Prevalence of Cytolethal Distending Toxin Production in *Campylobacter jejuni* and Relatedness of *Campylobacter* sp. *cdtB* Genes

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Campylobacter jejuni produces a toxin called cytolethal distending toxin (CDT). The genes encoding this toxin in *C. jejuni* 81-176 were cloned and sequenced. The nucleotide sequence of the genes revealed that there are three genes, *cdtA*, *cdtB*, and *cdtC*, encoding proteins with predicted sizes of 30,116, 28,989, and 21,157 Da, respectively. All three proteins were found to be related to the *Escherichia coli* CDT proteins, yet the amino acid sequences have diverged significantly. All three genes were required for toxic activity in a HeLa cell assay. HeLa cell assays of a variety of *C. jejuni* and *C. coli* strains suggested that most *C. jejuni* strains produce significantly higher CDT titers than do *C. coli* strains. Southern hybridization experiments demonstrated that the *cdtB* gene is present on a 6.0-kb *ClaI* fragment in all but one of the *C. jejuni* strains tested; the *cdtB* gene was on a 6.9-kb *ClaI* fragment in one strain. The *C. jejuni* 81-176 *cdtB* probe hybridized weakly to DNAs from *C. coli* strains. The *C. jejuni* 81-176 *cdtB* probe did not hybridize to DNAs from representative *C. fetus*, *C. lari*, *C. "upsaliensis,"* and *C. hyointestinalis* strains, although the HeLa cell assay indicated that these strains make CDT. PCR experiments indicated the probable presence of *cdtB* sequences in all of these *Campylobacter* species.

Campylobacter jejuni is established as one of the most frequent bacterial causes of diarrheal disease in humans throughout the world (6, 20, 33). The disease can be variable, but patients typically have watery diarrhea that often contains mucus and may progress to bloody diarrhea (5, 6). Whether specific disease symptoms correlate with specific virulence factors or pathogenic traits is not known. *C. jejuni* has been reported to produce toxins, although definitive reports of these toxins have not appeared, and it is not clear how many different toxins *C. jejuni* actually produces (11, 16, 18, 21, 23, 28). Consequently, the role of any toxin in pathogenesis has not been established.

Cytolethal distending toxin (CDT) production by *C. jejuni* was first described by Johnson and Lior (16) in 1988. CDT activity in culture supernatants caused several cultured cell lines, including HeLa and Vero cells, to become slowly distended over a 2- to 4-day period, after which the cells disintegrated. The activity was heat sensitive, trypsin sensitive, and nondialyzable (16). Johnson and Lior screened over 500 *C. jejuni* isolates obtained from many different countries and found that approximately 41% produced CDT. The CDT-positive isolates included representatives of all four *C. jejuni* biotypes and numerous serogroups. They also tested *C. coli*, *C. lari*, and *C. fetus* strains and showed that about 42% of these strains produced CDT (16). These *Campylobacter* species, as well as *C. hyointestinalis* and *C. "upsaliensis,"* have all been shown to cause diarrheal disease in humans, although they apparently cause the disease much less frequently in the United States than *C. jejuni* does (8, 10, 26, 32, 33, 37).

Johnson and Lior (13–15) also reported that a small number

of *Escherichia coli* and *Shigella* sp. isolates apparently produce a similar activity, also called CDT. The cloning and sequencing of *cdt* genes from two different *E. coli* strains was recently reported (30, 35). These reports indicate that CDT activity is produced from three adjacent genes which encode proteins of approximately 28, 30, and 20 kDa. The predicted amino acid sequences of the Cdt proteins confirmed that CDT is a novel toxin; none of the Cdt proteins exhibited similarity to any other proteins whose sequences are known.

The predicted Cdt amino acid sequences for the two *E. coli* strains exhibited considerable divergence. The *E. coli* CdtA, CdtB, and CdtC proteins shared 36, 55, and 34% identical amino acids and 17, 13, and 14% conserved amino acids, respectively (30, 35). These results suggest that considerable heterogeneity among *cdt* genes may exist.

We report here the cloning and sequencing of the *cdt* genes from *C. jejuni* 81-176, as well as the results of hybridization experiments designed to detect *cdtB* sequences in other *C. jejuni* and *C. coli* strains. In addition, HeLa cell assays were used to quantitate CDT production by a variety of *C. jejuni* and *C. coli* isolates. Finally, hybridization and PCR experiments were used to screen several related *Campylobacter* species for the presence of *cdtB* sequences. This is the first report of the isolation and characterization of any toxin genes from *Campylobacter* spp.

MATERIALS AND METHODS

Bacterial strains and media. *C. jejuni* 81-176 has already been described (2, 19). The other *C. jejuni* and the *C. coli* strains used in this study are listed in Table 1. Some of these strains have been reported elsewhere: *C. jejuni* C31 by Guerrant et al. (11); *C. jejuni* 79-193 and 84-49 by Blaser et al. (4); *C. jejuni* 84-19 by Blaser et al. (4) and Perez-Perez and Blaser (27); and *C. jejuni* G13, G14, and D1156 and *C. coli* D115 and D126 by Baig (1). *C. jejuni* isolates from animals were obtained from the Centers for Disease Control and Prevention (Atlanta, Ga.). *C. coli* 43473, 43482, 43485, and 43488; *C. fetus* ATCC 27374 (type strain); *C. hyointestinalis* ATCC 35217 (type strain); *C. lari* ATCC 43675; and *C. "upsaliensis"* ATCC 43954 were obtained from the American Type Culture Collection (Rockville, Md.). All other *C. coli* strains were obtained from the Centers for Disease Control and Prevention. All *Campylobacter* species were grown on brucella agar as previously described (29). *E. coli* SURE (Stratagene, La Jolla,

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TABLE 1. CDT production by *C. jejuni* and *C. coli* isolates

Organism	Source ^a	Mean titer ^b ± SD
<i>C. jejuni</i> strains		
81-176	Human, Minnesota	488 ± 195 (556, 640, 269)
82-16	Human, Connecticut	1,264 ± 592 (791, 1,928, 1,072)
84-142	Human, