




# Host-associated variability of the *cdtABC* operon, coding for the cytolethal distending toxin, in *Campylobacter jejuni*

Pedro Guirado<sup>1</sup> | Yaidelis Iglesias-Torrens<sup>2,3</sup> | Elisenda Miró<sup>2</sup> | Ferran Navarro<sup>2,3</sup>  | Camile Stephan-Otto Attolini<sup>4</sup> | Carlos Balsalobre<sup>1</sup>  | Cristina Madrid<sup>1</sup> 

<sup>1</sup>Departament de Genètica, Microbiologia i Estadística, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

<sup>2</sup>Hospital de la Santa Creu i Sant Pau and Institut d'Investigació Biomèdica Sant Pau (IIB Sant Pau), Barcelona, Spain

<sup>3</sup>Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Barcelona, Spain

<sup>4</sup>Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Barcelona, Spain

## Correspondence

Carlos Balsalobre and Cristina Madrid, Departament de Genètica, Microbiologia i Estadística, Universitat de Barcelona, Avda. Diagonal 643, Barcelona 08028, Spain.

Email: [cbalsalobre@ub.edu](mailto:cbalsalobre@ub.edu) and [cmadrid@ub.edu](mailto:cmadrid@ub.edu)

## Funding information

Catalonian government; European Regional Development Fund; Spanish Ministry of Economy and Competitiveness; Spanish Ministry of Science, Innovation and Universities; State Bureau of Investigation

## Abstract

*Campylobacter*, a major cause of food-borne gastroenteritis worldwide, colonize the gastrointestinal tract of a wide range of animals, being birds the main reservoir. The mechanisms involved in the interaction of *Campylobacter* with the different hosts are poorly understood. The cytolethal distending toxin, encoded in the *cdtABC* operon, is considered a pivotal virulence factor during human infection. Differences in the prevalence of *cdtABC* genes in *Campylobacter* isolates from three distinct origins (wild birds, broiler chickens and humans) prompted us to further characterize their allelic variability. The sequence of *cdtABC* is highly conserved among broiler and human isolates. A high diversity of *cdtABC* alleles was found among wild bird isolates, including several alleles that do not produce any functional CDT. These results suggest that specific variants of the *cdtABC* operon might define the host range of specific *Campylobacter jejuni* isolates. Moreover, our data indicate that PCR methodology is inaccurate to characterize the prevalence of the *cdt* genes, since negative PCR detection can be the result of divergences in the sequence used for primer design rather than indicating the absence of a specific gene.

## KEYWORDS

allelic variability, *Campylobacter*, cytolethal distending toxin, PCR detection, WGS

## 1 | INTRODUCTION

*Campylobacter* is a major enteropathogen worldwide, causing the most common zoonoses reported in humans in the European Union, the United States and Australia (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021; Kaakoush et al., 2015). In most cases, campylobacteriosis is self-limiting gastroenteritis. Severe cases, although rare, occur mostly among children younger than 5 years, elderly and immunocompromised patients (Kaakoush et al., 2015). Moreover, infection by

*Campylobacter* can trigger the autoimmune polyneuropathic disorder Guillain-Barré syndrome (Koga et al., 2006). *Campylobacter jejuni* is the species reported to cause most of the campylobacteriosis cases in humans (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021).

*Campylobacter jejuni* pathogenicity remains poorly understood. Among the array of bacterial products that contribute to its virulence, the cytolethal distending toxin (CDT) seems to play a pivotal role in the interaction of *Campylobacter* with the host. CDT blocks cell division, causing cell cycle arrest at the G<sub>2</sub> stage prior

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to mitosis (Whitehouse et al., 1998). CDT is a heteromeric AB-type genotoxin produced by several Gram-negative bacterial pathogens, including *Campylobacter*, enteropathogenic *Escherichia coli*, *Salmonella enterica* serovar Typhi, *Haemophilus duceryi* and *Shigella dysenteriae* (Jinadasa et al., 2011). The toxin consists of three subunits CdtA, CdtB and CdtC. CdtB is the active subunit whereas CdtA and CdtC are required for binding and delivery of CdtB within the target cell by a process that is still poorly understood (Lara-Tejero & Galán, 2001). CdtA/CdtC binds to the lipid rafts, cholesterol-rich microdomains within the host cell membranes (Lai et al., 2016). CdtB has DNaseI-like activity producing DNA double-strand breaks in the host cell that causes arrest of the cell cycle, cellular distension and cell death (Smith & Bayles, 2006). Although CDT is considered a crucial virulence factor during human infection, it remains unclear its role during the colonization of natural hosts, such as birds.

*Campylobacter* CDT toxin is encoded in the polycistronic operon *cdtABC*. Most *Campylobacter* epidemiological studies reporting prevalence of genes coding for potential virulence factors, including CDT, are based in the use of PCR for gene detection. Very disparate data on *cdtABC* prevalence among *C. jejuni* isolates from human patients suffering of gastroenteritis has been reported using this methodology. Some studies indicate a high prevalence of the *cdtA*, *cdtB* and *cdtC* genes, whereas other reports much lower prevalence, ranging from 100% (50/50) to 15% (3/20; Bang et al., 2003; de Melo et al., 2021; Iglesias-Torrens et al., 2018; Koolman et al., 2015; Pickett et al., 1996; Weis et al., 2014).

The allelic variability of the *cdtABC* genes in *Campylobacter* isolates from three distinct origins (wild birds, broiler chickens and humans) has been characterized. The present study aimed to evaluate the diversity of *cdt* genes among the isolates from different origins, and the possible role that this variability could play in *Campylobacter* host range specificity.

## 2 | MATERIALS AND METHODS

### 2.1 | Bacterial strains and culture conditions

The *C. jejuni* strains used in this study are part of a strain collection, previously described (Iglesias-Torrens et al., 2018), composed of isolates obtained from faeces of three different sources: human suffering symptomatic gastroenteritis (50), broiler chicken (50) and wild birds (50). Wild bird isolates were obtained from different species: yellow-legged gulls (*Larus michahellis*) from Barcelona (YLGB) and from Medes Islands (YLGMI); Audouin's gulls (*Larus audouinii*) from Ebro Delta (AGD) and Alboran Islands (AGA); feral pigeons (*Columba livia*) from Barcelona (FP); common ravens (*Corvus corax*) from Barcelona (CR); white storks (*Ciconia ciconia*) from Lleida (WS); and northern shoveler (*Spatula clypeata*) from Ebro Delta (NS). Isolates were cultured onto Columbia blood agar (CBA) plates (Sharlau) and incubated at 42°C for 48hr in microaerophilic conditions (CampyGen, Oxoid).

### Impacts

- The sequence of *cdtABC* operon, encoding the cytotoxic distending toxin in *Campylobacter jejuni*, is highly conserved among broiler and human isolates. Instead, a high diversity is found among wild bird isolates, including several alleles that do not produce any functional CDT.
- Sequence analysis reveals that the *cdtABC* operon seems to accumulate DNA modifications, particularly deletions, especially detected among wild bird isolates.
- The data obtained indicate that PCR methodology is inaccurate to characterize the prevalence of the *cdt* genes, since negative PCR detection can be the result of divergences in the sequence used for primer design rather than indicating the absence of the specific nucleotide sequences of a specific gene.

### 2.2 | PCR amplification

Genomic DNA was extracted from cultures grown on CBA plates using the InstaGene Matrix Kit (Bio-Rad Laboratories). PCR reactions (PCR Master Mix x2, ThermoScientific) were performed using 35 ng of DNA as a template and the specific primers for amplification of *cdtA*, *cdtB* and *cdtC* (Table 1) previously used in epidemiological studies (Martinez et al., 2006) and based in the genomic sequence of the reference *C. jejuni* strain 81-176. Amplified PCR products from *cdtB* gene of representative isolates showing different electrophoretic migration were purified using the E.Z.N.A Cycle Pure Kit (OMEGA Bio-tek) and sequenced using the same primers as those used to generate the fragment.

### 2.3 | Genome sequencing

Whole genome sequencing was performed for a selection of 47 *C. jejuni* isolates, including 12 isolates from broiler, 16 from human and 19 from wild birds. The selection was performed in order to sequence isolates from different origins, sequence types virulence and antibiotic resistance profiles (Iglesias-Torrens et al., 2018). The

TABLE 1 Primers used in this work

Primer	Sequence 5'-3'	PCR product (bp)
cdtA	Fw CTATTACTCCTATTACCCACC	422
	Rv AATTTGAACCGCTGTATTGCTC	
cdtB	Fw AGGAACTTTACCAAGAACAGCC	531
	Rv GGTGGAGTATAGGTTTGTGTC	
cdtC	Fw ACTCCTACTGGAGATTTGAAAG	339
	Rv CACAGCTGAAGTTGTTGTTGGC	

DNA extraction was carried out with the Wizard DNA-Purification kit (Promega). The DNA extracted was sent to LifeSequencing (Parc Científic, Universitat de València, Spain) to proceed with the DNA library preparation using the Nextera XT DNA Preparation kit and the NexSeq 500 platform. The BioProject has been deposited at NCBI GenBank, accession number PRJNA850915. To find the *cdtABC* nucleotide sequences in each genome we looked for the primers used in previous amplifications by doing the following. We used the “DNAStringSet” function from the Biostrings package (Pagès et al., 2022) in the R (R Core Team, 2020) programming environment (v.3.0.2) to read the primers and genomes in R. The “vmatchPattern” function was used to find all occurrences of the primers with at most 1 mismatch. The start and end positions and the corresponding strand were recorded.

## 2.4 | Phylogenetic analysis

Evolutionary analyses were conducted with MEGA X (MEGA v10.1.8) (Kumar et al., 2018). The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The evolutionary divergence between *cdtABC* sequences was inferred by estimating the distance matrix via the Maximum Composite Likelihood method (Tamura et al., 2004).

## 2.5 | Ethics approval

No ethical approval was obtained because this study did not involve human subjects, animals or tissue samples and only involved non-invasive procedures.

## 3 | RESULTS

### 3.1 | The prevalence of the *cdtA*, *cdtB* and *cdtC* genes is highly variable among *Campylobacter jejuni* isolates from wild birds

The prevalence of genes coding for *cdtA*, *cdtB* and *cdtC* in a collection of 150 isolates of *C. jejuni* from human suffering symptomatic gastroenteritis, broiler chicken and wild birds (see Materials and methods for details) was determined by PCR. The results indicated that the distribution of the *cdt* genes was very variable. The three *cdt* genes were found in all broiler chicken and human isolates. In contrast to what it was observed among human and broiler isolates, 22 out of 50 isolates from wild birds were negative for the detection of one or two of the *cdt* genes (Figure 1), and prevalence varies for each gene, being 58%, 94% and 90% for *cdtA*, *cdtB* and *cdtC*, respectively (Figure 1). The highest variability was detected for the *cdtA* gene. Hence, 14 isolates were *cdtA* negative, three isolates were *cdtAB* negative, four isolates were *cdtAC* negative and one was negative for *cdtC*.

Thirteen out of the fourteen *cdtA*-negative isolates belonged to the clonal complex ST-1,275 which has been described to be highly predominant among wild birds (Hughes et al., 2009; Sheppard et al., 2009). These isolates were collected from Audouin's gulls and yellow-legged gull from three distant location in the Spanish Mediterranean coast (Medes Islands, Ebro Delta, and Alboran Island). The remaining *cdtA*-negative isolate belonged to the singleton ST-8514 and it was collected from a common raven. Three out of four *cdtAC*-negative isolates from Audouin's gulls from Ebro Delta and Alboran Island also belonged to the CC ST-1275. The last *cdtAC*-negative isolate belonged to the singleton ST-996 and was collected from a northern shoveler in Ebro Delta. The three *cdtAB*-negative isolates belonged to the CC ST-952 and were collected from common ravens. The *cdtC*-negative isolate belonged to the CC ST-1034 and was collected from an Audouin's gull in Ebro Delta. Although all *cdt* gene negative isolates were collected from wild birds, it should be pointed out that *C. jejuni* isolates positive for the three *cdt* genes were also collected from wild birds at the same geographical locations. Our data show that a high diversity in the prevalence of the *cdt* genes was observed among *C. jejuni* isolates from wild birds when compared with isolates from broilers and humans.

