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Campylobacter jejuni Colonization in the Crow Gut Involves Many Deletions within the Cytolethal Distending Toxin Gene Cluster

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ABSTRACT *Campylobacter* spp. are major causes of gastroenteritis worldwide. The virulence potential of *Campylobacter* shed in crow feces obtained from a roost area in Bothell, Washington, was studied and compared with that from isolates from other parts of Washington and from a different crow species 7,000 miles away in Kolkata, India. *Campylobacter* organisms were isolated from 61% and 69% of the fecal samples obtained from Washington and Kolkata, respectively, and were confirmed to be *C. jejuni*. The cytolethal distending toxin (CDT) gene cluster from these isolates revealed a truncated sequence of approximately 1,350 bp. Sequencing of the gene cluster revealed two types of mutations: a 668-bp deletion across *cdtA* and *cdtB* and a 51-bp deletion within *cdtB*. Some strains had additional 20-bp deletions in *cdtB*. In either case, a functional toxin is not expected; a functional toxin is produced by the expression of three tandem genes, *cdtA*, *cdtB*, and *cdtC*. Reverse transcriptase PCR with total RNA extracted from the isolates showed no expression of *cdtB*. A toxin assay performed with these isolates on HeLa cells failed to show cytotoxic effects on the cells. However, the isolates were able to colonize the chicken ceca for a period of at least 4 weeks, similar to that of a clinical isolate. Other virulence gene markers, flagellin A and CadF, were present in 100% of the isolates. Our study suggests that crows carry the bacterium *C. jejuni* but with a dysfunctional toxin protein that is expected to drastically reduce its potential to cause diarrhea.

IMPORTANCE Campylobacters are a major cause of gastroenteritis in humans. Since outbreaks have most often been correlated with poultry or unpasteurized dairy products, contact with farm animals, or contaminated water, historically, the majority of the studies have been with campylobacter isolates from poultry, domestic animals, and human patients. However, the bacterium has a broad host range that includes birds. These reservoirs need to be investigated, because the identification of the source and a determination of the transmission routes for a pathogen are important for the development of evidence-based disease control programs. In this study, two species of the human-commensal crow, from two different geographical regions separated by 7,000 miles of land and water, have been examined for their ability to cause disease by shedding campylobacters. Our results show that the crow may not play a significant role in campylobacteriosis, because the campylobacter organisms they shed produce a nonfunctional toxin.

KEYWORDS *Campylobacter jejuni*, crows, cytolethal distending toxin, virulence determinants

Campylobacters are major cause of gastroenteritis in humans worldwide. Of the different species found within the genus that comprises Gram-negative spiral-shaped bacteria, the species *Campylobacter jejuni* is responsible for >88% of disease (1).

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In rare cases, the infection can have chronic sequelae, such as reactive arthritis and Guillain-Barre syndrome (GBS) (2).

Since outbreaks have most often been correlated with poultry or unpasteurized dairy products, contact with farm animals or household dogs and cats, or contaminated water, historically, the majority of the studies performed have been with *Campylobacter* isolates from poultry, domestic animals, and human patients (3). However, the bacterium has a broad host range. These reservoirs need to be investigated, because the identification of the source and a determination of the transmission routes of a pathogen are important for the development of disease control programs.

Birds appear to be appropriate reservoirs for campylobacters because of their relatively high body temperature (42°C) and the microaerophilic environment that is present within the deep crypts of the cecum where campylobacters colonize. Both conditions are optimal for the growth of the bacteria (4). Indeed, several bird species harbor *C. jejuni* organisms as a part of their normal gut flora (5). However, not all avian hosts that carry these bacteria play a significant role in the spread of the disease. Studies on isolates from Europe and Australia have shown that *C. jejuni* populations from different wild bird species were different from each other and were different from those typically found in domestic animals and humans (6). European starlings and graylag geese were shown to carry *Campylobacter* spp. that appeared to be highly host specific, possessing gene profiles that suggested a limited ability to infect humans or farm animals (7). On the other hand, black-headed gulls carry *C. jejuni* strains that are pathogenic to humans (8), and wild sandhill cranes were associated with an outbreak (9).

A few studies have looked into the pathogenic potential of *Campylobacter* spp. harbored by different species of crows (10–13) that live in close proximity to humans. The mainstay of such studies was the isolation of *C. jejuni* as the major strain, thus identifying a role for the crow in the spread of campylobacteriosis. Weis et al. (10) studied the virulence factor gene *flaA*, which encodes the flagellin protein responsible for motility and the invasion of the gastrointestinal tract, and the cytolethal distending toxin (*cdt*) gene cluster, which produces a toxin that results in cytotoxicity and ultimately the death of sensitive cells (14–16). The study showed that *flaA* and the *cdt* gene cluster were present in 46% and 20% of the strains, respectively (10). Shyaka et al. showed the presence of the virulence genes *flaA*, *flaB*, *ciaB*, *cadF*, *cdtB*, and *cdtC* in 100% (25/25) of the Japanese crow species *Corvus corone* and *Corvus macrorhynchos*; however, *cdtA* was present in only 1/25 (12). Thus, it appears that the CDT toxin may not have been expressed in a majority of the strains, since the expression of all three genes is necessary for the production of a functional toxin (14). We thus wanted to examine the *cdt* gene cluster in further detail, since this is the only toxin that has been verified in campylobacter and has been shown to cause inflammation in the intestines of mice and produce gastroenteritis (17, 18).

