceca such that luminal, mucosal, and intracellular microbes would be represented in the libraries. A modified QBiogene Fast Prep method was used to prepare DNA (QBiogene, Carlsbad, CA). Microbial cells were lysed in Boom L6 buffer by shaking with lysing matrix A at 5 m/s in a Fast Prep FP120 device for 30 s as described previously (6). DNA from the lysates was purified using phenol-chloroform extraction and ethanol precipitation.

ARISA. Automated ribosomal intergenic spacer analysis (ARISA) was performed as described previously, using primer set ITSF (5′-GTCGTAACAAGG TAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) (7, 9, 16). Primer ITSReub was 5′ end labeled with the 6-carboxyfluorescein (FAM) fluorochrome (Operon, Valencia, CA). Each 20-μl PCR mixture contained 4 ng template DNA. Similarity matrices were generated using the Dice coefficient, and cluster analysis was performed with the Mega 4.0 software program via the unweighted-pair group method using average linkages (UPGMA) (46).

OFRG analysis. Oligonucleotide fingerprinting of rRNA genes (OFRG) analysis was performed as previously described by Valinsky et al. (48). Briefly, bacterial 16S rRNA gene clone libraries were constructed in *Escherichia coli* DH5α using a USER Friendly cloning kit (New England Biolabs, Ipswich, MA) and PCR primers 27F (5′-GGGAAAGUAGRRTTTGATYHTGGYTCAG-3′) and 1492R (5′-GGAGACAUGBTACCTTGTTACGACTT-3′) (5, 27). PCR was performed in 20-μl reaction mixtures containing 50 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates (dNTPs), 0.5 mg/ml bovine serum albumin (BSA), 400 nM forward and reverse primers, 1 μl fecal DNA, and 1.75 U *Taq* DNA polymerase. Amplification was performed with an initial denaturation at 94°C for 5 min, cycling at 94°C for 30 s (denaturation), 48°C for 40 s (annealing), and 72°C for 60 s (extension), and a final elongation for 2 min at 72°C. The number of amplification cycles varied between 15 and 30 cycles for each DNA sample and was determined from the fewest cycles found to generate a PCR product barely visible when 5 μl was examined on an ethidium bromide-stained agarose gel. A 1,536-clone library was generated, with roughly 250 clones representing each of the treatments.

The 16S rRNA gene clone library was PCR amplified using primers UserOFRGFor2 (5′-TCGAGCTCAGGCGCGCCTTAATTAAGCTGA-3′) and UserOFRGRev2 (5′-GCCAAGCTTCCTGCAGGGTTTAAACGCTGA-3′) in reaction mixtures containing 50 mM Tris (pH 8.3), 0.5 mg/ml BSA, 2.5 mM $MgCl_2$, 250 μM dNTPs, 400 nM forward and reverse primers, 1 μl cells, and 1.75 U *Taq* DNA polymerase (5). Amplification was performed with an initial denaturation at 94°C for 10 min, 35 cycles of 94°C for 1 min and 72°C for 2 min, and a final elongation for 5 min at 72°C. Amplicons were arrayed onto nylon membranes with a multiblot replicator (V&P Scientific, Inc., San Diego, CA) as described previously (48). Membranes were hybridized overnight at 11°C with a set of 10-nucleotide (nt) bacterium-specific ^{33}P-labeled DNA probes (48). Two arrays were hybridized for each probe, stripped as described previously, and rehybridized with universal probe 27F (5′-AGRRTTTGATYBTGGYTCAG-3′). Hybridizations were visualized using a Typhoon variable mode imager (Amersham Biosciences, Pittsburgh, PA), and hybridization signals were analyzed with Image Quant TL image analysis software, version 2003 (Amersham Biosciences, Pittsburgh, PA). Fingerprints containing the designations N (neither positive nor negative hybridization event), 1 (positive hybridization event), and 0 (negative hybridization event) were generated based on control clone hybridization intensities and Bayesian classification (25). Clone fingerprints containing more than 10 uncertain (N) classifications were discarded from further analysis. OFRG fingerprints were clustered using greedy clique partitioning (GCPAT) (http://alglab1.cs.ucr.edu/OFRG/gcpw.php).

Sequence analysis. Clone sequencing was performed using primers UserOFRGFor2 (5′-TCGAGCTCAGGCGCGCCTTAATTAAGCTGA-3′), UserOFRGRev2 (5′-GCCAAGCTTCCTGCAGGGTTTAAACGCTGA-3′), 530F (5′-GTGCCAGCMGCCGCGG-3′), and 907R (5′-CCGTCAATTCMTTT RAGTTT-3′) (5, 27). Sequences were assembled and edited using Lasergene software (DNASTAR, Madison, WI). The sequences were compared to those in public databases by use of NCBI BLAST and Ribosomal Database Project II (RDP-II) (11). *Megamonas hypermegale* sequences were aligned using ClustalX, and phylogenetic analysis was performed using the neighbor-joining algorithm in Mega 4.0 with 1,000 bootstrap replications (46, 47). Sequence similarities were determined using PHYLIP DNAdist and distance-based operational taxonomic unit and richness determination (DOTUR) (15, 34).

Real-time PCR. Real-time SYBR PCR amplification was performed with an iCycler IQ5 optical system according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Reaction mixtures were composed of 1× iQ SYBR green supermix and 400 nM each primer. *Megamonas*-specific primers were designed for this study using the PRISE software program (17). The *Megamonas* type I (5′-ACTAAAGGAGGCCTAGTC-3′) and type II (5′-TCTAAAGGAG GCCTCTGAA-3′) forward primers were paired with the reverse primer MhypR2 (5′-CCCTAACAACAGAACTT-3′). Thermocycling required an initial

TABLE 2. Estimated amounts of antibiotic ingested per day per bird

Treatment group	Amt of antibiotic (mg) ingested per bird on:					Wk 1 daily avg (mg)
	Day 3	Day 4	Day 5	Day 6	Day 7	
VIR	0.32	4.6	1.8	0.6	2.3	1.9
ENR	0.13	0.3	1.3	1.6	1.8	1
NEO	NA[a]	7.5	7.5	11.3	9.8	9
VNC	6.7	13.2	15.0	34.7	25.3	19

[a] Water consumption was not measured.

denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, and a final elongation for 5 min at 72°C.

Species-specific amplification signals were normalized against the universal bacterial 16S copy number quantified using SYBR universal bacterial primers 27F (5′-AGRRTTTGATYBTGGYTCAG-3′) and 342R (5′-CTGCTGCSYCC CGTAC-3′) (27). Real-time PCR with the universal primers was performed with the *M. hypermegale* cycling parameters mentioned above, except a 56°C annealing temperature was used.

Intestinal response to antibiotic treatment. An experimental repeat (no *C. jejuni* colonization was obtained) generated intestinal samples checked for overt intestinal morphological changes due to antibiotic treatment. At necropsy, 1- to 2-cm lengths of ceca were removed from the animals and placed directly into 10% formamide. Tissue samples were allowed to fix for 6 h and then transferred to 70% ethanol. Cross sections of approximately 4 μm were processed in low-melting-point paraffin and stained with hematoxylin and eosin. Images were captured using a Nikon Eclipse e400 microscope with an Optronics Magnafire camera (Galeta, CA). Morphometric measurements of villus height and villus section area were determined with the aid of Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Measurements were taken for the four longest villi per animal, and means of villus height and area were determined for each animal and treatment.

Statistics. Student's *t* test, analysis of variance (ANOVA), Tukey's pairwise comparison, and Spearman's *D* nonparametric rank order test were performed using the Paleontological Statistics (PAST) software package for education and data analysis (20).

Nucleotide sequence accession numbers. Sequences were submitted to GenBank under accession numbers FJ440020 to FJ440103 and FJ489243 to FJ489251.

RESULTS

Antibiotic intake. Therapeutic antibiotic doses were achieved or exceeded for the virginiamycin (therapeutic dose of 0.04 g antibiotic/kg live bird weight), neomycin (therapeutic dose of 0.07 g/kg of live bird weight), and vancomycin (therapeutic dose of 0.02 g/kg live bird weight) groups (Table 2). Birds in the enrofloxacin (therapeutic dose of 0.05 g antibiotic/kg live bird weight) group consumed roughly a quarter of the therapeutic dose.

***Campylobacter* quantification.** *Campylobacter jejuni* levels in the ceca of 21-day-old poults were quantified by plate counts (Fig. 1). Virginiamycin-derived microbiota resulted in an 18-fold *C. jejuni* colonization increase over the level for the control treatments, whereas enrofloxacin-, neomycin-, and vancomycin-derived microbiota reduced *C. jejuni* colonization 1 log, 2 logs, and 4 logs, respectively (Fig. 1). ANOVA and Tukey's least significant difference (LSD) analyses indicated that the virginiamycin increase was statistically significant ($P < 0.001$).

ARISA results. Principal coordinates analysis of the microbiota detected four clusters (Fig. 2). One cluster was composed of samples from the IC, VIR, ENR, and NEO treatments. The other clusters were each composed of samples from individual treatments. The primary axis separated the IC, VIR, ENR, and

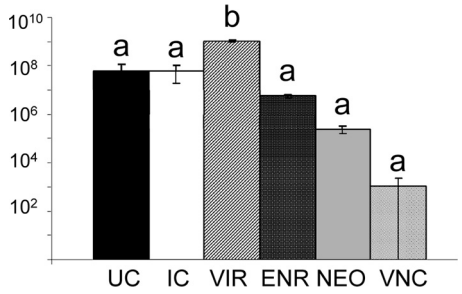

FIG. 1. *Campylobacter jejuni* plate counts from trial 2. Six treatments are represented: poults that received no adult cecal content (UC), poults that received adult cecal contents only (IC), and poults that received both cecal contents and therapeutic levels of one antibiotic, either virginiamycin (VIR), enrofloxacin (ENR), neomycin (NEO), or vancomycin (VNC). *C. jejuni* load was measured for four poults per treatment, with the exception of the VNC treatment (three poults). ANOVA generated an *F* statistic of 63.4 and a *P* value of <0.001. Tukey's least significant difference test identified two statistically significant groups (a and b), as noted above the data bars. Error bars indicate standard errors of the means.

NEO samples from the VNC, UC, and day-old-poult samples, indicating the greatest difference between these two groups. The secondary and tertiary axes separated the VNC, UC, and day-old-poult samples into different quadrants, indicating significant separation between the microbiota in these three groups (see Fig. S1 in the supplemental material).

OFRG results. Oligonucleotide fingerprinting of rRNA genes was used to analyze 1,536 16S clones from antibiotic

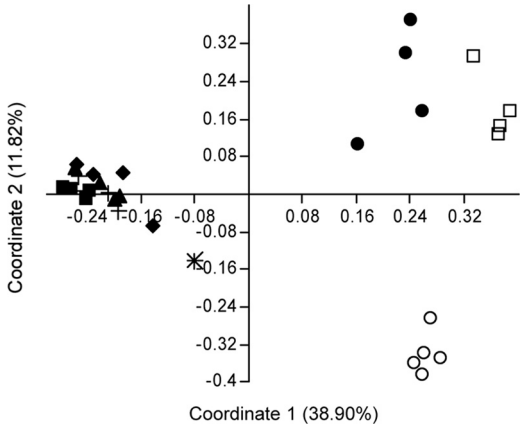

FIG. 2. ARISA fingerprint analyses of trials 1 and 2 combined. The Dice similarity index was used to estimate differences between fingerprints from 3-week-old poults. Treatments include the following: d1, poults killed at day 1 posthatch; UC, uninoculated controls; IC, controls inoculated with *Campylobacter*-free adult intestinal contents; ENR, inoculated and enrofloxacin-treated poults; VIR, inoculated and virginiamycin-treated poults; NEO, inoculated and neomycin-treated poults; and VNC, inoculated and vancomycin-treated poults. "Adult" indicates the *Campylobacter*-free adult intestinal inoculum. "Negative" indicates a no-template ARISA reaction. Principal component analysis was prepared from Dice similarity indices via the Paleontological Statistics software package. Microbiota from all 28 poults and the adult turkey inoculum are represented. ✳, adult; ○, day 1 posthatch; □, uninoculated control; +, inoculated control; ▲, virginiamycin derived; ■, enrofloxacin derived; ◆, neomycin derived; and ●, vancomycin derived. Alternate views of this graph are available in the Fig. S1 in the supplemental material.

