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Signature-Tagged Transposon Mutagenesis Studies Demonstrate the Dynamic Nature of Cecal Colonization of 2-Week-Old Chickens by *Campylobacter jejuni*†

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We have constructed plasmids to be used for in vitro signature-tagged mutagenesis (STM) of *Campylobacter jejuni* and used these to generate STM libraries in three different strains. Statistical analysis of the transposon insertion sites in the *C. jejuni* NCTC 11168 chromosome and the plasmids of strain 81-176 indicated that their distribution was not uniform. Visual inspection of the distribution suggested that deviation from uniformity was not due to preferential integration of the transposon into a limited number of hot spots but rather that there was a bias towards insertions around the origin. We screened pools of mutants from the STM libraries for their ability to colonize the ceca of 2-week-old chickens harboring a standardized gut flora. We observed high-frequency random loss of colonization proficient mutants. When cohoused birds were individually inoculated with different tagged mutants, random loss of colonization-proficient mutants was similarly observed, as was extensive bird-to-bird transmission of mutants. This indicates that the nature of campylobacter colonization in chickens is complex and dynamic, and we hypothesize that bottlenecks in the colonization process and between-bird transmission account for these observations.

Campylobacter jejuni is the most common bacterial cause of food-borne disease in the developed world, with an estimated 1 in 100 individuals in both the United States and the United Kingdom developing campylobacter-related illness each year (2). In developed countries, campylobacteriosis is common in neonates and young adults, with acute symptoms ranging from protracted watery diarrhea to bloody diarrhea with fever, abdominal cramps, and the presence of fecal leukocytes (10, 52). These symptoms usually present after an incubation period of 1 to 7 days and may persist for a week or more. Although the vast majority of cases are self limiting, campylobacter can cause severe postinfection complications, including bacteremia and polyneuropathies such as Guillain-Barré and Miller-Fisher syndromes.

C. jejuni is a thermophilic organism with an optimal growth temperature of 42°C, probably reflecting an adaptation to the avian gastrointestinal tract (44, 48). The bacterium is a common gut commensal of animals destined for human consumption, with fecal contamination of meat during processing a recognized route of transmission to humans (21). The commensal colonization of the domestic chicken is considered to be a major reservoir of *C. jejuni* in the food chain. It is estimated that 80% of raw chicken sold in the United Kingdom is contaminated with campylobacter (13), and studies suggest that the consumption of poultry is responsible for 50 to 70% of all endemic infections that are reported (11). Several approaches have been employed to reduce the incidence of campylobacter in commercial poultry production including increased biosecurity (the treatment of water, control of wild animals and birds, and broiler house cleansing and disinfection) and competitive exclusion (39). Successful development of these methodologies will be greatly enhanced by an understanding of the dynamics of colonization of chickens by *C. jejuni.*

Studies attempting to elucidate colonization factors of campylobacter have concentrated on testing the ability of a limited number of specific mutants of *C. jejuni* to colonize chickens, most commonly in the newborn chick model (3, 12, 31, 35, 36, 42, 54, 55). Chickens in the field, however, are believed to be colonized with campylobacter at around 2 weeks of age (43). A number of physiological changes occur in the 2 weeks posthatching; the gut flora in the 1-day-old chick is rudimentary, is still developing, and does not reflect the complexity of the flora present in older birds (47). Competition in vivo between campylobacter and the fully established gut flora is likely to be a key factor in colonization. A model for colonization in longer-term studies using 2-week-old birds with an established gut flora has therefore been developed, which we consider to be a better reflection of natural conditions (24).

Studies of campylobacter have been hampered by a lack of suitable genetic tools. Recently, however, several groups have developed methods for in vivo and in vitro transposon mutagenesis of *C. jejuni*. These include the development of an in vitro transposon-based signature-tagged mutagenesis (STM) system for *C. jejuni* (18), which the authors used to identify genes involved in colonization of the gastrointestinal tract of

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† Supplemental material for this article may be found at http://aem.asm.org/.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli		
DH5α	F$^-$ (ϕ80d*lacZ* Δ*M15*) Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA relA*	16
BL21(DE3)	F$^-$ *ompT* r_B^- m_B^-1 DE3	45
BW25141	F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) *lacIp-4000*(*lacI*^{q}) Δ(*phoB-phoR*) *580* λ$^-$ *galU95* Δ*uidA3*::*pir*$^+$ *recA1 endA9*(del-ins)::*FRT rph-1* Δ(*rhaD-rhaB*)*568 rrnB-3 hsdR514*	14
EC100D^{TM} *pir-116*	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG pir-116*(DHFR)	Epicentre Technologies
C. jejuni		
NCTC 11168	Clinical isolate used for genome sequencing	34
81-176	Clinical isolate	26
M1	Environmental isolate	D. G. Newell, gift
Plasmids		
pET29b+C9	pET29b+ carrying Q131R and E137K mutations in *Himar1* transposase	29
pRY111	Source of campylobacter *cat* cassette	53
pAJG39	pEnterprise2 with 0.6-kb fragment harbouring *ori*R6Kγ from EZ::TN<R6Kγori/KAN-2> cloned into the SalI site	This study
pAJG40-134	pAJG39 with 0.1-kb fragment harbouring an individual Holden DNA tag cloned into the PstI site	This study
pEnterprise2	pUC19 with 1.5-kb fragment harbouring *cat* and transposon from pEnterprise, cloned as a PmeI fragment into blunt-ended EcoRI and HindIII sites	D. R. Hendrixson, gift; reference 17
EZ::TN<R6Kγori/KAN-2>	Source of the R6Kγ origin of replication	Epicentre Technologies

the 1-day-old chick. STM is a powerful technique that enables a large number of mutants from a small number of animals to be screened simultaneously (20). We have developed an STM system for *C. jejuni*, with the added benefit of efficient rescue of DNA flanking the sites of insertion. We have also investigated the extent to which the *mariner*-based transposons can be said to insert into the *C. jejuni* chromosome randomly. We have attempted to use STM to study the colonization by *C. jejuni* of 2-week-old chickens as opposed to day-old chicks. We show that stochastic effects confound this approach; colonization-proficient mutants are readily lost from mixed-infection studies with simple or complex pools, and a high frequency of bird-to-bird transmission of mutants is observed in experiments where birds individually inoculated with different mutants are cohoused. These observations are presumably a consequence of bottlenecks in the colony establishment process and raise key questions regarding the population dynamics of campylobacter within the chicken and its environment.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *C. jejuni* was cultured at 42°C on Muller-Hinton (MH) agar supplemented with 5% horse blood (Sigma, Poole, United Kingdom) with 5-μg/ml trimethoprim under standard microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) in a MACS VA500 Variable Atmosphere Work Station (Don Whitley, Shipley, United Kingdom). Postmortem isolation of the tagged campylobacter strains was on campylobacter blood-free selective agar (CCDA; CM739; Oxoid, Basingstoke, United Kingdom) containing CCDA-selective supplement (SR155; Oxoid, Basingstoke, United Kingdom). *Escherichia coli* strains DH5α, BL21(DE3), BW25141, and EC100D λ*pir-116* were grown at 37°C on Luria-Bertani (LB) agar or in broth. Where necessary for selection, the medium was supplemented with chloramphenicol (10 μg/ml), ampicillin (100 μg/ml), or kanamycin (25 μg/ml). Long-term storage of bacteria was at −80°C in Microbank vials (Prolab Diagnostics, Neston, United Kingdom).

