Enumeration and Diversity of Campylobacters and Bacteriophages Isolated during the Rearing Cycles of Free-Range and Organic Chickens

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Campylobacters and *Campylobacter*-specific bacteriophages were isolated and enumerated during the rearing cycle of free-range (56 days) and organic chickens (73 days) at 3-day intervals from hatching until slaughter. In both flocks *Campylobacter jejuni* was the initial colonizer but *Campylobacter coli* was detected more frequently from 5 weeks of age. The diversity of the *Campylobacter* isolates was examined by pulsed-field gel electrophoresis of SmaI-digested genomic DNA and antimicrobial resistance typing. Bacteriophages were isolated from 51% (19 of 37 birds) of *Campylobacter*-positive organic birds ($\log_{10}$ 2.5 to $\log_{10}$ 5.7 PFU/g of cecal contents). The bacteriophages were all typical group III *Campylobacter* bacteriophages in terms of genomic size but could be characterized in terms of their host range and placed into five different groups. In contrast to the organic birds, anti-*Campylobacter* activity (bacteriocin-like) was observed in 26% (10 of 38 birds) of *Campylobacter*-positive free-range birds, and only one bacteriophage was isolated. Appearance of either bacteriophages or anti-*Campylobacter* activity was associated with changes in the levels of colonization and the predominant genotypes and species isolated. The frequency and potential influence of naturally occurring bacteriophages and/or inhibitory substances on the diversity and fluctuations of populations of campylobacters have not previously been reported in either free-range or organic chickens.

Campylobacter jejuni and *Campylobacter coli* are gram-negative bacteria that are ubiquitous in the environment (26). *Campylobacter* infection is the most frequent bacterial cause of enteric disease in England and Wales, with case estimates numbering over 359,000 for the year 2000 (1). The majority of these cases are due to *C. jejuni* (90%), with *C. coli* the second most prevalent species, contributing to around 26,000 cases in 2000 (12, 39). *C. jejuni* and *C. coli* can be isolated from a wide range of sources but are particularly prevalent in avian species including domestic poultry (9, 19). Approximately 80% of raw chicken meat sold in the United Kingdom has been found to be contaminated with *Campylobacter* (21). Human infection may therefore be acquired through the consumption of undercooked poultry contaminated with intestinal material during processing or through cross-contamination of other food products (18, 22). In one study the relative distribution of the two species on raw poultry meat was 27% for *C. coli* and 73% for *C. jejuni* (15).

Organically produced and free-range poultry are becoming an increasingly important sector of the retail chicken market in the United Kingdom and elsewhere. Organically produced birds are slaughtered at around 73 days old. There are strict rules regarding the use of antimicrobial substances; the birds are fed on organically produced feed and are allowed access to the outside environment. Free-range birds are slaughtered at around 56 days old and are generally raised with outside access

at low stocking densities. In contrast, intensively reared birds are slaughtered at 36 to 42 days old and are reared in an enclosed environment with higher stocking densities.

Once hatched, campylobacters are usually undetectable in intensively reared chickens until at least 10 days of age, with most becoming colonized with campylobacters after 2 to 3 weeks (10, 26). This may be due to the protective activity of maternal antibodies against *Campylobacter* colonization in the first few days (6, 33). Once one bird is colonized, all the flock become positive within a few days, probably through bird-to-bird transmission (26). The principal sites of colonization are the ceca, large intestine, and cloaca, where the levels of colonization can be high, in the range of 10^6 to 10^{10} CFU/g of excreta (3, 7, 37). Flock positivity is dependent on flock size (4). It is also dependent on the type of production system used. Positive flocks are generally more frequent among organic and free-range chickens than among intensively reared birds, possibly due to increased environmental exposure (14, 16). Consistent with exposure of the chickens to different environmental sources is the finding that organic and free-range chickens can be colonized with multiple *Campylobacter* isolates (26).

Campylobacter-specific bacteriophages have been isolated from broiler chickens (8, 13), retail poultry (2), and other sources including pig manure, abattoir effluents, and sewage (23, 35). Some of these bacteriophages have been characterized and form the basis of the United Kingdom phage typing scheme (11, 34). Owing to their environmental exposure, campylobacters colonizing extensively reared birds are more likely to be subject to challenge with bacteriophages. Bacteriophages have several characteristics that make them attractive as therapeutic agents or agents of biocontrol; these include their effectiveness in killing their target bacteria, their speci-

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ficity, natural residence in the environment, and the fact that they are self-replicating and self-limiting (38). Well-controlled animal models have demonstrated that phages can prevent or treat animals infected with certain pathogenic bacteria and may be good alternatives for the treatment of drug-resistant infections (5). However, despite their potential, there is little information available regarding the prevalence and influence of bacteriophages on campylobacters colonizing extensively reared poultry flocks.

In this study we describe the enumeration and diversity of campylobacters and bacteriophages isolated during the rearing cycle of free-range and organic chickens. The purpose of this work was to study interactions between campylobacters, bacteriophages, and other potential anti-*Campylobacter* agents in the course of rearing chicken flocks exposed to the environment.