When considering the ST-complex determined for each isolate, in most cases, the isolates belonging to a specific ST-complex shared the same pattern of *cdtABC* prevalence (Figure 1). The only exception was within the ST-1275 complex which was the most represented ST among wild birds (16 out of 50 isolates). All the ST-1,275 complex isolates were *cdtA* negative whereas only three of them were *cdtC* negative by PCR.

### 3.2 | Different *cdtB* alleles detected among the wild bird isolates

A puzzling observation was done during the PCR genotyping analysis of the different *cdt* genes. Most isolates PCR-positive for *cdtB* depicted a band that migrated as expected for the canonical *cdtB* sequence of the reference strain 81-176 (531bp). However, some isolates showed a band that migrated more than expected, indicating that the PCR amplified band carried a shorter sequence (Figure 2a). These results suggested the presence of different *cdtB* alleles among distinct isolates. Hence, from the 46 *cdtB*-positive wild bird isolates, 17 apparently carried the shorter *cdtB* allele, representing 36.9% of the *cdtB*-positive isolates. Sixteen isolates belonged to the CC ST-1275 and the remaining isolate to the singleton ST-8514 (Figure 2b). Remarkably, all the isolates that carried a shorter *cdtB* allele were PCR-negative for *cdtA*. It should be noticed that 100% of the broiler and human isolates carry a *cdtB* allele that by PCR amplification resembled the canonical allele of 81-176. The amplicons from the reference strain 81-176, the isolates W02, W54, carrying an apparently canonical allele; and W20, W25, W53, showing a shorter *cdtB* allele, were sequenced (Figure 2c). The amplicons from W20, W25 and W53 showed the same feature when compared to 81-176 amplicon, a deletion of 51 nucleotides, resulting in an amplicon of 480

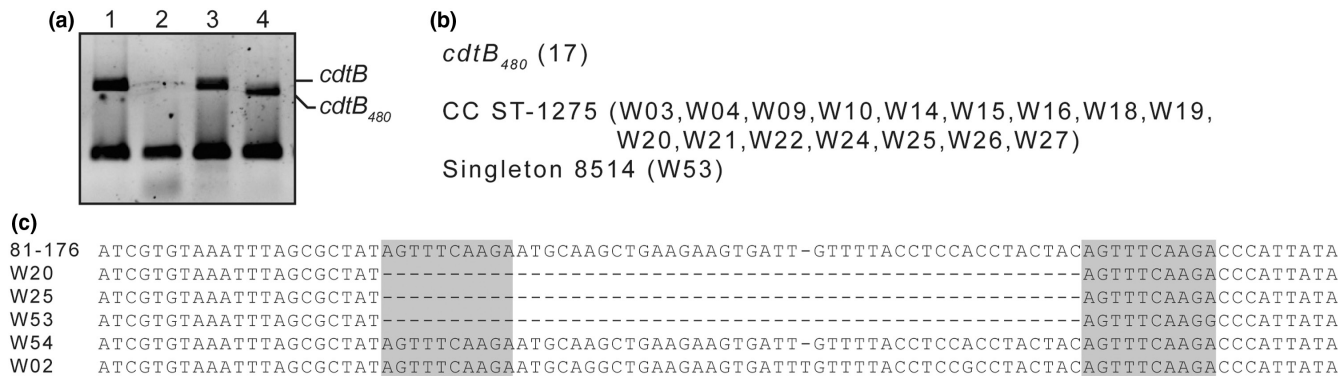
Strain	<i>cdtABC</i>	ST-CC (ST)	Strain	<i>cdtABC</i>	ST-CC (ST)	Strain	<i>cdtA</i>	<i>cdtB</i>	<i>cdtC</i>	ST-CC (ST)	Species	D4	D154	I6	D9	D668	D25	D51	D51 (PCR)	D17	D28	
H 02	21 (4664)	B 06	21 (883)	W30	45 (45)	YLGB																
H 06	21 (21)	B 07	21 (50)	W32	45 (45)	FP																
H 08	21 (3769)	B 14	21 (50)	W36	45 (45)	FP																
H 11	21 (21)	B 17	21 (883)	W37	45 (45)	FP																
H 12	21 (50)	B 20	21 (21)	W40	45 (45)	FP																
H 32	21 (21)	B 36	21 (21)	W43	45 (45)	FP																
H 34	21 (50)	B 46	21 (21)	W44	45 (45)	FP																
H 35	21 (50)	B 50	21 (50)	W47	45 (8512)	FP																
H 37	21 (883)	B 22	45 (652)	W29	48 (48)	YLGB																
H 52	21 (21)	B 27	45 (45)	W33	179 (179)	FP																
H 54	21 (1214)	B 31	45 (45)	W34	179 (2209)	FP																
H 59	21 (21)	B 42	45 (137)	W39	179 (2209)	FP																
H 60	21 (883)	B 09	48 (48)	W41	179 (220)	FP																
H 66	21 (50)	B 49	206 (46)	W46	179 (2209)	FP																
H 68	21 (19)	B 13	257 (2254)	W48	179 (2209)	FP																
H 73	21 (50)	B 52	257 (367)	W49	179 (2209)	FP																
H 58	42 (4016)	B 53	257 (367)	W28	354 (354)	YLGGM																
H 61	42 (459)	B 54	257 (367)	W54	354 (354)	CR																
H 48	45 (45)	B 55	257 (367)	W55	354 (354)	WS																
H 38	48 (48)	B 56	257 (367)	W56	354 (354)	WS																
H 71	49 (49)	B 57	257 (367)	W12	446 (3552)	AGA																
H 51	52 (52)	B 33	283 (267)	W50	952 (8513)	CR																
H 05	61 (61)	B 05	353 (400)	W51	952 (8513)	CR																
H 40	61 (61)	B 18	353 (400)	W52	952 (8513)	CR																
H 57	61 (61)	B 28	353 (5)	W05	1034 (4001)	AGD																
H 65	61 (61)	B 29	353 (5)	W03	1275 (1223)	AGD																
H 70	61 (61)	B 30	353 (400)	W04	1275 (1275)	AGD																
H 09	206 (572)	B 40	353 (400)	W09	1275 (1275)	AGD																
H 13	206 (572)	B 48	353 (356)	W10	1275 (1223)	AGD																
H 14	206 (572)	B 23	354 (354)	W14	1275 (1292)	AGA																
H 62	206 (572)	B 37	354 (8498)	W15	1275 (1223)	AGD																
H 64	206 (227)	B 45	354 (354)	W16	1275 (1292)	AGA																
H 03	257 (257)	B 08	464 (464)	W18	1275 (1223)	AGD																
H 50	257 (257)	B 15	464 (464)	W19	1275 (3629)	AGD																
H 56	257 (257)	B 21	574 (305)	W20	1275 (1275)	AGD																
H 67	257 (2254)	B 25	574 (305)	W21	1275 (1223)	AGD																
H 69	257 (2254)	B 04	607 (607)	W22	1275 (1268)	AGD																
H 36	353 (400)	B 19	607 (7110)	W24	1275 (637)	YLGGM																
H 63	353 (353)	B 24	607 (1707)	W25	1275 (8511)	YLGGM																
H 49	354 (354)	B 38	607 (904)	W26	1275 (3049)	YLGGM																
H 74	354 (354)	B 41	607 (607)	W27	1275 (637)	YLGGM																
H 19	443 (5799)	B 47	607 (904)	W02	S996	NS																
H 46	443 (51)	B 51	607 (7110)	W06	S4355	AGA																
H 18	464 (464)	B 02	S2334	W07	S4355	AGA																
H 04	607 (904)	B 10	S1710	W08	S4355	AGA																
H 01	S441	B 16	S531	W11	S1261	AGD																
H 07	S1710	B 26	S1710	W13	S1343	AGA																
H 33	S8479	B 35	S1710	W17	S2351	AGD																
H 53	S531	B 39	S7114	W23	S1343	AGA																
H 72	S441	B 44	S441	W53	S8514	CR																