DNA manipulations. Standard methods were used for molecular cloning (37). The plasmid pEnterprise2 (19), which contains a *mariner*-based transposon (derived from *Himar1*) on a pUC19 backbone, was used to construct the signature-tagged transposons. The *ori*R6kγ origin of replication was cloned into the SalI site of pEnterprise2 as a SalI fragment generated by PCR amplification from plasmid EZ::TN<R6Kγori/KAN-2> (Epicentre Technologies, Madison, WI) using the primers ajg200 and ajg201 (SalI sites incorporated into primers) to generate pAJG39. Primer sequences are listed in Table 2. Transposon tag plasmids pAJG40 to pAJG134 were generated by cloning unique DNA tags (20) into the PstI site of pAJG39 as PstI fragments. The tags were generated by PCR amplification from tag DNA generously donated by David Holden (Imperial College, London, United Kingdom), using the primers ajg204 and ajg205 (PstI sites incorporated into primers). Insertion of tags into pAJG39 was confirmed by restriction digestion with NotI (unique site introduced in ajg204). DNA sequencing was used to confirm that the resulting plasmids had individually identifiable $(NK)_{20}$ tag sequences.

Generation of an in vitro-based signature-tagged mutagenesis system for *C. jejuni*. Transposition reactions were performed as previously detailed (17), using total bacterial DNA (2 μg), transposon tag plasmid DNA (pAJG40 to pAJG134,

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5′–3′)
ajg200	AAAAAAAAGTCGACTAGATCCGAAGATCAGCA
ajg201	GGGGGGGGGTCGACATCCCTGGCTTGTTGTCC
ajg204	AAAAAAAACTGCAGGCGGCCGCTACAACCTCAAGCTT
ajg205	CCCCCCCCCTGCAGCATTCTAACCAAGCTT
ajg227	CCCGGGAATCATTTGAAG
ajg231	CTGCAGGCGGCCGCTACAACCTCAAGCTT
ajg232	CTGCAGCATTCTAACCAAGCTT
ajg287	GGGGGGATCCGTGAAGTATTACTCAATATCA
ajg288	GGGGGGATCCTCATTTCTTTGCTAAAAATTC
ajg354	GAATTCCCCGGGGTTATTTATTCAGCAAGTCTTG
ajg364	GGATCCCCCGGGCTCGGCGGTGTTCCTTTCCAAG
CC001	TAACTTGGAAAGGAACACCG
M13R	AGCGGATAACAATTTCACACAGGA

1 μg) and ~250 ng *Himar1* transposase purified as described by Lampe et al. (30) from *E. coli* harboring pET29b+C9, an expression vector containing the C9 hyperactive *Himar1* transposase (29). The transposed DNA was introduced into *C. jejuni* by natural transformation using a plate biphasic method adapted from van Vliet et al. (49). Transposon mutants were recovered on selective agar after growth under standard conditions for 60 to 72 h. Transposition/transformation efficiencies varied but yielded up to 5,000 colonies/μg of DNA. Mutant strains were assigned designations of T-M where T was the tag number (1 to 95) and M was the mutant number (1 to 50).

Determination of transposition insertion site by plasmid rescue. Chromosomal DNA (~1 μg) from an individual transposon mutant was prepared using the QIAGEN (Crawley, United Kingdom) Genomic DNA kit and digested overnight with an appropriate restriction enzyme; typically, we used AseI, BglII or SspI. DNA was extracted with phenol:chloroform and precipitated with ethanol. Fragments were circularized by ligation with 400 U of T4 DNA ligase (New England Biolabs, Hitchin, United Kingdom) for 16 h at 16°C in a final volume of 50 μl. DNA was precipitated with ethanol and electroporated into *E. coli* strain BW25141 or EC100D λ*pir-116* which contain the *pir* gene as a λ lysogen. Transformants were recovered by selection on LB agar plates containing chloramphenicol, with 48 to 72 h typically required for colonies to grow. A single transformant from each reaction was used to inoculate 10 ml of LB containing chloramphenicol and incubated overnight at 37°C with shaking; plasmid DNA was prepared from the entire culture with the QIAprep Spin Miniprep Kit (QIAGEN, Crawley, United Kingdom). DNA was recovered in elution buffer preheated to 70°C, precipitated with ethanol, and sequenced using primers reading outwards from the transposon. Insertion sites were matched to the genome of NCTC 11168 (34) and the tetracycline (pTet) and virulence (pVir) plasmids of 81-176 (5, 7).

DNA sequencing. DNA sequencing was performed at the University of Cambridge Department of Genetics by dye terminator chemistry. Primers were M13R to sequence the cloned signature tags and ajg227 or CC001 for transposon insertion sites.

Statistical analysis of insertion sites. The positions of the insertion sites were expressed as angles (ϕ) around the circular chromosomal or plasmid sequences (NCTC 11168, EMBL AL111168; 81-176 pVir, EMBL AF226280; and 81-176 pTet, EMBL AY394561) with 0° corresponding to the first base in the database sequence. The distribution of the insertion sites was tested against a null hypothesis of uniformity using the Kuiper statistic, K (28). P values were from Batschelet (8). Under the null hypothesis, insertion sites are equally spaced around the chromosome. Low P values indicate a significant deviation from uniformity.

Experimental animals. Specific-pathogen-free (SPF) Light Sussex chickens were hatched and reared at the Institute for Animal Health, Compton, United Kingdom. To standardize the initial gut flora of birds used in the 2-week-old infection model, chicks were inoculated orally on the day of hatching with 0.1 ml of campylobacter-free adult gut flora preparations. For this, 1 g cecal contents taken from a 50-week-old SPF chicken immediately postsacrifice was used to inoculate 10 ml LB broth and incubated in static culture for 24 h at 37°C. Inoculated birds were housed in high-biosecurity accommodations until they were used in colonization trials. Birds were fed a vegetable-based diet (Special Diet Services, Manea, Cambridgeshire, United Kingdom).