MATERIALS AND METHODS

Bacterial strains. *Campylobacter* reference strains were kindly supplied by the Campylobacter Reference Unit (Health Protection Agency, Colindale, London, United Kingdom) as described by Frost et al. (11).

Source of samples. A United Kingdom organic chicken farm was selected where a flock of approximately 6,000 birds were reared to organic standards. A similar number of birds were raised according to free-range standards on a second farm at a different geographic location. Before 2004, organic birds in the United Kingdom were reared from hatchlings taken from conventional breeder-layers. All birds in this study were Ross broilers from a commercial hatchery. Birds were reared on single species farms featuring open ranges with optional shelter provided.

Isolation of *Campylobacter* from organic and free-range chickens. Three birds were selected at random from organic and free-range flocks every 3 or 4 days from hatching until depopulation. Fresh excreta samples were collected from chicks up to 8 days of age. After this time three birds were removed from the flock, culled, and transported directly to the laboratory for dissection and analysis. The remaining flocks were depopulated for commercial processing at either day 73 for organic birds or day 56 for free-range birds. The ceca from sample birds were removed by sterile dissection, and the contents were collected for *Campylobacter* isolation. Serial dilutions were made by using Maximum Recovery diluent (catalogue no. CM733; Oxoid, Basingstoke, United Kingdom). Volumes (100 µl) of each dilution were then spread on the surface of modified cefoperazone charcoal deoxycholate agar (mCCDA) selective medium (CM739 and selective supplement SR155; Oxoid). The plates were then incubated at 42°C for 48 h under microaerobic conditions (5% O_2, 5% H_2, 10% CO_2, and 80% N_2) before being examined for typical *Campylobacter* colonies. Ten or more typical *Campylobacter* colonies were examined by Gram stain and wet mount and subcultured on blood agar made from blood agar base number 2 (CM 271; Oxoid), with 5% defibrinated horse blood (SR0050C; Oxoid) added. Oxidase and catalase tests were performed for confirmation of the *Campylobacter* isolates. The isolates were speciated by using both a conventional hippurate test and species-specific PCR with primers described by Linton et al. (24). To determine the limit of detection of one species of *Campylobacter* in the presence of another and to validate the enumeration data, we mixed *C. jejuni* and *C. coli* isolates at various ratios before reisolation and speciation from mCCDA plates.

Antimicrobial susceptibility. Antimicrobial sensitivity testing was performed by using the agar dilution and disk diffusion methods in accordance with National Committee for Clinical Standards guidelines (25). Breakpoint concentrations to distinguish antibiotic-resistant isolates were taken from those published by the U.S. National Antimicrobial Resistance Monitoring System or the British Society for Antimicrobial Chemotherapy. Standard test *C. jejuni* strains were employed to monitor antibiotic resistance and susceptibility. These strains are documented by Randall et al. (31) and form part of the panel of campylobacters used in the European Union project ARBADII. Antibiotics were purchased either from Sigma (Poole, United Kingdom) or pharmaceutical retail. The antibiotics selected for testing and quantities used in the disk assay (Oxoid) were as follows: penicillin, 10 µg; kanamycin, 30 µg; gentamicin, 10 µg; bacitracin, 10 µg; nalidixic acid, 30 µg; vancomycin, 30 µg; spiramycin, 100 µg; neomycin, 30 µg; ampicillin, 10 µg; tetracycline, 30 µg; ciprofloxacin, 5 µg; erythromycin, 15 µg; streptomycin, 10 µg; and chloramphenicol, 30 µg.

Isolation of phages. Cecal contents (1 g) were resuspended in 9 ml of SM buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, and 0.01% gelatin; Sigma Aldrich) and incubated at 4°C for 24 h with gentle agitation to allow phages to elute into the buffer. An aliquot of this suspension (1 ml) was then subjected to centrifugation at $3,000 \times g$ for 3 min to remove bulk debris, and the supernatant was then subjected to a further centrifugation step at $13,000 \times g$ for 5 min prior to filtration through a 0.2-µm-pore-size membrane filter (Minisart; Sartorius, Gottingen, Germany) to remove any remaining bacterial cells. The bacteriophages were isolated and enumerated as previously described (2).

Lytic spectra. Bacteria lawns were prepared by using each *Campylobacter* reference strain as previously described (2). In addition, each of the contemporary *Campylobacter* isolates from the organic or free-range birds was tested for sensitivity to the phages. Bacteriophages were plaque purified, and test suspensions (adjusted to contain approximately 10^8 PFU/ml) were applied as 10-µl spots to preprepared bacterial lawns and allowed to absorb into the top layer agar. The plates were then incubated for 24 h at 42°C under microaerobic conditions. Lysis of the *Campylobacter* reference strains was recorded if 20 or more plaques were visible (11).

Pulsed-field gel electrophoresis (PFGE). Genomic DNAs from *Campylobacter* isolates were prepared, digested with the SmaI restriction enzyme, and resolved by using a Bio-Rad CHEF-DRII system as described by Ribot et al. (32).

PFGE of phages to determine genome size. To determine the genome size of the bacteriophages, genomic DNA was prepared in agarose blocks as previously described (2).

RESULTS

Enumeration of campylobacters from organic and free-range chickens. Campylobacters were isolated from 68.5% (17 of 54) of the organic birds and 90% (38 of 42) of the free-range birds over the rearing cycles. Campylobacters were first isolated from the organic flock at 31 days of age in a single bird of the three sampled. In the free-range flock campylobacters were first isolated much earlier, at 8 days. The highest mean count of *Campylobacter* in the cecal contents of organic chickens (Fig. 1A) was $\log_{10}$ 9.7 CFU/g of cecum on day 38 while the lowest mean count was $\log_{10}$ 4.5 CFU/g of cecum on day 31 (first isolation). In free-range chickens the highest mean count of *Campylobacter* organisms in cecal contents was $\log_{10}$ 9.2 CFU/g on day 38 (Fig. 1B), while the lowest mean count was $\log_{10}$ 6.7 CFU/g recorded on day 56. Following initial colonization campylobacters were detected throughout the rearing period in both flocks, with the exception of a single bird at day 31 from the free-range flock. After day 45 the campylobacters enumerated from birds of the organic flock fell by $\log_{10}$ 2 over a 14-day period to reach minima of $\log_{10}$ 7.4 CFU/g of cecal contents at day 59 before recovering at day 69. A similar pattern was observed for the free-range birds after the 45-day sample point, although due to a 56-day rearing time for this flock the *Campylobacter* count did not recover (Fig. 1A and B).

Enumeration of *Campylobacter* bacteriophages in organic and free-range chicken. Bacteriophages were prevalent in the cecal contents of organic birds with 51% (19 of 37) of *Campylobacter*-positive birds also carrying phages. The numbers of bacteriophages present in the cecal contents of organic birds varied between $\log_{10}$ 2.5 and $\log_{10}$ 5.7 PFU/g of cecal contents. In contrast to the *Campylobacter* counts, the phage titers showed marked variation between birds collected at the same time point from the organic flock. The scatter plot in figure 1C represents the variation in the phage titers between individual birds taken at each time point. The phage titers show the greatest variability shortly after colonization and in later stages of the rearing period. All the phages isolated could be propagated on the *Campylobacter* universal propagation strain
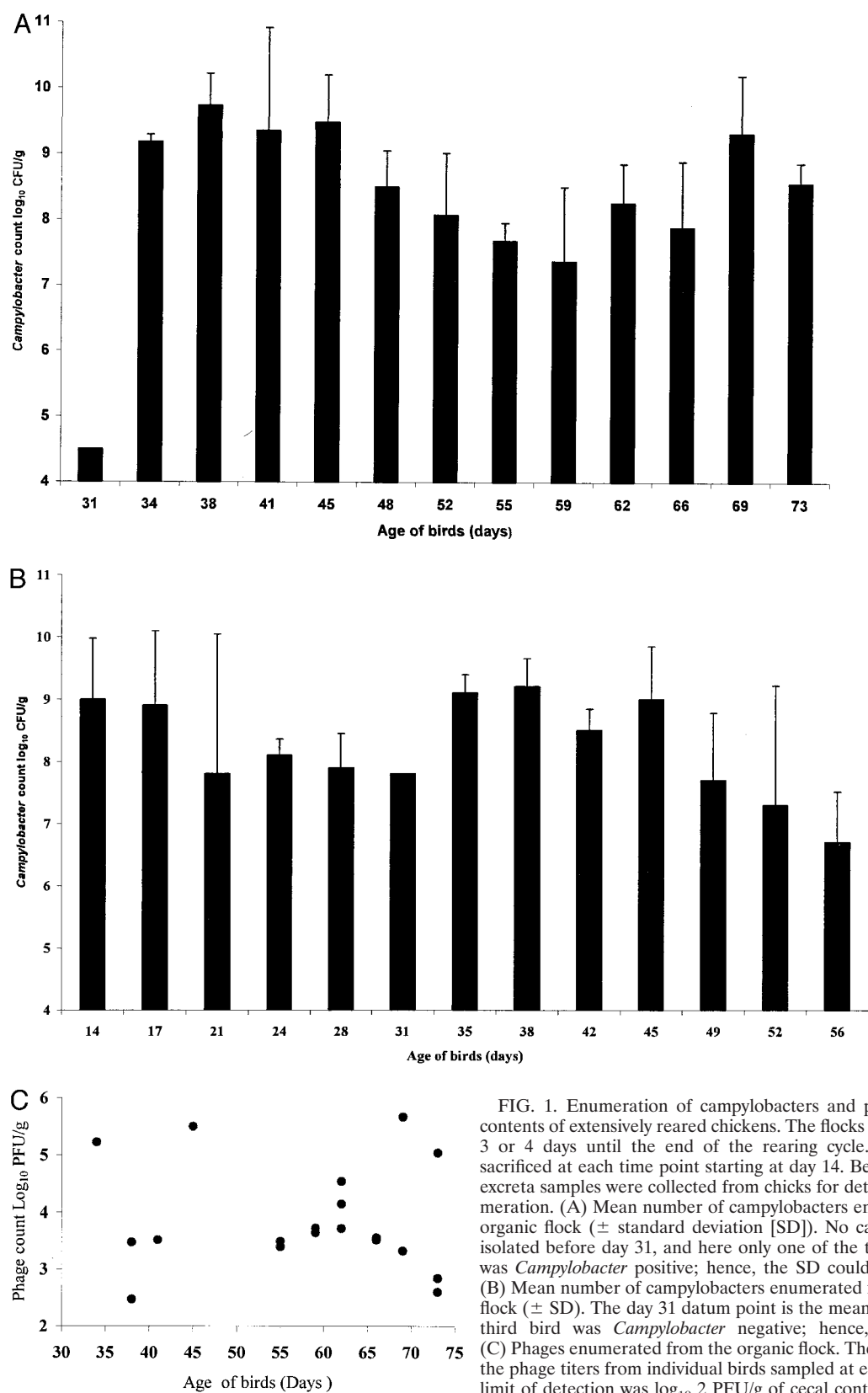
FIG. 1. Enumeration of campylobacters and phages in the cecal contents of extensively reared chickens. The flocks were sampled every 3 or 4 days until the end of the rearing cycle. Three birds were sacrificed at each time point starting at day 14. Before this date fresh excreta samples were collected from chicks for detection without enumeration. (A) Mean number of campylobacters enumerated from the organic flock ($\pm$ standard deviation [SD]). No campylobacters were isolated before day 31, and here only one of the three birds sampled was *Campylobacter* positive; hence, the SD could not be calculated. (B) Mean number of campylobacters enumerated from the free-range flock ($\pm$ SD). The day 31 datum point is the mean of two birds as the third bird was *Campylobacter* negative; hence, there is no SD. (C) Phages enumerated from the organic flock. The data points record the phage titers from individual birds sampled at each time point. The limit of detection was $\log_{10}$ 2 PFU/g of cecal contents.

TABLE 1. *Campylobacter* and bacteriophage isolation from cecal contents of three organically reared birds removed
from the flock at 3- or 4-day intervals over 73 days

Age (days)	Bird 1			Bird 2			Bird 3		
	Species isolated	PFGE MRP[a]	Phage isolated[b]	Species isolated	PFGE MRP[a]	Phage isolated[b]	Species isolated	PFGE MRP[a]	Phage isolated[b]
31	*C. jejuni*	OR1	−			−			−
34	*C. jejuni*	OR1	−	*C. jejuni*	OR1	−	*C. coli*	OR2	φ1A
38	*C. coli*	OR3	φ2A	*C. coli*	OR2	−	*C. coli*	OR2	φ3A
41	*C. coli*	OR2	−	*C. coli*	OR2	−	*C. coli*	OR2	φ4A
45	*C. coli*	OR4	−	*C. coli*	OR2	−	*C. coli*	OR4	φ5A
48	*C. coli*	OR2	−	*C. coli*	OR5	−	*C. coli*	OR2	−
52	*C. coli*	OR4	−	*C. coli*	OR4, 2	−	*C. coli*	OR2, 6, 7	−
55	*C. coli*	OR7	−	*C. coli*	OR2	φ6A	*C. coli*	OR7, 9	φ7A
59	*C. coli*	OR7	−	*C. coli*	OR6	φ8A	*C. coli*	OR2, 4	φ9A
62	*C. coli*	OR4, 5	φ10A	*C. coli*	OR2, 6, 10	φ11A	*C. coli*	OR4, 5	φ12A
66	*C. coli*	OR7	−	*C. coli*	OR7	φ13A	*C. coli*	OR7	φ14A
69	*C. coli*	OR6, 11, 13	φ15A	*C. coli*	OR12	φ16A	*C. coli*	OR8	−
73	*C. coli*	OR14	φ17A	*C. coli*	OR7, 15	φ18A	*C. coli*	OR11, 16	φ19A

[a] PFGE MRP was the arbitrary number given to each different PFGE MRP identified when genomic DNA was digested with the SmaI restriction enzyme.
[b] Bacteriophages were numbered according to order of isolation and with suffix A; −, no lytic activity.

(*C. jejuni* NCTC 12662). In contrast, zones of lysis were observed on primary bacterial lawns with the material that had passed through 0.2-μm-pore-size filters from *Campylobacter*-positive samples of the free-range flock (10 of 38 birds), but the majority of these could not be propagated as typical *Campylobacter* phages. A single bird from the free-range flock at day 21 produced bacteriophages that could be propagated.

Diversity of campylobacters. *C. coli* organisms were the predominant *Campylobacter* species isolated from both organic and free-range chickens. *C. jejuni* was isolated between days 31 and 34 from three birds of the organic flock (3 of 37 birds), but all subsequent isolates were *C. coli* (Table 1). Campylobacters were isolated from the free-range flock much earlier than the organic and were initially all *C. jejuni* from day 8 until replaced by *C. coli* at day 31 and returning towards the end of the rearing period (Table 2). Two *Campylobacter*-positive birds harbored both species (5%). The number of *Campylobacter*-positive birds with *C. coli* alone from the free-range flock was 50% (19 of 38 birds). The remaining 17 birds harbored *C. jejuni*

(45%). We investigated whether the presence of one *Campylobacter* species isolate could influence the recovery of another on mCCDA isolation plates by mixing the *C. jejuni* and *C. coli* isolates before plating and picking 100 individual colonies. There was no observable advantage for any species isolate over another in these experiments; the recovery rates were simply related to the input ratios of the two species.