Crows of the species *Corvus brachyrhynchos* in a wetland area within the University of Washington Bothell Campus, where every year during the winter months more than 15,000 crows congregate and roost from dusk to morning, provided an excellent opportunity to study their ability to carry *Campylobacter* and their potential to cause disease. In addition, crows from three other nonwetland areas within Washington were studied to understand if there was any niche specificity of the *Campylobacter* strains. The isolates were further compared with *Campylobacter* isolates from Kolkata, India, a highly populous city almost 7,000 miles away from Seattle, where the crows of the species *Corvus splendens* are abundant and live in close proximity to humans. The American crow is a larger species and is of a rich glossy black color, while the forehead, crown, throat, and upper breast of the Indian crow is of black color, with the neck and breast being a lighter gray-brown; it is also smaller in size. The virulence genes, *cdtABC* gene cluster, *flaA*, and *cadF*, a gene that encodes an adhesin that has been shown to be involved in colonization (19), were examined in all isolates. The goal of the study was to test if there were any differences in the virulence potential of the isolates from two

TABLE 1 Sampling sites, *Campylobacter* isolates, and virulence genes

Sampling site	No. of samples	No. of isolates		No. of isolates with virulence gene:		
		Campylobacter (confirmed by qPCR)	*C. jejuni* (confirmed by PCR)	*fla*	*cad*	*cdt*[a]
Washington, USA						
Crow roost in wetlands, Bothell	58	41	41	41	41	37
Mercer	8	2	2	2	2	2
Everett	9	4	4	4	4	4
Factoria	5	2	2	2	2	2
Kolkata, WB, India						
Rabindra Sarovar lake	8	7	7	7	7	7
NICED[b] campus	9	8	8	8	8	8
Talapark	7	5	5	5	5	5
Prinsep Ghat	12	5	5	4	5	5

[a]The smaller gene cluster (1,270 to 1,310 bp) found in the isolates.
[b]NICED, National Institute of Cholera and Enteric Diseases.

different parts of the world and subsequently determine their significance to public health.

RESULTS

Prevalence of *Campylobacter* and *Campylobacter jejuni* in different samples. Following their isolation under selective conditions and microscopic examination of their morphology, a genus-specific quantitative PCR (qPCR) assay confirmed that *Campylobacter* spp. were present in 61% (49/80) of the fecal samples collected from Bothell and neighboring areas that spanned a perimeter of 20 miles. Crow fecal samples collected from four sites in Kolkata during July through September 2016 revealed an overall presence of *Campylobacter* spp. in 69% (25/36) of the samples (Table 1). All of the crow campylobacter isolates were confirmed by PCR methods to be *C. jejuni*. In this study, no other species was isolated.

Determination of the presence of *cdtABC* virulence genes. The *cdt* gene cluster was present in 92% (45/49) of the Washington isolates and 100% (25/25) of the Kolkata isolates. However, a further analysis revealed all of these isolates to have a truncated gene cluster of approximately 1,350 bp when the primer set LYA-F and MII-R was used (Fig. 1). This primer set amplifies the entire *cdtABC* gene cluster but not the first 48 bp of *cdtA* or the last 39 nucleotides of *cdtC*, producing a 2,100-bp fragment in the wild-type *C. jejuni* strain (Fig. 2) (20). Amplification was not possible in two of the four isolates from Washington due to the degradation of the DNA, and the isolates could not be recovered from the freezer stocks.

Sequencing of the *cdt* gene cluster. The *cdt* gene cluster from *C. jejuni* was sequenced using the LYA-F forward and MII-R reverse primers (20). The sizes of the DNA sequences from 22 isolates varied between 1,330 and 1,372 bp, because there were additions and deletions of nucleotides within the sequences. The sequences were

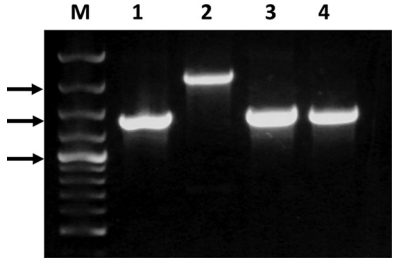

FIG 1 Detection of CDT gene cluster in *C. jejuni* strain ATCC 33560 (lane 2) and crow isolates B37, B3, and B50 (lanes 1, 3, and 4, respectively) by PCR using the primers LYA-F and MII-R. M, 1 kb plus molecular size ladder (GeneRuler). Arrows point to 2,000, 1,500, and 1,000 bp (top to bottom).

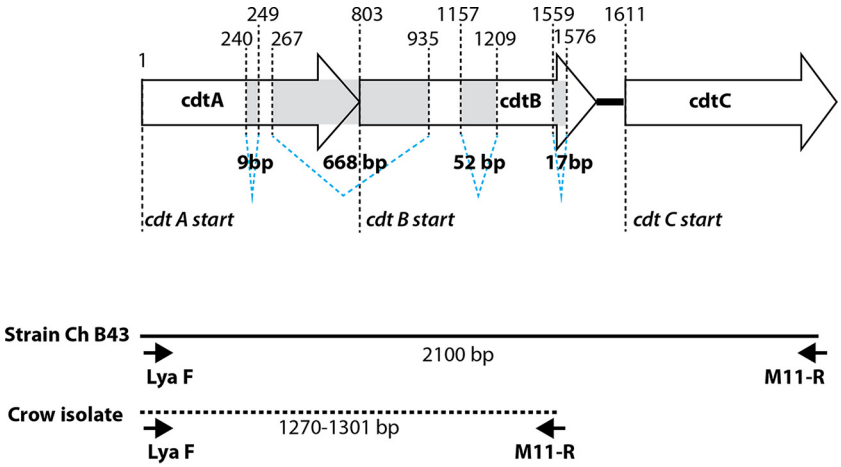

FIG 2 Schematic of *cdt* gene cluster. The arrows represent the three genes *cdtA*, *cdtB*, and *cdtC*. The gene cluster was sequenced with the primers LYA-F and MII-R in *C. jejuni* isolates from crows (represented by dashed line) when a region between 1,270 and 1,301 bp was obtained, depending on the isolate. The positions of the forward primer, LYA-F, and reverse primer, MII-R, are indicated in the *cdt* cluster from the wild-type strain ChB43 (GenBank accession no. AB274791), which is expected to give a nucleotide sequence of 2,100 bp (represented by a dark line). Numbers above the arrows are the nucleotide positions within the genes where the mutations occur, with 1 being the first base of the start codon of *cdtA*. The shaded regions are the deleted regions, with the number of bases deleted indicated below.