Colonization trials. Two-week-old birds with developed gut flora or 1-day-old chicks (without gut flora) were inoculated orally with 0.1 ml MH broth culture containing 8 $\log_{10}$ CFU of the campylobacter wild-type or mutant bacteria. The ability of strains to colonize the ceca was assessed by enumeration of bacteria present in serial dilutions of postmortem cecal samples resuspended in phosphate-buffered saline buffer at 1 g/ml plated onto CCDA medium. Colonizing ability is expressed as the mean bacterial load (of three chickens). The limit of detection was taken to be 100 CFU/g.

Screening of STM mutants for defects in colonization. Screens for the presence and absence of signature-tagged mutants from pools of bacteria were by a modification of the method of Hensel et al. (20). Confluent lawns of *C. jejuni* isolated from cecal contents were harvested into phosphate-buffered saline and thoroughly resuspended. Chromosomal DNA for first-round PCR was extracted using Genomic DNA Extraction kits (QIAGEN, Crawley, United Kingdom). Alternatively, 1 ml of campylobacter suspension was heated to 100°C for 15 min and centrifuged at 10,000 × *g* for 5 min; and 1 μl of supernatant was used as template for first-round PCR (this method was shown to be sufficient to detect the presence of all tags in test pools) (data not shown). First-round PCR products were concentrated with an Eppendorf Concentrator 5301, separated from primers by electrophoresis thorough 2.5% low-melting-point agarose (preparative grade for small fragments; Promega, Southampton, United Kingdom), and the DNA was excised. Radiolabeled probe was generated by second-round PCR using 1 μl of gel slice melted at 65°C as a template for PCR using [α-^{32}P]dCTP (Amersham Biosciences, Chalfont St. Giles, United Kingdom) and unlabeled dATP, dTTP, and dGTP (Invitrogen, Paisley, United Kingdom). All PCRs were with *Taq* DNA polymerase (Roche, Lewes, United Kingdom) and primers ajg231 and ajg232 (Table 2). The cycling protocol was 94°C for 5 min; 30 cycles, each consisting of 94°C for 30 s, 53°C for 45 s, and 72°C for 10 s; and a final extension of 72°C for 1 min. Invariant arms were removed by digestion with HindIII (Roche, Lewes, United Kingdom). The probe was denatured by the addition of a 1/10 volume 4 N NaOH and used immediately in hybridizations. Hybridization filters were prepared by spotting 50 ng of tag plasmids (pAJG40-134) in 1 μl onto positively charged nylon membrane (Roche, Lewes, United Kingdom), according to the manufacturer's instructions. Negative controls were irrelevant tag plasmids. Prehybridization (1 h) and hybridization overnight were carried out with PerfectHyb Plus (Sigma, Poole, United Kingdom) at 68°C. Washing was at 68°C in 0.1% sodium dodecyl sulfate–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (15 min) and twice in 0.1% sodium dodecyl sulfate–0.1× SSC (30 min each). Filters were used to expose Bio-Max MR film (Kodak, Hemel Hempstead, United Kingdom), typically for 4 h or overnight.

Motility assays. Motility assays were performed essentially as described by Silverman and Simon (41). Briefly, a platinum wire was dipped into a single colony and used to stab MH motility medium containing 0.4% agar. Motility was assessed by measuring colony diameter after incubation at 42°C for 16 h.

RESULTS

Production of signature-tagged transposons. To produce a signature-tagged transposon, it was necessary to include (i) flanking inverted repeats that would be efficiently recognized by a transposase in vitro, (ii) a selectable marker, (iii) a uniquely identifiable signature tag, and (iv) a quick reliable method for identifying the transposon insertion site in the chromosome. Criteria i and ii were satisfied by the kind donation of plasmid pEnterprise2 (19). This is a pUC-based plasmid carrying *mariner* transposon inverted repeats flanking a chloramphenicol resistance marker, *cat*. Criterion iii was fulfilled by the cloning of 40-bp $(NK)_{20}$ DNA tags provided by David Holden downstream of *cat*. Criterion iv was met by inserting an *ori*R6Kγ origin of replication into the transposon to facilitate plasmid rescue of the insertion sites. Initiation of DNA replication at this origin requires the π protein, the product of the *pir* gene (23). The gene encoding this protein is absent from the vector but can be supplied in *trans* by a chromosomal copy from the propagating *E. coli* strain. Insertion sites can be rescued by digestion of transposed chromosomal DNA, ligation to generate circular chromosomal fragments, and introducion of these into an *E. coli* strain carrying a copy of the *pir* gene, most commonly as a λ lysogen. Only fragments containing the *ori*R6Kγ and *cat*, hence the transposon, can replicate to confer chloramphenicol resistance on the host bacterium. Plasmid DNA can then be recovered and sequenced to determine the insertion site. We generated the series of plasmids pAJG40 to pAJG134, each identical apart from a unique signature tag. Sequencing of the plasmids indicated that the *ori*R6Kγ had inserted twice during cloning, but this duplication did not prevent rescue of plasmids.

Testing the STM plasmids in *C. jejuni* NCTC 11168. The ability of the tagged transposon plasmids to mutate *C. jejuni* was tested using strain NCTC 11168. *C. jejuni* NCTC 11168 was originally isolated by Martin Skirrow in 1977 from the feces of a patient with diarrhea (15), and the genome sequence of this strain has been previously reported (34). The donor transposon tag plasmid was incubated with recipient chromosomal DNA and purified transposase; subsequently, insertion sites were repaired and ligated, and transposed DNA was introduced into *C. jejuni* NCTC 11168. Bacteria in which homolo-

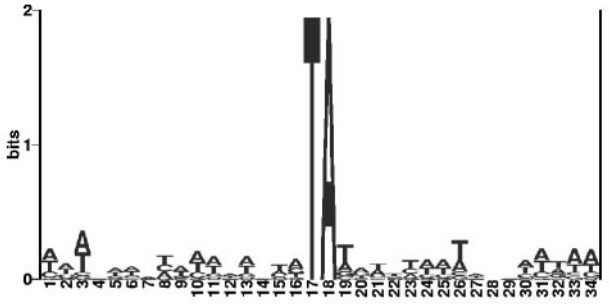

FIG. 1. Sequence logo (38) of insertion sites of the *mariner* transposon in the *C. jejuni* NCTC 11168 chromosome. *y* axis, bits of information for each position (maximum of 2 bits for a nucleotide sequence); *x* axis, nucleotide position (positions 17 and 18 correspond to the target TA dinucleotide). The height of the column indicates the degree of conservation with relative sizes of letters denoting base frequency at that position. The program WebLogo (http://www.bio.cam.ac.uk/cgi-bin/seqlogo/logo.cgi) was used to generate the graphic.

gous recombination had occurred were selected with chloramphenicol. The distribution of insertion sites was determined from randomly picked transformants by Southern hybridization (see Fig. S1 in the supplemental material) and plasmid rescue, followed by sequencing (see Table S1 in the supplemental material). Hybridizing fragments of a range of sizes and insertions throughout the chromosome were observed. We used 12 different tagged transposon plasmids to generate a 600-member mini-library of mutants in this strain.