The genetic diversity of the strains isolated from both flocks was examined by SmaI digestion of genomic DNA from five single-colony isolates for each bird, separated by PFGE to allow comparison. This revealed a highly diverse population of isolates. Each different SmaI-type macrorestriction pattern (MRP) was given an arbitrary number to allow comparison. There were 16 different MRPs in the organic flock and 9 different MRPs in the free-range flock. There were no common MRPs to both flocks, but certain MRPs dominated at different points in the rearing cycle and were then replaced by new isolates. While most individual birds were infected with indistinguishable isolates by PFGE, nine birds in the organic

TABLE 2. *Campylobacter* and bacteriophage isolation from cecal contents of three free-range birds removed
from the flock at 3- or 4-day intervals over 56 days

Age (days)	Bird 1			Bird 2			Bird 3		
	Species isolated	PFGE MRP[a]	Phage or lytic activity[b]	Species isolated	PFGE MRP[a]	Phage or lytic activity[b]	Species isolated	PFGE MRP[a]	Phage or lytic activity[b]
8[c]	*C. jejuni*	F1	−	*C. jejuni*	F1	−	*C. jejuni*	F1	−
14	*C. jejuni*	F2	−	*C. jejuni*	F2	−	*C. jejuni*	F2	−
17	*C. jejuni*	F2	−	*C. jejuni*	F2	−	*C. jejuni*	F2	−
21	*C. jejuni*	F2	−	*C. jejuni*	F2	−	*C. jejuni*	F2	φ1B
24	*C. jejuni*	F2	−	*C. jejuni*	F2	−	*C. jejuni*	F2	−
28	*C. jejuni*	F2	ZL	*C. jejuni*	F2	ZL	*C. jejuni*	F3	ZL
31	*C. jejuni*	F3	−	*C. jejuni* and *C. coli*	F3, 4	−			−
35	*C. coli*	F4	ZL	*C. coli*	F4	−	*C. coli*	F4	−
38	*C. coli*	F4	−	*C. coli*	F4	−	*C. coli*	F4	−
42	*C. coli*	F4, 5	−	*C. coli*	F4	−	*C. coli*	F4, 6	−
45	*C. coli*	F4	ZL	*C. coli*	F4	−	*C. coli*	F4	−
49	*C. coli*	F7, 8	ZL	*C. coli*	F4	ZL	*C. coli*	F4, 9	ZL
52	*C. coli*	F4	ZL	*C. coli*	F4	ZL	*C. jejuni* and *C. coli*	F2, 8	−
56	*C. jejuni*	F2	−	*C. coli*	F4	−	*C. coli*	F4	−

[a] PFGE MRP was the arbitrary number given to each different PFGE MRP identified when genomic DNA was digested with the SmaI restriction enzyme.
[b] Phage or lytic activity classified as follows: the single propagatable bacteriophage isolate was numbered φ1B, −, no lytic activity; ZL, non-propagatable zone of lysis.
[c] At early time points the campylobacters were not enumerated from ceca but isolated from fresh voided excreta.

TABLE 3. Antimicrobial resistance patterns of *Campylobacter* isolates from organic and free-range birds

Campylobacter MRP	Species	MICs (mg/liter) of antimicrobials (breakpoints)[a]											
		PEN (NA)	KAN (>16)	GEN (>16)	NAL (>32)	SPI (>10)	NEO (>25)	AMP (>32)	TET (>16)	CIP (>4)	ERY (>8)	STR (>16)	CHL (>32)
OR1	*C. jejuni*	5	>100	5	10	2	>100	5	>80	2	2	>100	10
OR2-3, OR6, OR11, OR13-14	*C. coli*	30	10	5	10	2	5	5–10	5	2	2–5	10	10
OR4, OR5, OR12	*C. coli*	50	10	5	10	2	5	35	5	2	2	30	10
OR7, OR9, OR10, OR16	*C. coli*	50	10	5	10	2	5	50	5	2	2–5	10	10
OR8	*C. coli*	30	20	5	10	2	5	5–10	5	2	2	10	10
OR15	*C. coli*	30	5	5	10	2	5	20	5	2	2	10	10
F1	*C. jejuni*	30	10	5	10	2	5	5–10	5	2	2	20	10
F2	*C. jejuni*	50	10	5	10	2	5	50	5	2	2	10	10
F3, F8	*C. jejuni*	100	10	5	10	2	5	>100	5	2	2	10	10
F4	*C. jejuni*	100	20	5	2	2	5	>100	>80	2	2	10	10
F5	*C. coli*	>100	10	5	>120	10	5	>100	>80	>10	2	50	10
F6	*C. coli*	30	10	5	10	2	5	10	50	2	2	10	10
F7	*C. jejuni*	50	20	5	2	2	5	>100	5	2	2	10	10
F9	*C. coli*	>100	10	5	10	2	5	>100	>80	>10	2	10	10
F10	*C. jejuni*	>100	10	5	5	2	5	>100	5	2	2	10	10

[a] PEN, penicillin; KAN, kanamycin; GEN, gentamicin; NAL, nalidixic acid; SPI, spiramycin; NEO, neomycin; AMP, ampicillin; TET, tetracyclin; CIP, ciprofloxacin; ERY, erythromycin; STR, streptomycin, CHL, chloramphenicol. Resistance-classified breakpoint concentrations are given in parentheses. NA, not available.

flock (23%) and seven birds in free-range flock (18%) had two or more different MRPs, indicating a mixed population of campylobacters within a single bird.