trimmed and aligned using Clustal W, and the final lengths of the sequences obtained were between 1,270 bp and 1,301 bp (Table 3). There was greater than 95% homology among the sequences. This length range was sufficient to observe the prominent mutations within the gene clusters in the *C. jejuni* isolates from crows. In all of the isolates, the first mutation was a deletion of 9 bp within *cdtA*, the second was a 668-bp deletion across *cdtA* and *cdtB* and a further 52-bp deletion within *cdtB* (Fig. 2; see also Fig. S1 in the supplemental material). The positions of the mutations are indicated in the *cdtABC* gene cluster from the strain *C. jejuni* ChB43 (21) that has an intact CDT toxin gene. Most of the strains had additional 17-bp deletions in *cdtB*. Of the 22 isolates sequenced, isolates B43, B69, B62, B31, and Kol 10 did not have the 17-bp deletion. In addition, there were several additions and deletions of 1 to 6 bp throughout the gene cluster that were not at conserved sites. Interestingly, isolates B69, B43, B42, and Kol 16 had the exact same sequences (Table 3).

Expression of *cdt* genes, experimental infection, and determination of toxin activity by the *cdt* gene cluster. To determine if the truncated *cdt* gene cluster was capable of expressing the genes, which are made as a single mRNA transcript, total RNA from these isolates was analyzed by reverse transcriptase PCR (RT-PCR). While an mRNA transcript was obtained from the clinical strain CS2 (Fig. 3, lanes 1 to 3), none were observed from two crow isolates, B37 and B50 (Fig. 3, lanes 4 to 9). Next, we wanted

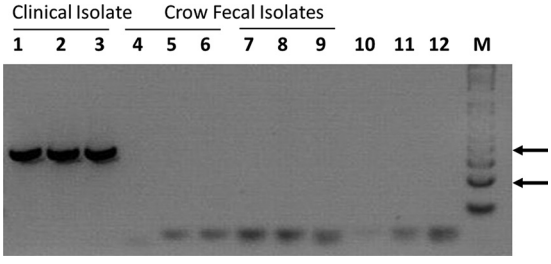

FIG 3 RT-PCR detection of CDT expression in *C. jejuni* clinical strain CS2 (lanes 1, 2, and 3) and crow isolates B37 (lanes 4, 5, and 6) and B50 (lanes 7, 8, and 9) using the primers DS15 and DS18 to amplify a 450-bp region overlapping *cdtA* and *cdtB*. Lanes 1 to 9 are the reverse transcribed products, lanes 10, 11, and 12 are extraction blank, RT negative control, and no template control, respectively. M, 1 kb plus ladder (Invitrogen). Arrows point to 400 and 200 bp (top to bottom).

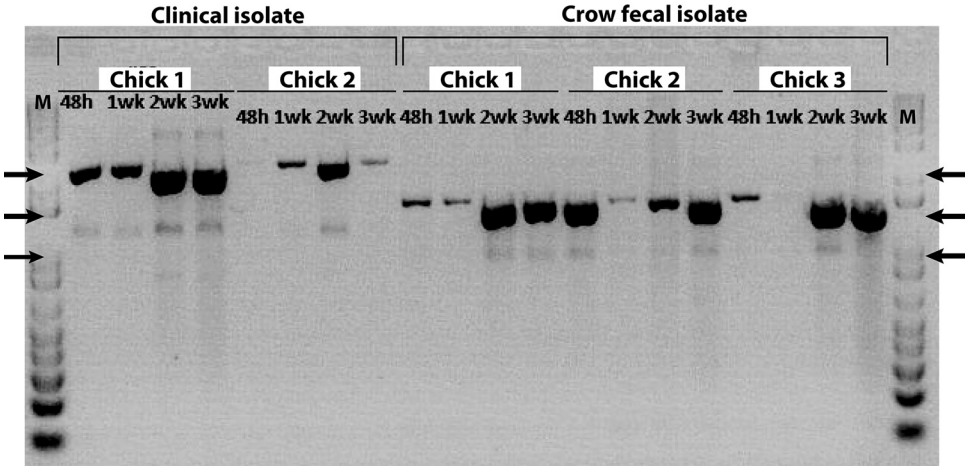

FIG 4 PCR detection of colonization of *C. jejuni* clinical strain CS2 and crow isolate strain B37 in the chicken gut using the *cdt* primers LYA-F and MII-R. The 2,100-bp band that amplified from DNA isolated from the fecal contents of the chicks that received the clinical strain CS2 is shown in the 2nd to 9th lanes. The 1,300-bp band that amplified from three chicks that received the isolate B37 is shown in the 10th to 21st lanes. The day/week that fecal DNA was tested from the respective chicks is indicated. M, 1 kb plus ladder (Invitrogen). Arrows point to 2,000, 1,650, and 1,000 bp (top to bottom).

to determine if the *cdt* mutants were capable of colonizing. The 1-day chicken model has often been used to study the colonization of campylobacter (22). Here, we used a newly developed method to compare the colonization of a crow fecal isolate that had a truncated *cdt* gene cluster (B37) with that of a human clinical isolate (CS2) in the chicken gut. When *cdt* gene cluster PCR was performed with DNA extracted from the feces of the chicks, no differences were observed in the densities of the amplicon bands in the 2-week or 3-week sampling times in any of the chicks (Fig. 4). The size of the band obtained from chicks infected with B37 was 1,350 bp, indicating the presence of a truncated *cdt* gene cluster. Chicks infected with CS2 had the normal gene cluster, that of approximately 2,100 bp. This suggests that the colonization of *C. jejuni* is not affected by the *cdt* gene cluster.