**FIGURE 1** Collection of *Campylobacter jejuni* isolates from human patients (H), broiler chicken (B) and wild birds (W). The strains are organized attending to the host and ST clonal complexes (ST-CC). Sequence types are also indicated (ST). The species of the wild bird strains are specified (YLGB: yellow-legged gulls from Barcelona; YLGM: yellow-legged gulls from Medes Islands; AGD: Audouin's gulls from Ebro Delta; AGA: Audouin's gulls from Alboran Islands; FP: feral pigeons; CR: common ravens; WS: white storks; NS: northern shoveler). *cdtA*, *cdtB* and *cdtC* positive strains by PCR amplification are indicated in colour background (green for human isolates, red for broiler chicken isolates and blue for wild bird isolates). The different nucleotide deletions and insertions determined by WGS or PCR analysis are indicated in yellow. The shadowed strain names (clear green, pink and clear blue) indicate the strains that the complete *cdtABC* operon sequence have been determined.

nucleotides (as compared with 531bp amplicon from 81-176), that explained the shorter band detected by PCR. From now, we will refer to this *cdtB* allele as *cdtB*<sub>480</sub>. Interestingly, in the genome of the strain 81-176, the 51 bp deleted sequence from *cdtB*<sub>480</sub> allele was flanked by two 10 bp direct repeats (Figure 2c), suggesting that the *cdtB*<sub>480</sub> allele was most probably generated by a site-specific recombination event.

### 3.3 | Whole-genome sequencing reveals a high diversity in the *cdtABC* locus among wild bird isolates

To further characterize the diversity within the *cdtABC* locus, whole-genome sequencing (WGS) was performed for 47 *C. jejuni* isolates, including 12 isolates from broiler, 16 from human and 19 from wild birds. The sequence of the *cdtABC* locus was characterized



**FIGURE 2** Identification of the *cdtB*<sub>480</sub> allele. (a) PCR amplification of *cdtB* gene from strains 81–176 (lane 1), W50 (lane 2), W54 (lane 3) and W54 (lane 4). The amplification of the housekeeping *gltA* gene was used as internal control. (b) Isolates that carry the *cdtB*<sub>480</sub> allele. (c) Sequences obtained from the PCR amplification of strains carrying the *cdtB*<sub>81-176</sub> or the *cdtB*<sub>480</sub> allele. Shadow square indicates the 10 bp direct repeats flanking the 51 bp deletion.

(Figure S1, Appendix S1). Surprisingly, sequences of the three ORFs of the *cdtABC* operon were found in all 19 isolates from wild birds, including isolates that were negative by PCR for *cdtA* (5 isolates), *cdtAB* (2 isolates), *cdtAC* (2 isolates) and *cdtC* (1 isolate; Figure 1). Sequence alignment of the *cdtABC* sequences from all 47 sequenced isolates and the reference strain 81–176, revealed that 38 of the isolates carried a *cdtABC* operon of similar length to the *cdtABC*<sub>81-176</sub>, whereas 9 isolates, all of them from wild birds, had suffer drastic DNA modifications, resulting in shorter *cdtABC* operons (Figure S1, Appendix S1).