Antimicrobial susceptibilities. *Campylobacter* isolates ($n \geq 5$) representative of each of the MRPs ascribed from the organic and free-range flocks were tested for their susceptibilities to 12 antibiotics prescribed in human and veterinary medicine. The susceptibility or resistance of each of these isolates to the antibiotics was determined by using agar dilution and agar disk diffusion methods. The test methods were consistent with each other and revealed differences in antibiotic sensitivity profiles between MRP groups but no variation was evident between *Campylobacter* isolates of the same MRP. The MICs of these antibiotics against the *Campylobacter* isolates are recorded in Table 3. There is a marked difference in the frequencies of antibiotic resistance between the two flocks, where multiple antibiotic-resistant types can be observed in the free-range flock, irrespective of the *Campylobacter* species, and were all but absent in the organic flock. The exception to this was the initial colonizing isolate of *C. jejuni* from the organic flock (MRP organic isolate 1 [OR1]), which was resistant to kanamycin, neomycin, tetracycline, and streptomycin. The antibiotic tolerances of the free-range flock isolates were sufficiently different to enable the discrimination of almost all the MRP groups with the exception of MRP free-range isolate 3 (F3) and F8.

Potential influence of phages and/or inhibitors on *Campylobacter* numbers and diversity. Bacteriophages were enumerated from diluted cecal material of organic birds by counting plaques produced on bacterial lawns. In most cases in the free-range birds, a clear zone of lysis was observed on the host bacteria lawn that could not be propagated. Moreover, the antimicrobial activity did not produce discreet plaques upon dilution. The origin of this anti-*Campylobacter* activity is not known but is reminiscent of bacteriocin activity. Tables 1 and 2 show the different strains (identified by their PFGE MRPs) isolated from individual birds at different time points to establish if there was any relationship between presence or absence

of phages or lytic activity and the replacement of an established resident strain. The results show the disappearance of *C. jejuni* MRP OR1 in the organic flock at the point when bacteriophages were first isolated. A variety of *C. coli* strains were isolated along with bacteriophages for the rest of the trial period. For the free-range flock, bacteriophages were isolated from a single bird at day 21 (Table 2). These bacteriophages did not proliferate or influence the recoverable campylobacters since no further bacteriophage isolates could be identified in this flock and *C. jejuni* isolates of the same MRP F2 persisted until day 28. At day 28 anti-*Campylobacter* activities (zones of lysis) were first observed, and this event was accompanied by the appearance of the MRP F3 in one of the three sample birds. At day 31 the F3 MRP was present in two birds and in one in conjunction with the first appearance of *C. coli* MRP F4. The third sample bird at day 31 had no detectable *Campylobacter* colonization ($<\log_{10} 2$ CFU/g of cecal contents). By day 35 *C. jejuni* had been replaced by *C. coli* in all sample birds and did not reappear until the anti-*Campylobacter* activity was absent near the end of the rearing period.

Bacteriophage characteristics. Bacteriophages from 19 organic birds and one free-range bird were propagated on *Campylobacter* strain NCTC 12662. PFGE revealed all the phages to have a genome size of approximately 140 kb and, therefore, to be typical of the group III phage described by Sails et al. (34). Representative phage isolates were sequentially plaque purified and characterized by examination of their lytic profiles against 13 National Collection of Type Cultures reference *Campylobacter* strains that represent the various *Campylobacter* classes of phage-typing scheme adopted in the United Kingdom (11). These experiments revealed that the 19 bacteriophages isolated from organic birds could be grouped into five distinct host lytic profiles (Table 4). The single phage isolate from the free-range flock, φ1B, had a distinct profile from the organic isolates. Those within each lytic group were probably closely related phages. Only phage isolate φ18A (from bird 2 on day 73) was capable of propagation on any of