To verify whether the crow isolates were able to produce a functional toxin, a toxin assay was performed using HeLa cells. HeLa cells exposed to phosphate-buffered saline (PBS) or a B37 *C. jejuni* lysate did not show any cytotoxic effects after 72 h of exposure (Fig. 5A and B). However, HeLa cells exposed to sonicates of the *C. jejuni* wild-type clinical isolate, CS2, showed cell distensions characteristic of CDT activity (Fig. 5C).

Determination of the presence of other virulence genes. All crow isolates (100%) had *flaA* genes as determined by the presence of 1,728-bp products produced with primer set flaF and flaR or the 328-bp products formed with primer set FLA242FU and FLA625RU (Table 2). Another gene, *cadF*, which encodes a protein responsible for colonization, was also present in 100% of the isolates.

***flaA* SVR sequence analysis.** The short variable regions (SVRs) of the *flaA* genes from 32 representative isolates (23 from Washington and 9 from Kolkata) were sequenced to determine if there was any phylogenetic relationship between the isolates (Fig. 6). The choice of 32 crow isolates was based on a representation of at least 2 isolates from each round of collection. Thirty-two isolates clustered into 9 groups (at least 2 isolates were present in a group), and 10 isolates were present singly. The two most diverse sequences differed in 44 of 268 nucleotides. Alleles of the *flaA* SVR sequence showed linkage among subpopulations of *Campylobacter* isolates within the Washington area, as also with isolates from Kolkata. Thus, within 10 clusters (Fig. 6), several had sequences that were from isolates from Washington as well as from Kolkata (e.g., B23, B21, and Kol6; B54CVA and Kol 35; B30 and Kol 5; and B6 and Kol15). Typically, collections from one date clustered together (e.g., B36, B37, and B39; B42 and B44; and B62 and B61), and this is not surprising, since cohabitation of the crows at a roost likely leads to the transmission of some of the same strains.

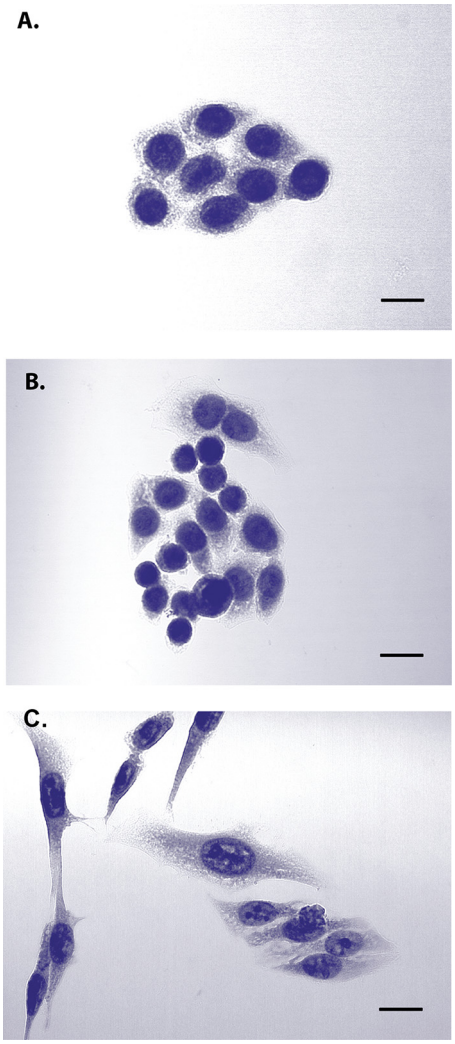

FIG 5 Stained HeLa cells exposed to PBS (A) or filter-sterilized supernatant fractions of cell sonicates of the *C. jejuni* isolate B37 (B) or *C. jejuni* wild-type clinical isolate CS2 (C). The CS2 strain produced cytoplasmic and nuclear distensions, characteristic of CDT activity. Bars, 20 μm.

DISCUSSION

Despite being separated by over 7,000 miles, two species of crows that are distinct in appearance and size shed only the species *C. jejuni*. This was confirmed by using two well-established PCR protocols that targeted different genes. *C. jejuni* was isolated almost at the same frequency from Kolkata samples (69%) as from samples from the greater Seattle area (61%). Other studies have reported the presence of campylobacters in 21 to 68% of fecal samples from crows (10, 12), and our results are in agreement with those, although Weis et al. also found *Campylobacter coli* and *C. lari* isolates, based on 16S rRNA gene sequence analysis (10).

The most unexpected result was that of the CDT gene cluster. In all of the 74 isolates in which the cluster was detected, irrespective of whether their origin was in the United States or Eastern India, PCR amplicons of the same size were obtained, at approximately 1,350 bp instead of the full-length 2,100 bp found in the wild type. The sequencing of 22 representative isolates that were chosen on the basis of the location and/or collection date revealed the same types of mutations within the gene. The 3 sets of deletions occurred in the exact same base positions in all of the 22 isolates (Fig. 2). CDT is a tripartite protein formed by the expression of three tandem genes, *cdtA*, *cdtB*, and *cdtC* (14), where *cdtB* encodes the active component of the toxin and *cdtA* and *cdtC* are

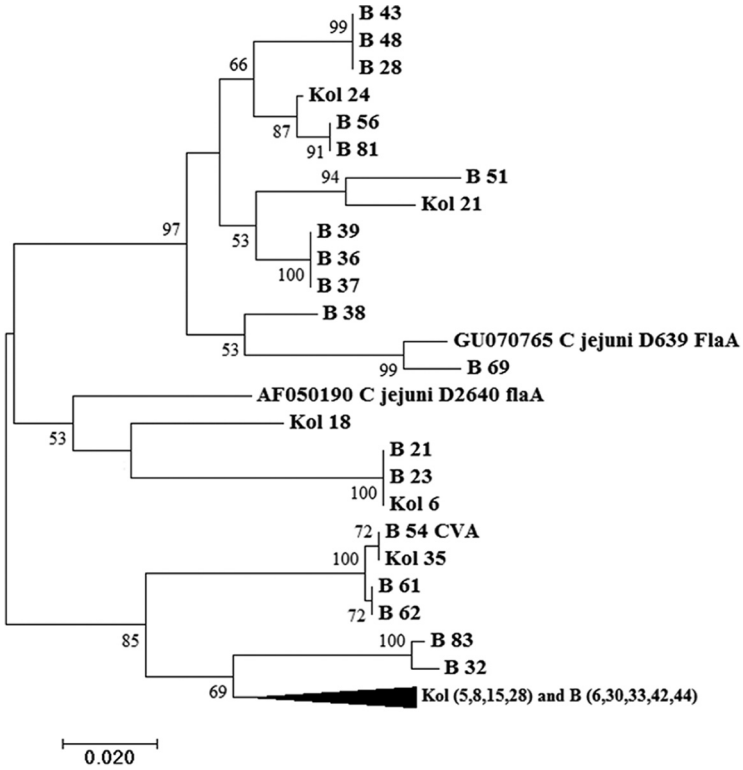

FIG 6 Analysis of *flaA* SVR. The short variable region (SVR) of the *flaA* gene, comprising 268 bp, was analyzed in 32 representative isolates, including 23 representative crow isolates from different batches of sampling from Washington and 9 isolates from Kolkata. Isolates from Bothell and other Washington areas are indicated by the prefix B and those from Kolkata are indicated by Kol. Sequences were aligned, and a bootstrap consensus tree was created with MEGA 7.0.14 (5% divergence).

responsible for the binding and internalization of the toxin (14). Since the bulk of the *cdtA* gene and the first 126 bp, including the start codon, of the *cdtB* gene are deleted, it is unlikely that the *Campylobacter* isolates from the crows would be able to produce a full-length *cdtA* or *cdtB* gene product and thus a functional toxin. Indeed, no mRNA transcript was obtained from these isolates for the gene cluster in RT-PCR assays. This also indicates that no other variant of the *cdtABC* gene cluster is expressed. The crow isolates were unable to produce distension of HeLa cells when examined *in vitro* in toxin assays. Other studies have reported similar observations. One study showed that while a purified CdtA subunit protein having a 43-amino-acid deletion can bind to HeLa cells well, it cannot form the functional CDT holotoxin with wild-type CdtB and CdtC to cause G_2 cell cycle arrest (23). Another study showed that *C. jejuni* isolates lacking CdtB were not able to adhere or invade HeLa cells as well as the wild-type strains and did not show any cytotoxicity (18). The same study reported that when mice were inoculated with cell culture supernatants from these strains that lacked CdtB, they failed to show any histopathology in the gastrointestinal tract. However, crow *Campylobacter* isolates can colonize chicken ceca very well, and this was validated in our chick infection model. Whether such strains can produce gastroenteritis in humans remains to be determined. One study reported that while *C. jejuni* mutants lacking CdtB can colonize NF-κB-deficient mice well, they cause significantly less gastritis than the wild type (17). *C. jejuni* strains can remain colonized in these immunodeficient mice for much longer times than in immunocompetent ones (17). There are two reports where *Campylobacter* organisms that possessed the exact same patterns of deletion were isolated from diarrhea patients (22, 24). One of the reports stated that the isolates were recovered from immunocompromised patients (22). In any case, this smaller fragment was observed in 2.7% (4/147) and 1% (4/325) of the total numbers of patients screened.

None of the reports clarified whether this was the sole strain of *Campylobacter* present in the patients. In chickens, it has been reported that different strains are able to colonize the intestine simultaneously (25); thus, it cannot be ruled out that another strain of *Campylobacter* was responsible for the diarrhea in these patients, especially since no other major toxin has been characterized in these bacteria (26). The likelihood of polymicrobial infections, which are common in settings such as Kolkata, in these diarrheal patients also cannot be ruled out (27). Since patients suffering from campylobacteriosis clearly harbor circulating antibodies to the entire CDT (22), it is unlikely that crow isolates are able to cause diarrhea. In the absence of a suitable animal model, it becomes difficult to test this. Two other reports have presented results on the *cdt* gene cluster from crow campylobacters (10, 12). Of 25 crow *C. jejuni* isolates that were isolated from the species *Corvus corone* and *Corvus macrorhynchos* from Japan, only one had all three genes, while *cdtA* was missing in the other 24. No sequencing of the genes was performed. The study conducted by Weis et al. (10) on *C. brachyrhynchos* used the primer set GNW and LPF-X (18, 20), which amplifies a portion of the gene cluster, generating a product of 1,215 bp in size. On the basis of this, they reported a presence of *cdtA*, *cdtB*, and *cdtC* in 20% (12/59) of the strains. Since the amplicon was not sequenced, it is not clear if the mutations were encompassed within this 1,215-bp product from the *cdt* gene cluster.