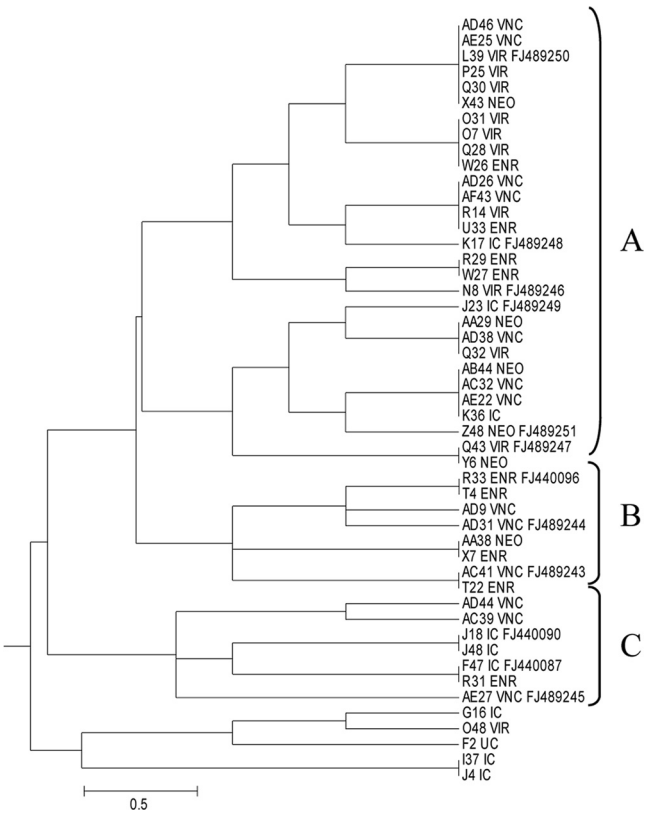

FIG. 3. Dendrogram generated from fingerprints of *Megamonas hypermegale* 16S genes cloned from six microbiota treatments. Fingerprints were clustered using the greedy clique partitioning algorithm. Labels consist of the clone identifier followed by the treatment designation. The sequenced clones display the appropriate GenBank accession number. Clusters B and C are composed of clones from *Campylobacter jejuni*-protective treatments. Cluster A includes *Megamonas* clones from all treatments. A full dendrogram of all 1,200 fingerprints is provided in Fig. S2 in the supplemental material.

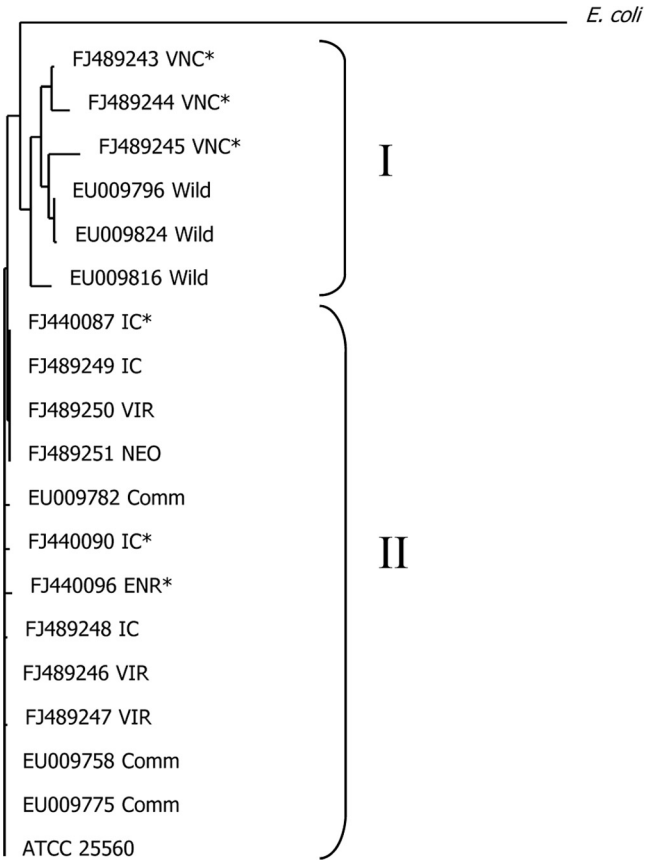

FIG. 4. *Megamonas hypermegale* sequence analysis. Type I, *Megamonas* 16S clones derived from the vancomycin treatment and isolated from wild turkeys in a previous publication. Type II, *Megamonas* clones derived from the inoculated control, virginiamycin, enrofloxacin, and neomycin treatments and isolated from 18-week-old commercially raised turkeys in a previous publication. Types I and II are different at the 97% sequence identity level, as determined by DOTUR analysis. Sequences denoted by an asterisk are derived from Fig. 3, clusters B and C.