TABLE 4. Lytic spectra of phages against reference strains

Strain or phage		Lytic pattern[a]					
	Species	1	2	3	4	5	6
Campylobacter reference strain							
PT1 (C605)	*C. jejuni*	R	R	R	S	S	R
PT2 (C682)	*C. jejuni*	R	R	R	R	R	R
PT5 (C856)	*C. jejuni*	S	R	S	R	S	S
PT6 (C594)	*C. jejuni*	R	S	S	S	S	R
PT19 (C11288)	*C. jejuni*	S	S	S	S	S	S
PT33 (C1312)	*C. jejuni*	S	S	S	S	S	S
PT34 (C13503)	*C. jejuni*	S	R	S	R	S	S
PT35 (C13553)	*C. jejuni*	S	S	S	S	S	R
PT44 (C10131)	*C. jejuni*	R	R	R	R	R	R
NCTC 12661	*C. jejuni*	R	S	S	S	S	R
NCTC 12662	*C. jejuni*	S	S	S	S	S	R
NCTC 12668	*C. coli*	R	R	R	R	R	R
NCTC 11168	*C. jejuni*	R	S	S	S	S	R
Phages[b]		φ1A, φ2A, φ3A, φ7A	φ4A, φ5A, φ9A, φ10A, φ11A	φ6A, φ17A	φ8A, φ12A, φ13A, φ15A	φ14A, φ16A, φ18A, φ19A	φ1B

[a] Lytic activity patterns produced by the bacteriophage isolates. S, susceptable to bacteriophage (≥20 plaques visible on each reference strain), R, resistant to bacteriophage (≤20 plaques on each reference strain).
[b] Phages producing the same lytic pattern were grouped together.

the campylobacters isolated from the same source at the same time (*C. coli* OR15).

DISCUSSION

As anticipated for birds that are exposed to the environment, both the organic and free-range flocks examined in this study were colonized by campylobacters, and the colonization was evident at the point of slaughter (range, $\log_{10}$ 5.2 to $\log_{10}$ 10.8 CFU/g of cecal contents). However, genotyping by using PFGE profiles from SmaI-digested DNA revealed that the strains isolated changed as the birds aged. Intensively reared flocks in Europe have been shown to be colonized with a limited number of *C. jejuni* subtypes, but greater diversity is common in conventional flocks from other countries, such as the United States and Australia, and may be associated with greater exposure to the environment (26). The results presented here show a surprisingly high incidence of *C. coli* in both types of birds (92% in organic birds and around 43% in free-range birds), although in each case *C. jejuni* was the first species to colonize. Studies with intensively reared birds generally show the opposite proportions of the two species, with 80 to 90% of isolates being *C. jejuni* and the remainder being *C. coli* (21, 40). To validate our observations, we examined whether any species isolate had a selective growth advantage over another on the isolation medium. The recovery rates were simply related to the input ratios of the two species; we therefore presume the species dominance we note is a reflection of the *Campylobacter* populations of the cecal contents of the sample birds. Petersen et al. (29) reported that adult hens were more readily colonized by *C. coli*, and so our results may be a result of the extended rearing periods of free-range and organic birds as well as their environmental exposure.

There was a notable difference in the incidence of antibiotic resistance between the organic and free-range flocks. The use of antibiotics in the rearing of organic chickens is prohibited, and as might have been anticipated, the organic flock was

colonized by campylobacters susceptible to the majority of the antibiotics tested. However, it is not clear why antibiotic resistance was evident in the free-range flock since antimicrobials as growth promoters have been banned in the European Union since 1998 and since no veterinary treatment was prescribed in the course of rearing the flock. Cross-resistance to several antibiotics could arise through a common mechanism. For example, through changes in the function or expression of a broad specificity efflux pump, such as *cmeB* recently reported for *C. jejuni* that affects susceptibility to ampicillin, erythromycin, ciprofloxin, and tetracycline (30). However, the susceptibility and resistance profiles of the free-range *Campylobacter* isolates deduced from the antibiotic MICs and sensitivity to antibiotic disks could distinguish almost all the MRP groups, and given that these groups are genotypically distinct and represent more than one species, the evidence would suggest that the flock had been exposed to a diverse set of environmental campylobacters. It is plausible that the vicinity of the range represents a microenvironment due to the impact of rearing successive flocks. During the course of these operations, it would seem likely that veterinary intervention via antibiotic therapy may be required on occasion and that the selection for antibiotic resistance results from these instances. It is common practice to rotate the ranges so that they can be left fallow for a period of time. How effective this procedure is should now be examined with respect to the antibiotic resistance and colonization potential of the *Campylobacter* populations present.

Bacteriophages were prevalent in the birds from the organic flock in relatively high numbers (up to $\log_{10}$ 5.5 PFU/g of cecal contents), with frequent isolation after their first appearance at day 34, only 3 days after initial colonization by campylobacters. In contrast, the bacteriophages were infrequent in the free-range birds. Although all the bacteriophages isolated were typical of group III *Campylobacter* phages characterized by Sails et al. (34) in terms of their genome size, they could be differentiated on the basis of their host ranges. The variation in the cecal phage titers from individual birds sampled through-

out the rearing period may also be indicative of nonsynchronous changes in the populations. The diversity could arise from the acquisition of phages from multiple environmental sources, or, alternatively, once acquired, the phages could adapt within the chicken intestinal tract to maximize the use of the host campylobacters available to them. It is evident that the phages would have encountered several campylobacter genotypes in the course of the rearing period of the organic flock. The bacteriophages isolated toward the end of the rearing period were capable of replication on additional *Campylobacter* reference hosts compared to those isolated earlier. Most notably, the later isolates could replicate on the *C. jejuni* strain C605 representative of PT1, which are distinguished in the present typing scheme by their inability to support replication of 14 out of 16 of the typing phages (11). However, despite their broad virulence against *C. jejuni*, none of these bacteriophages was active against the *C. coli* reference strain NCTC 12668 or, indeed, the majority of the *C. coli* that were frequently isolated from birds of the same flock. Moreover, the first appearance of bacteriophages in a single bird of the organic flock at day 34 coincided with the appearance of *C. coli* as the dominant species instead of *C. jejuni*. This was preceded by the finding that *C. coli* was the dominant species in all three birds sampled at day 38.