It will be worth finding out if other bird species share the same pattern of deletions in their *cdt* gene clusters The *C. jejuni* organisms detected from sandhill cranes implicated in outbreaks (9) have intact *cdt* gene clusters and were 99 to 100% identical to a GBS patient isolate, ICDCCJ07001 (28 and our unpublished data). One of the public health implications would be that the crow does not have a role in the disease and in fact may even be modifying a wild-type *C. jejuni* strain acquired in the environment, which it then sheds back into the environment but with a defective major toxin gene. Recent molecular epidemiological reports indicate that genotype diversity is being continuously generated in a bacterial population (29). One or more genetic mechanisms may be facilitating this, and these include intragenomic events such as rearrangements, point mutations, deletions, duplications, and inversions, as well as horizontal gene transfer (reviewed by Feil et al. [30]). In *C. jejuni*, there is substantial evidence for recombination via interstrain genetic exchange, as well as intragenomic alterations that occur *in vivo* during *C. jejuni* infection (29, 31). The considerable genome plasticity observed for this pathogen, which we saw in analyzing the *cdt* gene cluster and *flaA* gene, may explain its ability to survive in multiple hosts and hostile environments. One estimate reports that the rate of recombination is of a magnitude similar to that for the rate of mutation (31). Furthermore, several studies have shown vertical transmission to be nonexistent in chickens, and even if there is, it is not a significant source for the spread of an allele among the progenies (32). Taken together, it appears that the crow-adapted *C. jejuni* possessing a truncated gene cluster is likely to have come from genomic rearrangements through recombination or mutation. It is also possible that this strain of *Campylobacter* has evolved to persist in the environment, enabling it to be transmitted from the environment to wild bird hosts such as crows, thereby reducing the evolutionary cost of a pathogen killing its host and the creation of a virulent strain. However, such strains would have to evolve in the same way in two distant and highly varied geographical regions, generating identical mutations within the gene cluster. Our future studies will explore the latter hypothesis by investigating whether such strains are better able to persist in the environment than other strains.

Finally, a phylogenetic analysis, based on *flaA* SVR sequencing, showed diverse nucleotide sequences among the 32 isolates that were sequenced. Twenty distinct sequences were obtained. This is not surprising, since *Campylobacter* organisms are weakly clonal (33). However, enough sequences of the SVR may not have been deposited in GenBank databases to draw conclusive results. It is worth noting that the isolates came from two very different geographical areas. Kolkata is a densely populated city in India, a country reported to have one of the largest numbers of diarrheal cases

in children globally (27). On the other hand, greater Seattle, especially from where the fecal samples were collected, is a pristine and clean city. These results will be examined with another technique such as pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST). However, the results add further confirmation to the observation reported by Griekspoor et al. that it is the avian host species rather than the geographic origin of the isolates that is the determinant of the genotype of *C. jejuni* (6).

In conclusion, we report that *C. jejuni* isolates from crows have a highly truncated CDT gene that produces a nonfunctional toxin. The result may have a significant public health implication in terms of the spread of a nonvirulent strain of *Campylobacter*.

MATERIALS AND METHODS

Sample collection. In the crow roost areas within the wetland present on the UW Bothell Campus, crow fecal samples were collected by spreading plastic sheets on the ground underneath the trees where the crows roosted, in the evening prior to the day of collection. Fresh fecal samples, ideally 10 to 30 droppings, from free-flying crows were collected the following morning with sterile swabs and placed in sterile vials kept on ice. This method is similar to those used by several other groups, one of which was from the same site (10, 34, 35). Samples in Bothell wetlands were collected from August 2014 to April 2015. From nonroost areas, such as parking lots or garbage dump areas within the greater Seattle area, samples were collected between September 2015 and December 2015. Crows were attracted by spreading bread crumbs and were subsequently observed to ensure crow droppings from different birds were collected. Fecal samples were collected from 4 sites in Kolkata, India, from July 2016 to September 2016, with the distance between the 2 farthest points being 10 miles (Table 1). Spots in Kolkata were carefully reviewed prior to collection, such that crows were the only birds in those areas. Fecal samples were collected as described above. It took less than 1 h to obtain 10 to 15 fresh fecal samples, and each sample came from a single crow as determined by observing the crows. Samples were collected with sterile swabs, placed in sterile plastic tubes kept on ice, and processed within 2 h of collection.

***Campylobacter* isolation.** *Campylobacter* was isolated by direct plating of the feces on sheep blood agar plates (SBAP) containing specific antibiotics. Briefly, a 100-mg fecal sample was diluted in 500 μl PBS until a fluid suspension was obtained. From each suspension, 30 μl was plated on CVA blood agar plates (Hardy Diagnostics, Santa Clara, CA) containing the antimicrobials cefoperazone, vancomycin, and amphotericin B. The remaining sample was stored at 80°C in PBS. Kolkata isolates were plated on blood agar plates containing the antibiotics bacitracin, cefazolin sodium, colistin sulfate, cycloheximide, and novobiocin.

The plates were incubated at 37°C under microaerophilic conditions either by using a CampyGen pouch (Oxoid Limited, Hampshire, UK) or by placing them in a double gas incubator (Heraeus, Germany) that maintained microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) for up to 7 days, with first observation after 2 days. White-to-translucent colonies were plated again on Columbia blood agar plates and incubated under microaerophilic conditions for 48 h. Initially, the isolates were examined microscopically for a spiral-shaped morphology. Presumptive positives were verified by extracting the DNA and performing qPCR as described below.

The isolates that were positive by PCR were stored at −80°C in tryptic soy yeast (TSY) containing 15% glycerol.

Chick colonization model. The method was developed by the U.S. EPA and is described elsewhere (D. Lye, J. Lu, I. Struewing, E. Villegas, C. Ju, and T. Grube, submitted for publication). Briefly, suspensions of *C. jejuni* clinical strain CS2 (obtained from a fecal sample of a patient diagnosed with *C. jejuni* infection) and crow fecal isolate B37 were prepared by harvesting lawns of bacteria after 48 to 72 h on SBAP in sterile tap water. The suspensions were adjusted to obtain bacteria at 1×10^7 CFU/ml, dispensed in containers of 50 ml tap water, and placed into individual chick cages. One-day-old chicks were obtained from fertilized chick eggs (premium fertile eggs; Charles Rivers Labs, North Franklin, CT). The volume of water ingested by each chick was monitored after 24 h. Total bacterial numbers ingested were estimated from the volumes ingested. Control chicks received only sterile tap water.

Fecal samples were collected at four time points (48 h and 1, 2, and 3 weeks). Approximately 1.3 mg of the fecal specimens was used to isolate *Campylobacter* as described earlier. All animal experiments were approved by the U.S. EPA animal facility oversight of the Institutional Animal Care and Use Committee.

DNA extraction. Chick feces (~0.25 mg) were lysed in microtubes containing 300 μl of tissue and cell lysis solution (Epicenter Biotechnologies, Madison, WI) by using a Mini-Beadbeater-16 (BioSpec Products, Inc., Bartlesville, OK) twice for 30 s. The mixtures were centrifuged at 10,000 $\times$ *g* for 8 min, and the supernatants were transferred to sterile tubes. DNA was extracted and purified by using the MasterPure complete DNA purification kit (Epicenter Biotechnologies) as per the manufacturer's instructions. DNA concentrations were estimated with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). DNA extracts were stored at −20°C until qPCR and PCR were performed.

A rapid DNA isolation method was used to extract DNA from the presumptive positive *Campylobacter* isolates obtained from the crow feces. A colony was picked directly from the plate and suspended in 50 μl Prepman sample buffer (Life Technologies, Foster City, CA). The suspension was heated for 10 min at 95°C, and 2 μl of the supernatant was used in a qPCR/PCR mixture volume of 25 μl. For larger preparations, DNA was extracted from colonies of pure culture streaked on half of an agar plate, which

TABLE 2 *Campylobacter*-specific primers used in the study

Primer	Sequence (5′→3′)	Gene and/or species (product size [bp])	Reference
CampF2	CACGTGCTACAATGGCATAT	*Campylobacter* spp. 16S rRNA	36
CampR2	GGCTTCATGCTCTCGAGTT		
CampP2 (probe)	FAM-CAGAGAACAATCCGAACTGGGACA		
hipOU	GAAGAGGTTTGGGTGGTG	*C. jejuni* (735)	37
hipOR	AGCTAGCTTCGCATAATAACTTG		
flaA-F	GGATTTCGTATTAACACAAATGGTGC	*fla* (1,728)	40
flaA-R	CTGTAGTAATCTTAAAACATTTTG		
Fla242 FU	CTATGGATGAGCAATT(AT)AAAAT	*fla* (383)	41
FLA625RU	CAAG(AT)CCTGTTCC(AT)ACTGAAG		
LYA-F	CTTTATGCATGTTCTTCTAAATTT	*cdt* gene cluster	20
MII-R	GTTAAAGGTGGGGTTATAATCATT		
cadFU	TTGAAGGTAATTTAGATATG	*cadF* (400)	43
cadFR	CTAATACCTAAAGTTGAAAC		
Forward			
lpxA *C. coli*	AGACAAATAAGAGAGAATCAG	*C. coli lpx* (391)	38
lpxA *C. jejuni*	ACAACTTGGTGACGATGTTGTA	*C. jejuni lpx* (331)	
lpxA *C. lari*	TRCCAAATGTTAAAATAGGCGA	*C. lari lpx* (233)	
lpxA *C. upsaliensis*	AAGTCGTATATTTTCYTACGCTTGTGTG	*C. upsaliensis lpx* (206)	
Reverse			
lpxAARKK2 M	CAATCATGDGCDATATGASAATAHGCCAT		
DS18	CCTTGTGATGCAAGCAATC	*cdtA-cdtB* (450)	39
DS15	ACACTCCATTTGCTTTCTG		

were subsequently suspended in 200 μl of Prepman sample buffer. The supernatants containing the DNA could be stored at −20°C for at least one year without any degradation.

Verification of *Campylobacter* genus by qPCR and species by PCR. The presence of *Campylobacter* spp. was verified by a qPCR method based on the 16S rRNA gene (36). A concentration of 10 pmol was used for each of campF2 and campR2 primers and the campP2 probe (6-carboxyfluorescein [FAM]) (Table 2) together with 12.5 μl of iTaq universal probes supermix (Bio-Rad, Hercules, CA) and 2 μl of sample DNA in a final volume of 25 μl. The qPCR amplification was performed in a MiniOpticon iCycler (Bio-Rad) as follows: 1 cycle at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C, with a final cycle of 5 min at 72°C. For determining the presence of *C. jejuni*, a PCR based on the *hipO* gene (37) was used. A multiplex PCR method was performed with some isolates using the lipid A gene (*lpxA*) according to the method of Klena et al. (38) to see if species other than *C. jejuni*, specifically, *C. coli*, *C. lari*, and *C. upsaliensis*, were present in the feces samples. The primers and the annealing temperatures are shown in Table 2.

Detection of virulence genes. The presence of the *cdt* gene cluster, *flaA*, and *cadF* in the 70 isolates was determined by PCR using the primer sets described in Table 2. A primer concentration of 0.6 μM, together with JumpStart REDTaq ReadyMix reaction mix (Sigma-Aldrich, St. Louis, MO) that contained deoxynucleoside triphosphates (dNTPs), Mg^{2+}, and *Taq* polymerase, was used for all PCRs. Further details of the thermocycling conditions can be found in the respective references in Table 2. The PCR products were detected on 1% agarose gels. The PCR products were verified by sequencing.

Sequencing of the *cdt* gene cluster. The amplified PCR products from the *cdtABC* gene cluster were purified using the GeneJET PCR purification system (Thermo Fisher Scientific) and were sequenced using the same primers as those used to generate the fragment. Sanger sequencing was performed by Eurofins Genomics (Louisville, KY). Altogether, 22 isolates were sequenced, including isolates from Kolkata, India, and from Bothell, Everett, Mercer Island, and Factoria, Washington.