The optimal tree of the evolutionary analyses of the *cdtABC* operons (Figure 3a) showed that most of the operons from broiler and human isolates formed a very compact cluster (cluster I in Figure 3a). Only four wild bird isolates were found scattered within this cluster I, whereas most of the *cdtABC* operons from wild bird isolates were distant from the broiler/human cluster. The four wild bird isolates founded within the broiler/human cluster I were W12, a ST-446 isolated from AGA; W29 a ST-48 isolated from YLGB and W28 and W56, two ST-354 isolated from YLGM and WS, respectively. The four isolates were determined as *cdtABC* positive by PCR detection.

The rest of *cdtABC* operons from wild birds were grouped in three different clusters. Cluster II was characterized by carrying no deletions or 1 bp deletion, cluster III by having deletions shorter than 50bp in total and cluster IV by carrying several deletions causing an overall deletion of more than 700bp.

The evolutionary divergence between *cdtABC* sequences was also estimated and the resulting heat map showed the differences between most of the *cdtABC* operons from wild bird isolates respect those isolated from broiler and human (Figure 3b). As expected, similar clustering of the *cdtABC* operons as observed in the optimal tree (Figure 3a) was found.

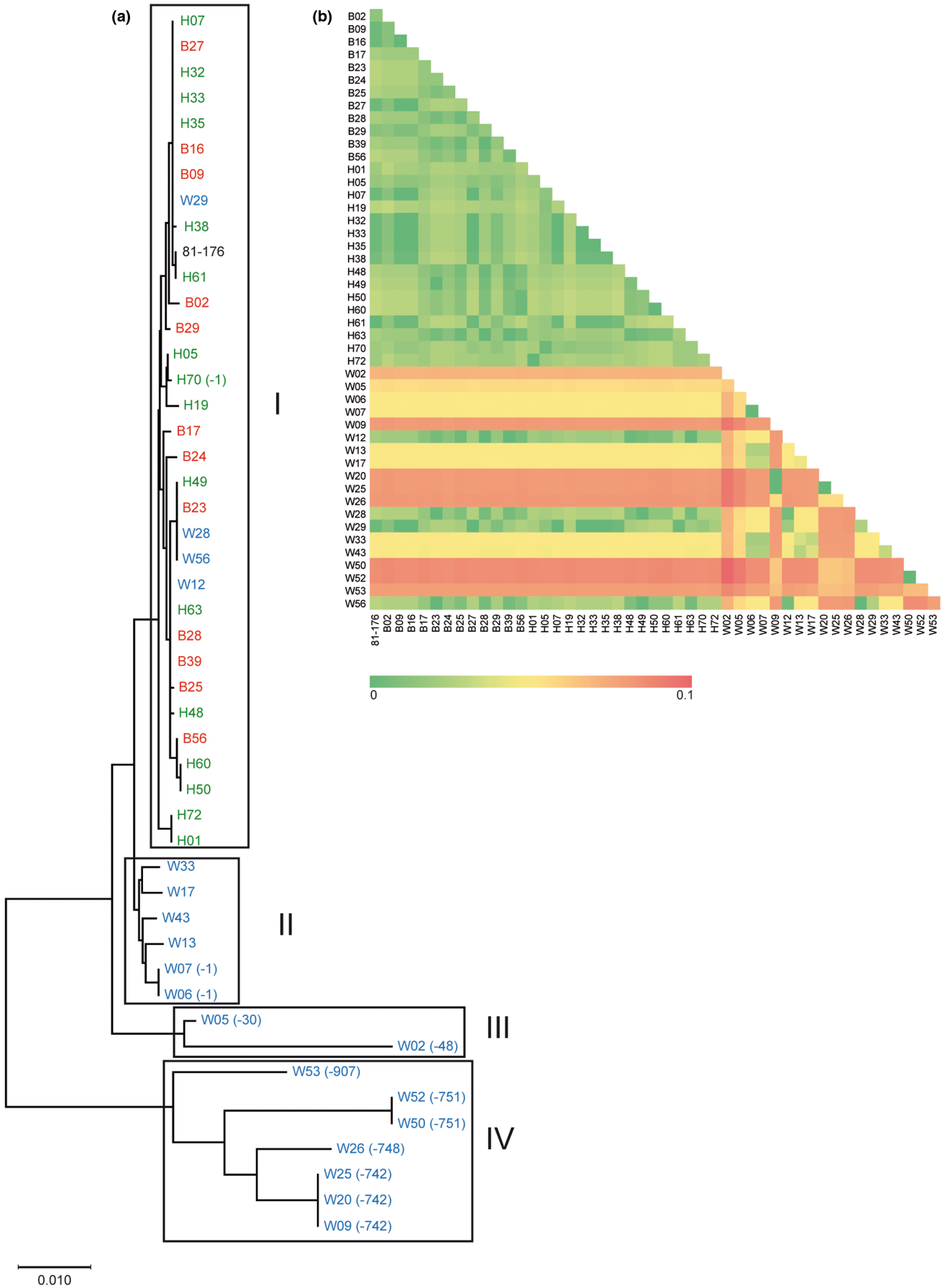
The single nucleotide polymorphism (SNP) distribution respect to the *cdtABC*<sub>81-176</sub> for the 38 isolates with a presumably intact *cdtABC* operon, corresponding to cluster I and II (Figure 3a), is shown in Figure 4a. Overall, it was manifest that *cdtA* accumulated higher number of SNPs as compared with *cdtB* and *cdtC*. The frequency of

nucleotide substitution for *C. jejuni* isolates from broilers, humans and wild birds, was 0.48%, 0.53% and 0.53% for *cdtA*, 0.25%, 0.16% and 0.65% for *cdtB*, and 0.06%, 0.09% and 0.77% for *cdtC*, respectively (Figure 4b). Moreover, it was also patent that a group of wild bird isolates (W06, W07, W13, W17, W33, W43), corresponding to cluster II, carried *cdtB* and *cdtC* genes with a higher degree of variation than the isolates from cluster I. The results point out that the *cdtABC* operons from wild birds showed a higher variability (0.64%) as compared with the *cdtABC* operons from broiler and human isolates (0.28% in both groups; Figure 4b). The nucleotide substitution frequency was also calculated for the arbitrarily chosen *ciaB*, *cadF* and *glnA* genes, revealing similar divergence among the three groups of isolates.