There is no strong sequence conservation at the insertion sites. To analyze the primary sequence at the insertion site, a sequence logo (38) was constructed for 16 bases either side of the 5′-TA-3′ target using the 42 sequenced NCTC 11168 sites (Fig. 1). Apart from 100% conservation of 5′-TA-3′ at the insertion site, there was no sequence preference in the analyzed range except for a general bias towards A and T residues that would be expected from the *C. jejuni* genome A+T content (69.4%) (34).

Transposon insertions are not distributed uniformly around the recipient DNA. The distribution of transposon inserts in NCTC 11168 was plotted (see Fig. S2 in the supplemental material) and analyzed with the Kuiper statistic, which tests for any deviation from uniformity. Because the transposon only integrates at a 5′-TA-3′ dinucleotide, Kuiper statistics were also calculated considering only 5′-TA-3′ locations to rule out the possibility of bias in the distribution of 5′-TA-3′. There was evidence for deviation from uniformity (Table 3). Visual inspection suggested a bias towards an extended region of the target molecule in the vicinity of the origin, although insertions were located throughout the chromosome (see Fig. S2 in the supplemental material).

TABLE 3. Kuiper statistics for distribution of transposon insertion sites in *C. jejuni* DNA[a]

Target DNA	Statistic		
	N	K_{all}	K_{TA}
NCTC 11168	42	2.537 ($P < 0.001$)	2.537 ($P < 0.001$)
81-176 pVir	19	1.617 ($P < 0.1$)	1.614 ($P < 0.1$)
81-176 pTet	17	1.794 ($P < 0.05$)	1.782 ($P < 0.05$)

[a] N, number of samples; K_{all}, Kuiper statistic considering all nucleotides; K_{TA}, Kuiper statistic considering 5′-TA-3′ only; *P*, level of significance.

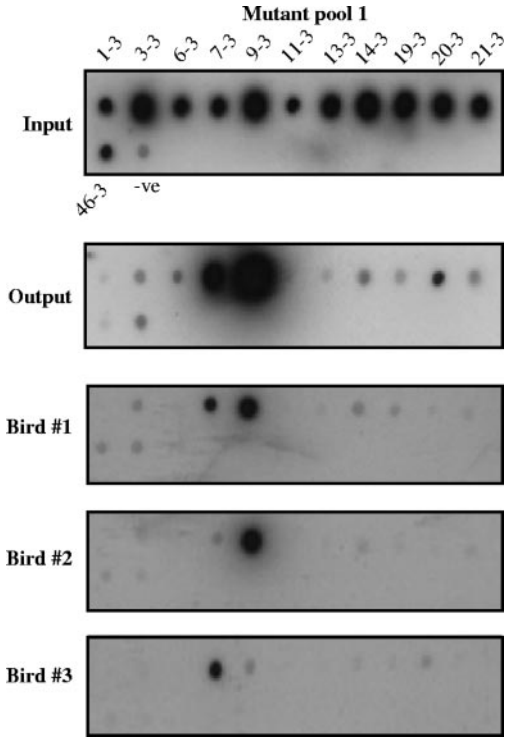

FIG. 2. Representative blot showing presence of signature tags in a *C. jejuni* 81–176 mutant pool experiment. Input, tags present in inoculum; Output, tags recovered from cecal contents at day 7 postinoculation (pool of three birds); (-ve), negative control (irrelevant tag on filter). Also shown are the hybridizations from bacteria recovered from individual birds.

Colonization of 2-week-old chickens by *C. jejuni* 81-176. *C. jejuni* NCTC 11168 colonizes chickens with low efficiency soon after infection (24). We therefore sought a strain that was transformable and would efficiently colonize and persist in our model. We chose *C. jejuni* 81-176, one of the best-characterized strains of campylobacter. *C. jejuni* 81-176 was isolated from an outbreak in Minnesota and has been shown in two volunteer feeding studies to cause diarrheal disease (10, 26), as well as colonizing the avian gastrointestinal tract in a 1-day-old chick model of infection (18). SPF Light Sussex chickens harboring a standardized normal gut flora were orally inoculated at 2 weeks of age with 8 $\log_{10}$ CFU of *C. jejuni* 81-176. After 7 days, all chickens were colonized with bacterial numbers in the ceca of around 9 $\log_{10}$ CFU/g of cecal contents. The colonized birds appeared healthy and showed no signs of disease, in agreement with different models for this and other strains (9, 18, 42).

High-frequency, random loss of *C. jejuni* 81-176 mutants from pools. We generated a 700-member mutant library in *C. jejuni* 81-176 using 14 signature tags. This library was used to screen for mutants defective in colonization of the ceca of 2-week-old birds carrying adult gut flora. We constructed pools containing 12 to 13 differently tagged transposon mutants of *C. jejuni* 81-176 by mixing bacterial cultures at equivalent optical densities. Pools were used to orally inoculate three 2-week-old

TABLE 4. Presence of tagged mutants in two experiments sampling cecal contents at up to day 4 postinoculation with a pool of 12 *C. jejuni* 81-176 signature-tagged mutants (pooled output from three birds per time point)[a]

Expt and day	Mutant											
	1-3	3-3	7-3	9-3	11-3	13-3	14-3	19-3	20-3	21-3	30-3	46-3
Expt 1												
Day 1	+	+	+	+	−	+	+	+	+	+	+	−
Day 2	+	+	+	+	−	+	+	+	+	+	+	−
Day 3	+	+	+	+	−	−	−	+	−	+	−	−
Day 4	−	+	+	+	−	+	−	+	−	−	−	−
Expt 2												
Day 1	+	+	−	+	−	+	+	+	+	+	−	−
Day 2	+	+	−	+	−	+	−	+	+	−	−	−
Day 3	−	−	−	−	−	−	−	+	+	−	−	−

[a] All mutants were present in input pools (not shown). +, tag detected; −, tag not detected.

SPF Light Sussex chickens, carrying a normal gut flora, with 8 $\log_{10}$ total mutant campylobacters (input pool). Cecal contents were collected at day 7 postinfection, and the presence of tagged mutants was assessed. We screened 6 pools; typically, 60 to 85% of mutants were absent from the output pool at day 7 postinfection (Fig. 2).

This frequency of mutant loss was high, and we suspected that random loss of colonization proficient mutants was occurring. To investigate this possibility, we chose one pool of 12 mutants to analyze in detail. The pooled output in this experiment contained only two tagged mutants, designated 7-3 and 9-3 (Fig. 2). First we determined the mutants present in the three individual birds; bird no. 1 had both mutants 7-3 and 9-3, bird no. 2 had only 7-3, and bird no. 3 had only 9-3 (Fig. 2). Next, we investigated the extent to which the time after inoculation affected recovery of each mutant. In these experiments, three birds per time point were inoculated with the same pool of mutants, with mutant 6-3 replaced by 30-3, and housed in the same cage. Three birds were taken at each time point for determination of mutants present in the ceca. We observed an increase in loss of mutants as time progressed (Table 4). Interestingly, a repeat of this experiment produced a different data set, with different mutants present or absent on different days. Moreover, the mutants present at day 7 in the original experiment (7-3 and 9-3) were absent from this repeat at day 3. The between-bird and between-experiment variation suggested that there was nothing specific about the different mutants that resulted in their presence or absence, i.e., the experiments were not simply demonstrating a high frequency of genuine noncolonizing mutants. To confirm this, the transposon insertion site from each mutant was determined, and each mutant was tested for its ability to colonize 2-week-old chickens and 1-day-old chicks in pure culture (Table 5). All these mutants colonized to wild-type or near-wild-type levels in both models, with the exception of 11-3 (Student's *t* test; $P < 0.01$). The transposon insertion in this mutant mapped to *rpoN*, consistent with previous studies showing the requirement of this gene for colonization (18). During studies with other pools, we identified a mutant that was consistently absent from output pools. This mutant had a transposon insertion in the gene *kpsC* and colonized in pure culture at $<1 \times 10^2$ CFU/g compared to wild-type at $(5.0 \pm 1.2) \times 10^8$ CFU/g. KpsC mutants are expected to be defective in high-molecular-weight capsular polysaccharide synthesis and virulence (6, 24, 25), confirming and emphasizing the importance of the capsule in colonization.

Distribution of transposon insertions in *C. jejuni* 81-176 and its endogenous plasmids. *C. jejuni* 81-176 contains two endogenous plamids, pVir and pTet (4, 7). During our studies with the *C. jejuni* 81-176 transposon mutants, we observed insertion in

TABLE 5. Transposon insertion sites and colonizing ability of 81-176 and M1 transposon mutants in the 2-week-old chicken and 1-day old chick colonization models[a]

Strain and mutant	Insert site	Predicted function and/or homologue(s)	Colonization (CFU/g)	
			2-week-old chicken [(mean ± SEM)/10^9]	1-day-old chick [(mean ± SEM)/10^{11}]
81-176				
1-3	pTet *cpp4*	Unknown	0.45 ± 0.24	1.6 ± 0.45
3-3	pTet *cpp33*	Unknown	0.48 ± 0.11	4.1 ± 0.49
6-3	*cj1113*	Similar to *Haemophilus influenzae* gene HI0077	2.3 ± 1.89	1.4 ± 0.38
7-3	pTet *tetO*	Tetracycline resistance	0.69 ± 0.08	ND
9-3	pVir *cjp40*	Unknown	0.58 ± 0.31	1.9 ± 0.046
11-3	*cj0670* (*rpoN*)	Sigma factor	$<1 \times 10^2$	0.00047 ± 0.00023
13-3	HS23.07	Putative sugar transferase	2.7 ± 0.96	2.4 ± 0.285
14-3	*cj0411*	Putative ATP/GTP-binding protein	1.6 ± 0.92	1.7 ± 0.69
19-3	pVir *cjp42*	Unknown	3.1 ± 1.7	2.1 ± 1.0
20-3	pTet *cpp44*	Similar to *Helicobacter pylori cag* island protein	2.4 ± 1.6	2.8 ± 0.24
21-3	pTet *cpp9*	Unknown	0.99 ± 0.76	4.4 ± 0.12
30-3	*cj0690c*	Possible DNA methylase	2.9 ± 1.29	2.4 ± 1.6
46-3	pTet *cpp47*	Unknown	3.6 ± 0.93	3.8 ± 0.97
M1				
23-1	*cj1126c*	PglB (glycosyltransferase)	$<1.0 \times 10^2$	ND
30-1	*cj0252*	MoaC (molybdenum cofactor biosynthesis)	0.39 ± 0.073	ND
38-4	*cj0337c*	MotA (flagellar motor protein)	$<1.0 \times 10^2$	ND
42-1	Intergenic	Insertion between $tRNA_{ser}$ and ORF of unknown function	2.5 ± 1.6	ND
88-4	*cj1565c*	PflA (required for flagellar function)	$<1.0 \times 10^2$	ND

[a] The colonization level is that at day 7, following inoculation with a single mutant at day 0. ORF, open reading frame; ND, not done. HS23.07, BX545858 in EMBL.

TABLE 6. Presence of tagged mutants[a]

Presence of mutants	Mutant													
	4-1	5-1	10-1	22-1	23-1	24-1	30-1	33-1	35-1	37-1	38-1	42-1	43-1	84-1
Cecum														
Day 1	+	+	+	+	−	+	−	+	+	+	+	+	−	+
Day 3	+	−	−	+	−	+	−	−	+	+	+	−	−	−
Day 7	+	−	−	+	−	+	−	−	+	+	+	−	−	−
Day 7														
Bird 1	+	−	−	+	−	+	−	−	+	−	+	−	−	−
Bird 2	+	−	−	+	−	−	−	−	−	+	+	−	−	−
Bird 3	+	−	−	+	−	−	−	−	+	+	+	−	−	−

[a] Presence of tagged mutants in a screen of a pool of 14 *C. jejuni* M1 STMs at days 1, 3, and 7 postinoculation. Three birds per time point were infected on day 0 with a pool of mutants and housed in the same cage. The presence of tags was assessed in pooled bacteria isolated from cecal contents and in individual birds at day 7.

both plasmids, even though genomic DNA was prepared using a QIAGEN Genomic DNA Preparation kit. Our results showed that the insertions were distributed approximately 1:1:1 with respect to chromosome:pTet:pVir (data not shown), in contrast to the size ratio between these molecules, ca. 1,600: 45:37 (4, 7, 34). This may indicate preferential insertion into plasmid DNA or may be a consequence of a higher copy number of the plasmids with respect to the chromosome. The copy number of these plasmids has not been reported. Previous studies (17, 18) have used *mariner* mutagenesis to generate both untagged and signature-tagged mutant libraries in a *C. jejuni* 81-176 variant which contained only pVir, having lost pTet (D. Hendrixson, personal communication). Interestingly, Hendrixson et al., who had previously tested for randomness of insertion using only Southern hybridization (17), also noted transposon insertion in pVir in a subsequent study (19), although no information on the frequency of this insertion was presented. Similarly to insertions in the genome of NCTC 11168, the distribution of insertion in pTet and pVir was not uniform (Table 3; see Fig. S2 in the supplemental material).