dissection trial 2. Clones fell primarily into the *Clostridiales*, *Bacteroidetes*, and *Proteobacteria* taxa (see Fig. S2 in the supplemental material). Two small clusters were composed of clones from the *C. jejuni*-suppressive treatment but not the *C. jejuni*-conducive treatment. Sequence analysis indicated that the cluster was composed of *Megamonas hypermegale* 16S genes (Fig. 3, clusters B and C). An adjacent cluster (cluster A) was composed of 16S clones from both conducive and suppressive treatments. Sequence analysis of the *Megamonas* 16S clones identified two subtypes, type I, containing *Megamonas* clones from the VNC treatments, and type II, containing clones from the IC, VIR, ENR, and NEO treatments (Fig. 2 and 3). These two subtypes were distinct operational taxonomic units (OTUs) at the 97% sequence similarity level as determined by DOTUR analysis and had both previously been detected in turkey cecal microbiota (Fig. 4) (37).

***Megamonas hypermegale* quantification.** Real-time PCR was used to quantify *M. hypermegale* in the cecal contents (Table 3). *Megamonas* spp. were relatively abundant in all antibiotic dissection treatments that received the adult inoculum (Table 3). Type I and type II *Megamonas hypermegale* subtypes showed different responses to the antibiotic treatments, and Student's *t* test indicated significantly higher loads of *M. hypermegale* type II than of type I in all but the IC and VNC treatments (Fig. 4 and Table 3). Spearman *D* nonparametric rank order tests between *C. jejuni* and *Megamonas* type I ($P = 0.188$) and

TABLE 3. Quantification of *Megamonas hypermegale* types I and II in ceca of poults from six antibiotic dissection treatments

Treatment group	No. of real-time target genes/10,000 16S gene copies (SE)[a]		P^b
	M. hypermegale type I	*M. hypermegale* type II	
UC	0	2.2 (0.7) a	0.0300
IC	325 (188) a	5,493 (2,659) a	0.1006
VIR	13 (3) a	2,381 (542) a	0.0047
ENR	0 (0.01) a	2,282 (661) a	0.0136
NEO	32 (10) a	4,088 (1,212) a	0.0155
VNC	978 (164) b	93 (5.1) a	0.0058

[a] One-way ANOVA generated an *F* statistic of 13.55 and a *P* value of 0.0001 for *M. hypermegale* type I and an *F* statistic of 2.706 and a *P* value of 0.0563 for *M. hypermegale* type II. Tukey's least significant difference test identified two statistically significant groups (a and b).

[b] Student's *t* test.

between *C. jejuni* and *Megamonas* type II ($P = 0.323$) did not identify a correlation.

Morphometric measurements. Neither cecal villus height nor villus area differed significantly between treatments (data not shown).

DISCUSSION

In the current work, antibiotic treatment was used to generate subsets of the microbiota from a *Campylobacter*-free turkey flock, and these subsets were tested *in vivo* for competitive exclusion capability. The four antibiotics were chosen for their historic use in commercial poultry production and/or their various modes of action (Table 1). In addition, these antibiotics are controversial because of their potential influence on human health. Virginiamycin is a growth-promoting antibiotic commonly used in poultry production in the United States as well as a therapeutic treatment for necrotic enteritis and aflatoxosis (1, 2). However, virginiamycin also causes cross-resistance to quinupristin-dalfopristin, the drug of last resort that is used to treat patients with vancomycin-resistant *Enterococcus faecium* or methicillin-resistant *Staphylococcus aureus* infections (8, 12). Use of the antibiotic avoparcin for growth promotion was banned in Europe in 1997 because a statistically significant association exists between the use of the antibiotic in poultry production and the occurrence of vancomycin-resistant *Enterococcus* species (26). Subsequent to the ban, significant reductions have been observed in the poultry carriage rates of vancomycin-resistant enterococci (49). Enrofloxacin (Baytril) is a fluoroquinolone used to treat poultry for *Escherichia coli* and *Pasteurella* infections. Treatment of poultry with various fluoroquinolones, including enrofloxacin, induces ciprofloxacin cross-resistance in a variety of intestinal pathogens (23). Ciprofloxacin is the drug of choice for treatment of campylobacteriosis; however, recent increases in ciprofloxacin resistance are reducing the utility of this drug for efficacious treatment (13, 52).