Bacteriophages were much less prevalent in the free-range flock, with just one phage isolated. More notable within this group was the incidence of samples that exhibited a lytic activity against campylobacters but which could not be propagated. These lytic zones may have been formed by bacteriocins or other anticampylobacter metabolites produced by the cecal flora (bacteria were removed during the phage isolation but soluble or particulate material of <0.2-μm size would remain). Bacteriocins are a heterogeneous group of proteins or peptides produced by bacteria that kill other bacteria. Many bacteria isolated from chicken intestines can produce bacteriocins in vitro, but whether they have any effect in vivo is largely unproven (20). Antagonistic activity of several bacteria against campylobacters has been shown (7, 17, 36), although it is not clear whether this activity is due to bacteriocins, to production of metabolites such as organic acids, or to a combination of factors. Competitive exclusion can be applied to control the growth of microorganisms in livestock. However, success has been variable for reducing levels of *Campylobacter* colonization (27). Some of the competitive exclusion agents tested were reported to reduce *Campylobacter* colonization and produce anti-*Campylobacter* metabolites in experimental birds (36).

The lytic activity observed in this study became evident at day 28 and preceded a decline in numbers of campylobacters and a change in the dominant species from *C. jejuni* to *C. coli* at day 31. To establish cause and effect for these activities will require further experimentation.

The fact that virtually none of the phages could lyse *Campylobacter* strains isolated from the same source was consistent with other studies of phage-*Campylobacter* ecosystems (2). There are several possible explanations. One possibility is that the campylobacters have acquired resistance to the phages. However, if this were the case, the phage numbers and frequency of isolation would be expected to decline with time. The frequency of isolation certainly did not decline in the organic birds, but too few bacteriophages were isolated from

free-range birds to draw any firm conclusion in this regard. A second possibility is that their specific host *Campylobacter* was actually present in the ceca of the birds but in a mixed culture with a phage-resistant partner. The true host may be present but in such inferior numbers that the frequency of isolation would be rare when the populations were sampled at random. It was notable that a few plaques were observed at the threshold of sensitivity on some of these otherwise nonpermissive *Campylobacter* hosts. It is possible these plaques represent a subpopulation of bacteriophages that have adapted to new hosts. A single phage isolate, φ18A, found late in the rearing period (day 73) was virulent against *C. coli* (OR15) from the organic flock. This phage may have specifically evolved to utilize the *C. coli* host that had been present in the flock since day 52, since it is otherwise indistinguishable in its host range from phage isolates φ14A, φ16A, and φ19A.

This is the first attempt to understand the complex ecology of campylobacters in free-range and organic poultry and the influence of natural predators, such as phages and bacteria producing bacteriocins and or anti-*Campylobacter* metabolites, on the succession of strains that inhabit the intestinal tract. It has not previously been appreciated that such dramatic changes in *Campylobacter* type occur during the life of a broiler chicken and that bacteriophages and bacteriocins may influence which campylobacters are present ultimately on the final poultry meat product. It is of particular note that most intensively reared broilers are killed at approximately 35 days old, which is the time at which we have observed the succession of *C. jejuni* by *C. coli* as the dominant species in the free-range and organic flocks. It is possibly for this reason that *C. jejuni* is the most frequent species isolated from conventional barn-reared broiler chickens (21, 28) and, by inference, the species to which the human population is exposed. Organic and free-range birds are reared over a longer period and are at the same time exposed to a wider variety of environmental campylobacters and to other microbes producing metabolites that may be differentially prejudicial to the campylobacters colonizing the intestinal tracts of the chickens. Environmental campylobacters may therefore independently succeed resident populations through competitive advantage or through the synergistic action of antimicrobial agents that have the potential to provoke changes in the dominance of the campylobacters present in chicken ceca.

Our observations would suggest that not only are there changes in strain type but also the dominant species changes to *C. coli.* In a recent surveillance study in England and Wales, *C. jejuni* was reported to be responsible for more than 12 times the number of cases of human campylobacterosis compared to *C. coli* (12). However, *C. coli* is still a significant cause of human food-borne diarrheal illness even if the risk factors associated with it may be different (39). As the organic and free-range sectors of the market increase, it will be interesting to see whether the number of human cases of *C. coli* also increase proportionally.

An understanding of the complex ecology of bacteriophages and their hosts is essential to the implementation of potential phage therapy applications to reduce numbers of *Campylobacter* organisms in chicken. Bacteriophages isolated in this study could potentially be used as such therapeutic agents or be incorporated into phage-typing schemes.

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