Colonization of 2-week-old chickens with *C. jejuni* M1. Due to the high frequency of transposon insertion into the plasmids of 81-176, for continued studies into the dynamics of colonization, it was desirable to use a plasmid-free strain. We generated a 3,000-member mutant library with 60 signature tags in *C. jejuni* strain M1, a natural poultry isolate with no reported plasmids, which has been shown to both colonize the chicken and cause disease in humans (D. Newell, personal communication). We ascertained whether *C. jejuni* M1 could colonize the gastrointestinal tract of chickens using our 2-week-old chicken model of infection. After 7 days, all chickens were colonized with bacterial numbers in the ceca of around 9 $\log_{10}$ CFU/g of cecal contents. The colonized birds appeared healthy and showed no signs of disease.

High-frequency, random loss of *C. jejuni* M1 mutants from pools. We constructed a pool of 14 differently tagged transposon mutants of *C. jejuni* M1 and conducted colonization trials as described for 81-176. A similar pattern of mutant loss was observed: 57% of mutants were lost, loss of mutants increased over time, and there was between-bird variation in recovered mutants (Table 6).

Loss of colonization-proficient strains is observed in large groups of individually inoculated birds housed together. To determine whether the observed stochastic mutant loss was a consequence of the complexity of the pool of mutants, 25 individually identifiable (wing-banded) birds were each individually inoculated with 8 $\log_{10}$ bacteria of a different *C. jejuni* M1-tagged mutant. The 25 individually inoculated birds were then housed together in a single cage. The presence of different tagged mutants in the ceca of each bird was determined at day 7 postinfection (Table 7). All birds remained campylobacter positive but not necessarily with the mutant with which they were originally inoculated. In some cases, birds carried more than one tagged mutant. Interestingly, two particular mutants (43-4 and 75-4) were present in the majority of birds. It is possible that these mutants had a colonization advantage, had adapted in vivo, were more transmissible, or were better able to survive in the cage environment. In contrast, some mutants were not present in any bird; it is possible that these were less fit for colonization. Alternatively, the predominance of some mutants and lack of others could have been due to stochastic processes, as observed with the pool experiments previously described. To address this, the transposon insertion site from each mutant was determined (Table 8), and the apparently noncolonizing mutants were assessed for motility and their ability to colonize groups of three birds in pure culture. Of these 11 mutants, 8 had wild-type motility, mutant 57-4 (*leuD*) had reduced motility, and mutants 38-4 (*motA*) and 88-4 (*pflA*) were nonmotile (data not shown). Nine mutants were colonization proficient (data not shown), while 38-4 (*motA*) and 88-4 (*pflA*) failed to colonize (Table 5). This is consistent with previous studies showing the nonmotile phenotype of a *pflA* mutant (53) and the requirement of these two genes for colonization (18).

These results imply that there is considerable bird-to-bird transmission of the bacteria, and demonstrates that despite an initial inoculum of 8 $\log_{10}$ bacteria, replacement of one colonizing strain by another (to the limit of detection of this assay) within 7 days can be observed when a mixed population of mutants is present within a cage.

Loss of colonization-proficient strains is observed in small groups of individually inoculated birds housed together. The high frequency of colonization-proficient mutant loss in the individually inoculated bird experiment described above could have been a consequence of the number of different mutants present within the cage. To investigate this possibility, we reduced the

TABLE 7. Presence of tagged mutants in the ceca of 25 individual birds at day 7 postinfection[a]

Mutant	Tag(s) present at day 7																								
	04	22	23	24	30	33	35	36	37	38	42	43	50	51	53	54	55	57	60	61	62	75	84	85	88
4-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
22-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−
23-4	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	−	+	−
24-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	+	−
30-4	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
33-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
35-12	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−	+	−	−	−
36-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−
37-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−
38-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	−	−	−
42-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	+	−
43-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−
50-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
51-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	+	−
53-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−
54-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	+	−	−	−
55-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	−	+	−
57-4	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	+	+	−	+	−
60-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	+	−	−	−	−
61-4	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	−	−	−
62-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
75-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
84-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−
85-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
88-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−

[a] All birds received 8 $\log_{10}$ CFU of a single M1 signature-tagged mutant (left-hand column) on day 0 and were housed in the same cage. +, tag detected; −, tag not detected.

complexity by using smaller groups of four or five identifiable birds each individually inoculated with 8 $\log_{10}$ bacteria of a different *C. jejuni* M1-tagged mutant. Birds in a group were housed together in a single cage and the presence of different tagged mutants in the ceca was determined at day 7 postinfection by combining campylobacters recovered from cecal contents of all birds in a group (Table 9). Three mutants, designated 23-1, 30-1, and 42-1, were not detected. When assayed in pure culture, only

TABLE 8. Transposon insertion sites of M1 mutants

Mutant	Insert site	Predicted function
4-4	Not done	Not applicable
22-4	*cj1095*	Integral membrane protein, weak similarity to *E. coli* apolipoprotein *N*-acetyl transferase
23-4	*cj1506c*	Probable methyl-accepting chemotaxis protein-type signal transduction protein
24-4	*cj1334*	Unknown
30-4	*cj1520*	Unknown
33-4	*cj1528*	Pseudogene in 11168; probable transmembrane protein similar to *E. coli* C4-dicarboxylate anaerobic carrier in M1 (data not shown)
35-12	Intergenic *cj1191-dctA*	Cj1191c, possible signal-transduction sensor protein; DctA, probable C4-dicarboxylate transport protein
36-4	*cj1194*	Possible phosphate permease
37-4	HS41.24[a]	Probable capsular nucleotidyl-sugar pyranose mutase
38-4	*cj0337c*	MotA; flagellar motor protein
42-4	*cj0631c*	Possible ribonuclease
43-4	*cj0970*	Unknown
50-4	*cj1471c*	Probable type II secretion system E protein
51-4	*cj0013*	IlvD; probable dihydroxy acid dehydratase
53-4	*cj1544c*	Probable integral membrane protein of unknown function
54-4	*cj0948c*	Possible transmembrane transport protein
55-4	—[b]	Unknown
57-4	*cj1716c*	LeuD; probable 3-isopropylmalate dehydratase small subunit
60-4	*cj0893c*	RpsA; 30S ribosomal protein S1
61-4	—[c]	Possible cytochrome *c*
62-4	*cj1633*	Unknown
75-4	Unmatched	Unique 308-bp open reading frame of unknown function
84-4	*cj1468*	Probable integral membrane protein
85-4	*cj1256c*	Possible membrane protein
88-4	*cj1565c*	PflA; required for flagellar function

[a] Match to locus from *C. jejuni* strain 176.83 (EMBL, BX545857).
[b] —, match to locus from *C. jejuni* strain 11828 (GenBank, AF343914).
[c] —, open reading frame of 1,971 bp whose translation matches *Campylobacter lari* strain RM2100 locus (GenPept, EAL54972).

TABLE 9. Results of a colonization screen of *C. jejuni* M1 STMs[a]

Pen		Mutant												
		4-1	5-1	10-1	22-1	23-1	24-1	30-1	33-1	35-1	37-1	38-1	42-1	43-1	84-1
1	In	*	*	*										*	*
	Out	+	+	+										+	+
2	In				*	*	*		*				*		
	Out				+	−	+		+				−		
3	In							*		*	*	*			
	Out							−		+	+	+			

[a] Chickens were housed in three pens of four or five birds apiece, each bird receiving a single tagged mutant. In each case, the first line shows the mutant used to inoculate (In) and the second line shows the pooled output (Out) on day 7 postinfection. *, mutant inoculated; +, tag detected; −, tag not detected.

one of these failed to colonize (Table 5); its transposon insertion site was mapped to *pglB*. This gene is required for N-linked glycosylation in *C. jejuni*, and disruption of its function has been shown to affect the immunoreactivity of membrane proteins (51), decrease adherence to and invasion of INT407 cells, and prevent colonization in a mouse model (46). Mutant 30-1, which was reduced in its ability to colonize (Student's *t* test; $P < 0.02$) had an insertion in *moaC*, the gene putatively encoding molybdenum cofactor biosynthesis protein C. The molybdoenzyme encoded by *cj0379* is essential for colonization of the avian gastrointestinal tract (33); hence, it may be predicted that the *moaC* mutant would be colonization defective. However, the insertion in 30-1 is at the extreme 3′ end of the *moaC* gene (data not shown), potentially resulting in a partially functional protein which could account for the reduced colonization ability of this mutant.

To determine whether transmission of campylobacter between birds had occurred, the mutants present in individual birds in one of the groups were assessed (Table 10). Interestingly, one mutant, 22-1 was present in all birds, three birds had two mutants, and three birds did not have the mutant with which they were initially inoculated. A repeat of this experiment yielded a different pattern of recovery, with different mutants present and/or absent in different chickens (Table 10). Strikingly, mutant 22-1 did not predominate in the repeat, suggesting that its predominance in the first experiment was not necessarily a consequence of an increased fitness, and once again indicating that stochastic effects were at play.

TABLE 10. Presence of mutants in the ceca at day 7 in individual-inoculation, mixed-cage colonization trials[a]

Bird		Expt 1 and mutant					Expt 2 and mutant				
		22-1	23-1	24-1	33-1	42-1	22-1	23-1	24-1	33-1	42-1
1	In	*					*				
	Out	+	−	−	−	−	+	−	−	−	−
2	In		*					*			
	Out	+	−	−	+	−	−	−	+	−	−
3	In			*					*		
	Out	+	−	+	−	−	−	−	+	−	−
4	In				*					*	
	Out	+	−	+	−	−	−	−	−	+	−
5	In					*					*
	Out	+	−	−	−	−	−	−	+	−	−

[a] In, strain used to infect; Out, tag recovered at day 7; *, mutant inoculated; +, tag detected; −, tag not detected.

DISCUSSION

Several studies have attempted to elucidate colonization factors of campylobacter by testing the ability of a limited number of specific mutants of *C. jejuni* to colonize chickens, most commonly in the newborn chick model (31, 35, 54, 55). Recently, Hendrixson and DiRita applied in vitro STM with the aim of identifying *C. jejuni* colonization factors in 1-day-old chicks (18). Chickens in the field are colonized at 2 weeks of age (22) and possess a more complex gut flora than newborn birds (47). We have attempted to use STM to study the colonization of 2-week-old chickens by *C. jejuni*. We generated STM libraries in three strains of *C. jejuni* using a modified *mariner* transposon system and have screened pools of mutants from two of these libraries through our chicken model of colonization, with intriguing results.

In our study, Southern hybridization analysis of transposon mutants suggested random insertion, by virtue of different-sized hybridizing fragments. Moreover, sequencing of the insertion sites indicated integration of the transposon into a different site in each mutant. However, statistical analysis of the insertion sites in the *C. jejuni* NCTC 11168 chromosome and the plasmids of strain 81-176 indicated that their distribution was not uniform. Visual inspection of the distribution suggested that deviation from uniformity was not due to preferential integration of the transposon into a limited number of hot spots, rather, that there was a bias towards an extended region of the target DNA molecule in the vicinity of the origin in the NCTC 11168 chromosome (34). This preference could be due to target site selection by the transposon during the in vitro transposition reaction and/or preferential homologous recombination between the transposed and bacterial DNAs. Recombination may be favored near the origin in dividing cells, as DNA replication will increase the relative copy number of this region. The level of significance for deviation from uniformity is greater for insertions around the chromosome of NCTC 11168 than for the plasmids of 81-176. This may reflect a difference in insertion site distribution between these DNA molecules or be a consequence of the smaller sample size for the plasmid species. In addition to the DNA structural preferences seen for the Tc1/*mariner* element *Sleeping Beauty*, Vigdal

et al. reported a preference for a short palindromic AT repeat (5′-ATATATAT-3′; the underlined TA is the insertion site), although the conservation was relatively weak (50). The insertion sites for our *mariner* transposon did not show a similar preference. Despite the nonuniform distribution of transposon insertion sites, mutants were isolated from locations distributed around the bacterial DNA, demonstrating that these *mariner* STM plasmids offer a reasonably efficient means of generating random tagged mutants of *C. jejuni*. The observation that the *mariner* transposon integrates at a high frequency into the plasmids of 81-176 should be taken into account for future mutagenesis studies, i.e., to cover the genome of a plasmid-bearing strain, the number of mutants generated may need to be significantly higher.