C. jejuni colonization was inhibited to various levels by differently derived microbial subsets. Morphometric analysis of the gross structure of the cecal villi did not differ between treatments; however, fingerprint analysis of the antibiotic-derived consortia indicated that community composition was unique to each of the treatments (Fig. 2). Communities harbored by day-old poults, vancomycin-treated poults, and the 3-week-old uninoculated controls were all different from one another and very different from the other three antibiotic-derived communities (Fig. 2). As initial cecal communities are known to be dominated by lactobacilli, clostridia, and enterobacteria, principal component analysis (PCA) and clustering results are consistent with predicted vancomycin resistance shown by aerobes (53). In contrast, ARISA patterns from virginiamycin-derived communities clustered with the adult and inoculated control (IC) profiles, suggesting predominantly anaerobic communities (Fig. 2). This result was a bit unexpected, as virginiamycin and vancomycin are predicted to have similar ranges of activity (Table 1). However, the necessarily limited scope of antibiotic sensitivity testing for different bacterial species appears to have masked the true sensitivity ranges *in vivo*.

A repeat of the animal trial was performed to determine the repeatability of community selection using antibiotics *in vivo*. Fingerprint analysis of the antibiotic-derived consortia indicated that community composition was unique to each of the trials (see Fig. S3 in the supplemental material). It is not surprising to note that, independent of antibiotic treatment, ultimate microbiota composition is dependent on the initial community. This is demonstrated by the clustering of communities by experiment (e.g., clusters I and II).

In 1979, *M. hypermegale* was suggested as a competitive exclusion strain against *Salmonella enterica* (3). On the basis of 16S sequence, *M. hypermegale*, previously named *Bacteroides hypermegas*, was recently reassigned to the phylum *Firmicutes*, family *Acidaminococcaceae* (21, 32). *M. hypermegale* cells are large, up to 15 μm long, are obligately anaerobic, require fermentable sugars, and produce acetic and propionic acids (21). This analysis of the antibiotic-derived communities indicated two small clusters of *Megamonas hypermegale* 16S genes containing only clones from *C. jejuni*-suppressive treatments (Fig. 3, clusters B and C). DOTUR analysis of the *Megamonas* sequences indicated two subtypes at the 97% sequence identity level (Fig. 4). One type was composed of *M. hypermegale* sequences from the vancomycin-derived community, and the second type contained *M. hypermegale* sequences from the remaining treatments. The *Megamonas hypermegale* type strain, ATCC 25560, belongs to *Megamonas* type II (Fig. 4) (10). Examination of *Megamonas* sequences from a previous study indicated the presence of *Megamonas* type I in wild turkeys (GenBank accession no. EU009816, EU009796, and EU009824) and *Megamonas* type II in commercial birds (GenBank accession no. EU009775, EU009758, and EU009782) (Fig. 4).

To confirm whether *M. hypermegale* colonization correlated with *C. jejuni* suppression, real-time quantification was performed on the antibiotic-treated populations. *Megamonas* type I was significantly enriched in the VNC (*C. jejuni* suppressive) treatments compared to the levels for the other treatments, while *Megamonas* type II was (not significantly) suppressed (Table 3). Spearman *D* analysis of *C. jejuni* and *Megamonas* loads revealed no direct correlation. However, the analysis was performed across antibiotic treatments. If *Megamonas* is one of a group of organisms involved in CE, this correlation may have been obscured. Further perusal of the antibiotic dissection data, perhaps by combining trial results and metagenomic analysis, is needed to identify more microbes associated with *C. jejuni* exclusion. In addition, it will be necessary to isolate both *Megamonas* types and perform *in vivo* competition experiments against *C. jejuni*, as only the presence of *M. hypermegale* type I appears to correlate with *C. jejuni* suppression.

The preliminary results described here suggest a correlation between vancomycin-derived microbiota, including *M. hypermegale* type I, and *C. jejuni* suppression. In addition, a correlation is suggested between virginiamycin-derived cecal microbiota and the enhanced ability of *C. jejuni* to colonize (Fig. 1). In light of growth-promoting virginiamycin use on commercial farms in the United States, it will be interesting to determine whether type I *Megamonas* strains are virginiamycin sensitive.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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