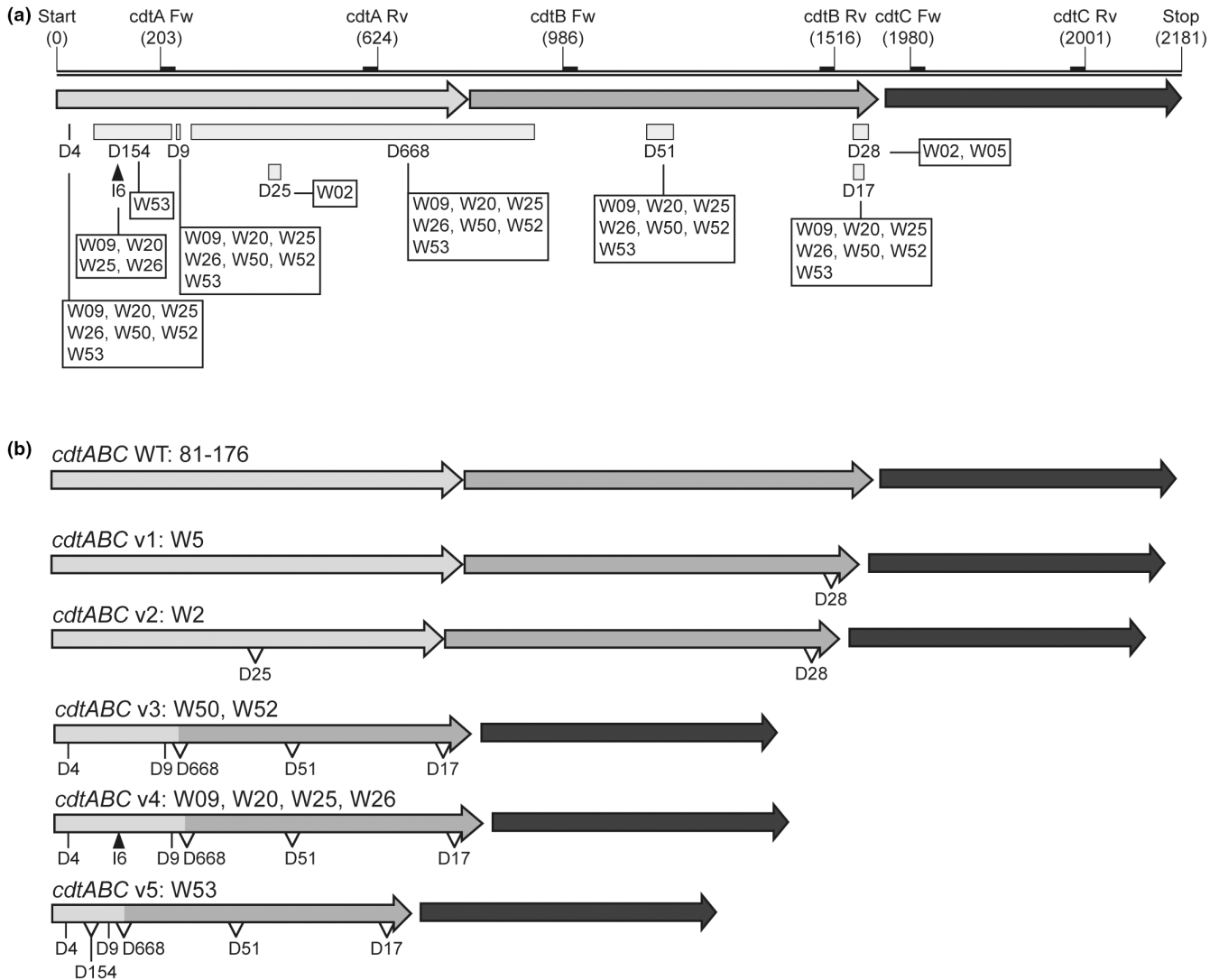
### 3.4 | Several *Campylobacter jejuni* isolates from wild birds carry nonfunctional *cdtABC* operons

The analysis of the nucleotide sequence of the *cdtABC* operons from wild bird isolates showed that this operon is highly variable and prompted to accumulate DNA modifications. When considering deletion and insertion larger than 3bp, several modifications were identified within the *cdtABC* operon. Remarkably, the modifications occurred within the *cdtA* and *cdtB* genes. No deletions and insertions larger than 3bp were found within *cdtC* (Figure 5a, Figure S1, Appendix S1). Among *cdtA* genes, four deletions were detected, D4, D9, D25 and D154 of 4, 9, 25 and 154bp, respectively. An insertion of 6 bp (I6) was also identified in four *cdtA* alleles. Within the *cdtB* gene three specific deletions were detected D17, D28 and the above described D51. Interestingly, six isolates carried a large deletion of 668bp (D668) that affected both *cdtA* and *cdtB* genes.

Considering the mentioned modifications, five *cdtABC* operon variants were identified (Figure 5b). The *cdtABC* v1 has only a deletion (D28) within *cdtB*. The *cdtABC* v2 seems to derive from variant 1 since in addition to D28, an additional deletion (D25) was found in *cdtA*. The other three variants underwent drastic modifications,







**FIGURE 5** Rearrangements detected within the *cdtABC* operons. (a) Schematic representation of the *cdtABC* gene cluster. The arrows indicate the sequences of the three *cdtA*, *cdtB* and *cdtC* genes. The relative position of primers used for PCR amplification of each gene is indicated. The different deletions and insertions found are represented, indicating the number of nucleotides affected and the wild bird isolates that carries each of them. (b) Representation of the five variants of the *cdtABC* operon identified as compared with the *cdtABC* operon of strain 81-176. The deletions and insertions determined in each of the variants are showed, as well as the isolates where have been characterized.