RNA extraction and RT-PCR. Total RNA was extracted from the isolates and the chicken cecal contents using TRIzol reagent (Thermo Fisher Scientific). RNA was quantitated by a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Since the *cdtABC* gene cluster is transcribed as a single mRNA transcript (39), primers DS18 and DS15 (Table 2), which amplify a region within *cdtA* and *cdtB*, were used to determine the expression of *cdtA* and *cdtB* as described by Hickey et al. (39). RT-PCR was performed using the Applied Biosystems high-capacity cDNA reverse transcription kit as per the manufacturer's instructions (Thermo Fisher Scientific). The products were analyzed on 1.5% agarose gels.

CDT assay on HeLa cell line. The CDT assay was done as described previously (17, 18), with minor modifications. Briefly, two different *C. jejuni* isolates, a crow environmental isolate B37 (CDT mutant) and a clinical strain CS2, were grown overnight on SBAP as described above. Bacterial cells were harvested in PBS, pelleted by centrifugation at 4,150 rpm, and resuspended in 4 ml of F12K (ATCC, Manassas, VA) to an average optical density at 600 nm (OD_{600}) of 1.61 ($\sim 4.15 \times 10^7$ CFU · ml^{-1}). Samples were then lysed by sonication on ice (four 30-s bursts with 30-s intervals between bursts at 40% amplitude). Cell debris and unlysed bacteria were removed by centrifugation at 4,150 rpm for 20 min at 5°C, and the samples were sterilized by filtration using 0.22-μm-pore-size filters.

HeLa S3 cells (ATCC, Manassas, VA), suspended in F12K medium with 10% fetal bovine serum (FBS), were seeded into each chamber of Lab-Tek 2 chamber slides at a concentration of 10^3 cells/ml. After

TABLE 3 *Campylobacter* isolates with their corresponding *cdtABC* gene cluster and *fla* SVR sequence accession numbers

Isolate	Sampling site	Accession no.	
		cdtABC	*flaA* SVR
B3	Bothell	KY643496	
B19	Bothell	KY643497	
B23	Bothell	KY643499	KY643521
B28	Bothell	KY643500	KY643522
B31	Bothell	KY643501	
B37	Bothell	KY643502	KY643526
B42	Bothell	KY643503	KY643529
B43	Bothell	KY643504	KY643530
B50	Bothell	KY643505	
B62	Factoria	KY643506	KY643538
B68	Mercer	KY643507	
B69	Mercer	KY643508	KY643539
B81	Everett	KY643509	KY643540
B83	Everett	KY643510	KY643518
Kol 6	Kolkata	KY643498	KY643548
Kol 8	Kolkata	KY643511	KY643549
Kol 10	Kolkata	KY643512	
Kol 12	Kolkata	KY643513	
Kol 15	Kolkata	KY643514	KY643542
Kol 16	Kolkata	KY643515	
Kol 18	Kolkata	KY643516	KY643541
Kol 24	Kolkata	KY643517	KY643544
B6	Bothell		KY643536
B21	Bothell		KY643520
B30	Bothell		KY643523
B32	Bothell		KY643519
B33	Bothell		KY643524
B36	Bothell		KY643525
B38	Bothell		KY643527
B39	Bothell		KY643528
B44	Bothell		KY643531
B48	Bothell		KY643532
B51	Bothell		KY643533
B54CVA	Bothell		KY643534
B56	Bothell		KY643535
B61	Factoria		KY643537
Kol 5	Kolkata		KY643547
Kol 21	Kolkata		KY643543
Kol 28	Kolkata		KY643545
Kol 35	Kolkata		KY643546

incubation of the cells for 1 h at 37°C and 5% CO_2, undiluted and 1:2 dilutions of the filter-sterilized sonicates were added to each chamber. The slides were incubated for 72 h at 37°C in 5% CO_2. Each slide was washed with 1× Hanks' balanced salt solution (HBSS) and stained using the Hema 3 stain kit (Fisher Diagnostics, Middletown, VA). Cells were observed for any morphological changes by using a confocal microscope (Zeiss, Oberkochen, Germany). Wells in which greater than 50% of the HeLa cells were enlarged were considered positive. Images were taken using a monochrome digital camera.

Molecular typing. The *flaA* gene was amplified using the primer set flaA-F and flaA-R as described earlier (40). The 1,728-bp fragment generated was sequenced using the same primers as those used to generate the fragment. The forward primer *flaA* 242FU, which is used for *flaA* SVR sequencing, was also sometimes used to resolve some of the ambiguities (41). From the entire *flaA* sequence, a 268-nucleotide region between nucleotides 301 and 569 that encompasses the SVR, was used for allelic comparisons. In all, 32 fecal isolates (23 from Washington and 9 from Kolkata) were sequenced.

Phylogenetic analysis. The *flaA* sequences as well as the *cdtABC* sequences were analyzed and compared to reference sequences from the National Center for Biotechnology and Information (NCBI) using the search tool BLAST (42). For alignment and tree building, the Mega6 suite of programs was used, and unique alleles were aligned by Clustal W. The maximum composite likelihood model and the neighbor-joining statistical method were used for phylogeny reconstruction, while the test of phylogeny was conducted by the bootstrap method to generate a data set of 1,000 bootstrap replicates from the alignment of unique alleles for the *fla* SVR sequences. Branches with less than 50% bootstrap support or with branch lengths that were not significantly different from zero were collapsed.

Accession number(s). The GenBank accession numbers of the *cdt* gene cluster of unique alleles from *Campylobacter* strains isolated in this study can be found in Table 3. The nucleotide sequences of *fla* SVR

from crow isolates were also submitted to the GenBank and their accession numbers, KY643518 to KY643549, can be found in Table 3.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01893-17.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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