Our data from mixed mutant inoculum pools indicate that traditional STM approaches are an inefficient way to study the relationship between *C. jejuni* and its avian host, due to high-frequency random loss of colonization-proficient mutants. Similar observations with campylobacter mixed-inoculum colonization experiments have been reported by other groups. A recent STM study identified genes involved in campylobacter colonization of the gastrointestinal tract of chicks between 12 and 36 h old (18), hereafter referred to as the day-old chick model. The authors also reported a high frequency of mutant loss (20 to 50 mutants from pools of 74-82), although they do not state how often this occurred. To address this problem, they adopted an iterative STM screen in which mutants absent from output pools were combined to produce new input pools, which were rescreened. Potential noncolonizers were then individually assessed for their ability to colonize the gastrointestinal tract of 1-day-old chicks. A colonization-defective phenotype was assigned to any mutant that colonized at least 10-fold lower than the wild type. Of 187 putative mutants from the STM screen, only 29 were assigned this phenotype. These observations are in broad agreement with our studies, in which we saw 60 to 85% of mutants lost from pools of 12-14. The authors do not discuss in detail the significance of the random loss of mutants, but we believe this phenomenon is evidence for a complex population dynamic of this bacterium in its host. Our data clearly show that loss of mutants is not hierarchical (i.e., dependent solely on colonization ability) but has a significant stochastic component.

A prime candidate for a confounding factor in these studies is the possibility of population bottlenecks during transit through the gastrointestinal tract prior to establishment of bacterial colonies in the ceca. With mixed mutant pool inocula, we can imagine that the stochastic loss of colonization-proficient mutants is a consequence of a population crash early in the process, for example, by exposure to the low pH of the gizzard. In an attempt to overcome this, we used an individual inoculation approach where up to 25 individual birds were inoculated with 8 $\log_{10}$ CFU of different single mutants and housed together in a single cage. In this model, we expected that the high single-mutant dose would enable establishment of cecal colonies in individual birds, should the mutant be colonization proficient. Thus, a large number of mutants could be assessed in a single experiment by performing STM analysis of the pooled output from all birds in a cage. Simply assessing the bacterial load in each bird by plate count would not be sufficient, due to the possibility of transmission of bacteria between birds. Contrary to expectations, colonization-proficient mutants were absent at day 7 postinfection even when a small number (i.e., five birds) were housed together. Upon analysis of individually identifiable birds, it was clear that extensive bird-to-bird transmission of mutants had occurred; some birds harbored more than one mutant, and some carried a mutant with which they were not inoculated, despite this mutant being present in other birds. Variation between experiments using the same mutants demonstrated the random nature of this phenomenon, as opposed to it being a consequence of any phenotype of the mutants. Particularly striking was the observation that in one experiment using five individually inoculated birds, mutant M1-22-1 was present in all the birds in the cage and yet when the experiment was repeated it was present only in the bird that originally received it. This demonstrated that this mutant was no more able than the others to spread between birds in the cage. Korolik et al. reported that displacement of one strain by a different strain could occur within 7 days in experimental infections (27). They hypothesize that the displacing strain was more proficient at colonization. Our study suggests that differences in colonization proficiency are not required for this phenomenon. The random nature of the transmission reported in the present study may be explained in part by the coprophagic nature of chickens; bacteria present in feces will be readily ingested, potentially reseeding colonies in the gastrointestinal tract. With a number of different mutants in different birds in a cage, this transmission is not predictable and is likely to be dependent not only on phenotypic differences between mutant bacteria, but also on many other factors, including the number of bacteria ingested at any time (with and without food), the makeup of any preexisting colony within the ingesting animal, and physiological variation between birds (amount and consistency of feces shed). Interestingly, Achen et al. reported significant variation in shedding of campylobacter between birds infected with the same bacterial strain (1). Competition with the developed gut flora in our model, while more representative of colonization in nature, is likely to be yet another complicating factor.

We hypothesize that bottlenecks during colonization within a bird and bird-to-bird transmission of campylobacters are responsible for high-frequency random loss of colonization-proficient mutants in mixed infection studies. These results differ markedly from those seen when STM libraries of *Salmonella enterica* serovar Typhimurium are used in chickens under similar experimental conditions (32) and presumably reflect a fundamental difference in the nature of the interaction between these bacteria and the host. It is important to note that in the present study, colonization was assessed by enumerating bacteria in the ceca. There is a possibility that campylobacters are retained elsewhere in the gastrointestinal tract, potentially seeding cecal colonies. Our observation that loss of mutants from pools increases over time is consistent with this model; at early time points, it may be expected that the bacteria present are more representative of those used for the initial inoculum. Over time, stochastic effects influence the population of mutants within the cage, resulting in a limited number of mutants coming to predominate. The use of an early time point in an STM screen would, however, prevent the discovery of genetic factors important for long-term colonization of the avian gastrointestinal tract by campylobacter. Similarly, the day-old

chick model of Hendrixson and DiRita (18) may fail to detect factors important for campylobacter to colonize in the presence of an adult gut flora and could be a less-stringent measure of assessing colonizing ability. Indeed the authors report that *rpoN* and *pflA* transposon mutants of 81-176 were able to colonize day-old chicks, albeit at a reduced level (18), in contrast with our 2-week model in the present study where 81-176 *rpoN* and M1 *pflA* mutants were not detectable 7 days postinoculation (Table 5).

The observations from this study have implications for the way colonization by single mutants of campylobacter is interpreted. While it has long been known that flock-wide colonization progresses rapidly following an initial introduction (40), even where the number of birds is in the thousands, transmission effects in colonization studies have rarely been addressed. Most work to date has assessed the colonization potential of mutants by measuring the bacterial load in more than one animal per cage. This enables statistical comparisons to be made between groups and is also required to comply with animal welfare regulations. Our data suggest the possibility that significant amounts of bacterial transmission between birds occur, potentially resulting in a continuous reseeding of birds within the cage. If so, measures of colonization may reflect the ability of a strain not merely to establish and persist in the gastrointestinal tract, but also to spread to and persist in the cage. Modifications to existing protocols, such as frequent cage-bed cleaning or housing a single animal in a cage, may reduce this complication but are likely to be difficult practically. To address the extent of this recycling phenomenon, we are beginning to study the spatiotemporal population dynamics of essentially wild-type bacteria within and between birds housed together. We anticipate that these studies will enable a quantitative analysis of transmission dynamics and should inform research into contamination of commercial flocks and control strategies such as competitive exclusion.

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