*cdtABC* since the predicted proteins are of same length as the one produced by the 81-176 strain. The only exception was the isolates W06 and W07 that carried a *cdtB* gene encoding a slightly shorter protein of 259 amino acids. The *cdtABC* from the two isolates in cluster III encoded a shorter CdtB toxin, since had a theoretical length of 145 and 179 residues for W02 and W05, respectively. Similarly, the CdtC subunit from both isolates was also shorter with 82 and 40 as compared with the 189 residues of CdtC<sub>81-176</sub>. CdtA was apparently expressed in W05 whereas was non-expressed in W02 by the presence of a codon stop in the third codon.

The sequence of the *cdtABC* operon from the the seven *C. jejuni* isolates from cluster IV indicated that these isolates did not produce any functional CDT toxin since apparently any of the three subunits could be produced. SNPs and the deletions and insertions in the

sequences caused early stop codons within *cdtA* and *cdtC*, and the accumulated deletions did not allow generation of a CdtB protein.

## 4 | DISCUSSION

In this research, the existing variability within the *cdtABC* operon has been studied in a collection of *C. jejuni* isolates from three different origins: broiler, human and wild birds. Our data shows that the *cdtABC* operon seems to be highly conserved among broiler and human isolates whereas a great diversity was found among wild bird isolates as shown by the prevalence of the three *cdt* genes by PCR, the fragment length polymorphism of the *cdtB* amplicon and the *cdtABC* sequence analysis from a selected group



**TABLE 2** Summary of the results of PCR amplification, sequence homology with the primers used, deletions and insertions determined, total bp and predicted AA from *cdtA*, *cdtB* and *cdtC* genes of wild bird isolates, grouped in clusters as defined in Figure 3a

			PCR	Primers	Deletions	Insertions	bp	AA
<i>cdtA</i>	WT	81–174	POS	22/22 22/22			807	268
	I	W12	POS	22/22 22/22			807	268
		W28	POS	22/22 22/22			807	268
		W29	POS	22/22 22/22			807	268
		W56	POS	22/22 22/22			807	268
		W06	POS	22/22 22/22			807	268
	II	W07	POS	22/22 22/22			807	268
		W13	POS	22/22 22/22			807	268
		W17	POS	22/22 22/22			807	268
		W33	POS	22/22 22/22			807	268
		W43	POS	22/22 22/22			807	268
	III	W02	NEG	20/22 20/22	D25, 1(-1)	6 (+1)	787	1
		W05	POS	22/22 22/22			807	268
	IV	W09	NEG	20/22 ND	D4, D9, D668	1 (+1), 1 (+6)	567	11
		W20	NEG	20/22 ND	D4, D9, D668	1 (+1), 1 (+6)	567	11
		W25	NEG	20/22 ND	D4, D9, D668	1 (+1), 1 (+6)	567	11
		W26	NEG	20/22 ND	D4, D9, D668	1 (+1), 1 (+6)	256	11
		W50	NEG	20/22 ND	D4, D9, D668	1 (+1)	250	11
		W52	NEG	20/22 ND	D4, D9, D668	1 (+1)	250	11
		W53	NEG	ND/ND	D4, D154, D9, D668, 1 (-1)		94	11
<i>cdtB</i>	WT	81–174	POS	22/22 22/22			798	265
	I	W12	POS	22/22 22/22			798	265
		W28	POS	22/22 22/22			798	265
		W29	POS	22/22 22/22			798	265
		W56	POS	22/22 22/22			798	265
		W06	POS	22/22 22/22	1 (-1)		797	259
	II	W07	POS	22/22 22/00	1 (-1)		797	259
		W13	POS	22/22 22/22			798	265
		W17	POS	22/22 22/22			798	265
		W33	POS	22/22 22/22			798	265
		W43	POS	22/22 22/22			798	265
	III	W02	POS	22/22 22/22	D28	2 (+1)	772	145
		W05	POS	22/22 21/22	D28	1 (+1)	771	179
	IV	W09	POS	20/22 21/22	D51, D17	1 (+1)	607	NO
		W20	POS	20/22 21/22	D51, D17	1 (+1)	606	NO
		W25	POS	20/22 21/22	D51, D17	1 (+1)	606	NO
		W26	POS	20/22 21/22	D51, D17, 1 (-2), 2 (-1)	1 (+1)	601	NO
		W50	NEG	20/22 20/22	D51, D17, 1 (-1)	2 (+1)	605	NO
		W52	NEG	20/22 20/22	D51, D17, 1 (-1)	2 (+1)	605	NO
		W53	POS	21/22 20/22	2 (-1), D51, D17		602	NO

(Continues)

TABLE 2 (Continued)

			PCR	Primers	Deletions	Insertions	bp	AA
cdtC	WT	81-174	POS	22/22 22/22			570	189
		I	POS	22/22 22/22			570	189
		W12	POS	22/22 22/22			570	189
		W28	POS	22/22 22/22			570	189
		W29	POS	22/22 22/22			570	189
		W56	POS	22/22 22/22			570	189
	II	W06	POS	22/22 22/22			570	189
		W07	POS	22/22 22/22			570	189
		W13	POS	22/22 22/22			570	189
		W17	POS	22/22 22/22			570	189
		W33	POS	22/22 22/22			570	189
	III	W43	POS	21/22 22/22			570	189
		W02	NEG	19/22 21/22	1 (-3) 2 (-1)	3 (+1)	568	82
		W05	NEG	19/22 21/22	1 (-3) 2 (-1)	2 (+1)	567	40
	IV	W09	NEG	22/22 19/22	2 (-2)		567	44
		W20	POS	22/22 19/22	2 (-2)		567	44
		W25	POS	22/22 19/22	2 (-2)		567	44
		W26	POS	22/22 20/22	1 (-1), 2 (-2)	1 (+1)	566	11
		W50	POS	21/22 21/22	1 (-1), 2 (-2)		565	11
		W52	POS	21/22 21/22	1 (-1), 2 (-2)		565	11
W53		POS	21/22 22/22	2 (-1)	1 (+2)	568	122	

of isolates. The high conservation of *cdtABC* among broiler and human *C. jejuni* isolates is consistent with being the chicken meat the most common transmission route of *C. jejuni* to humans via food cross-contamination. The *cdtABC* operon from most of the wild bird isolates showed modifications when compared with the *cdtABC*<sub>81-176</sub> operon highly prevalent among broiler, suggesting that the *cdtABC*<sub>81-176</sub> does not seem to promote efficient colonization among bird other than chicken. Evolutionary analyses of the *cdtABC* sequences grouped some wild bird isolates (W12, W28, W29 and W56) with the human and broiler isolates, which is not surprising since cross-transmission among the three ecological niches studied has been demonstrated (Hald et al., 2016). W12, W28 and W56 isolates belong to clonal complexes ST-354 and ST-446 that have been previously associated with broiler chicken and humans (Cobo-Díaz et al., 2021; Jolley et al., 2018). We do not find isolates of this clonal complexes among the wild bird isolates grouped in clusters II, III or IV. W29, also grouped with human and broiler, was isolated from a sea gull (*L. audouinii*) in the city of Barcelona. Most wild birds that live in urban environment have feeding habits that include feeding in urban refuse dumps.

Among the isolates carrying *cdtABC* operons that did not suffer drastic DNA modifications a higher number of SNPs were detected in *cdtA* as compared with *cdtB* and *cdtC*. Interestingly, *cdtA* was the most commonly missing gene when the prevalence of the three *cdt* genes was determined by PCR. In a previous report, it was shown that addition of CdtB and CdtC to human culture cells has a cytotoxic action as effective as when the three subunits were added, whereas addition of CdtA and CdtB did not induce any damage,

suggesting that CdtA is not essential for the CDT action (Smith & Bayles, 2006). The frequency of nucleotide substitution for *cdtA* is similar among the isolates from clusters I and II independently of its origin, whereas *cdtB* and *cdtC* showed low variability among human and broiler and much higher among wild birds. These results suggest a correlation between certain *cdtABC* alleles and efficient colonization of specific hosts.

Analysis of the complete sequence of the *cdtABC* operons provide an explanation to the negative detection of *cdt* genes by PCR. All isolates that were negative for one or more *cdt* genes carries *cdtABC* alleles were deletions or SNPs affect the hybridization with the specific primers used. Our data indicate that PCR amplification fails when at least two SNPs are found within the primer-hybridizing sequence. These results question the convenience of using PCR-genotyping to determine gene prevalence of the *cdt* genes.

Several deletions were found among *cdtABC* operon from wild bird isolates defining five different allelic variants. The deletions D51 and D668 were previously reported in isolates from human patients (AbuOun et al., 2005; Kabir et al., 2011). In our isolate collection, we did not detect the indicated deletions among the isolates from broiler or human patients. In AbuOun et al. (2005), the only *C. jejuni* strains carrying the D51 and D668 were isolated from patients with underlying health problems that could induce an immunocompromised status. In fact, those patients develop *Campylobacter* bacteremia. The same deletions (D51 and D668) together with D9 and D17 were detected in *C. jejuni* isolates from crows (Sen et al., 2018). We detected those deletions not only in crow isolates but also in isolates from different wild birds,

belonging to different clonal complex, and living in different ecological niches.

Our data indicates that isolates circulating among wild birds have specific characteristics, regarding the *cdtABC* operon, which differs from the isolates colonizing broiler and human. These findings suggest that *cdtABC* might be a cellular factor involved in host specificity attending to the high degree of conservation among broiler and the relevant differences detected among the wild bird isolates. Moreover, the fact that many wild bird isolates carry apparently nonfunctional CDT toxin suggest that *C. jejuni* from wild bird strains might be less virulent to humans.

#### AUTHOR CONTRIBUTIONS

P.G, E.M., Y. I-T., F.N., C.S-O.A, C.M. and C.B. contributed to conceptualization and formal analysis. P.G, Y. I-T., E.M., C.M. and C.B. performed the investigation. C.S-O.A performed the WGS analysis. The original draft was written by C.B. and C.M. All the authors reviewed the manuscript. C.B. and C.M. contributed to project administration and funding acquisition. All the authors have read and agreed to the published version of the manuscript.

#### ACKNOWLEDGEMENTS

We deeply thank Francesc Mestres (University of Barcelona) for his helpful assistance with the evolutionary analysis and the fruitful discussion about the results described in this manuscript.

#### FUNDING INFORMATION

This research was funded by the Spanish Ministry of Economy and Competitiveness (grant AGL2013-45339R), the Spanish Ministry of Science, Innovation and Universities (MCIU), State Bureau of Investigation (AIE) and European Regional Development Fund (FEDER) (grant PGC2018-096958-B-I00) and the Catalanian government (grant 2017SGR499). During this work, a trainee teaching and research staff supported PG grant (Universitat de Barcelona).

#### CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

#### DATA AVAILABILITY STATEMENT

Research data are not shared.

#### ORCID

Ferran Navarro  <https://orcid.org/0000-0002-4302-2838>

Carlos Balsalobre  <https://orcid.org/0000-0002-4147-219X>

Cristina Madrid  <https://orcid.org/0000-0002-5861-3980>

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Guirado, P., Iglesias-Torrens, Y., Miró, E., Navarro, F., Attolini, C.-O., Balsalobre, C., & Madrid, C. (2022). Host-associated variability of the *cdtABC* operon, coding for the cytolethal distending toxin, in *Campylobacter jejuni*. *Zoonoses and Public Health*, *00*, 1–12. <https://doi.org/10.1111/zph.12994>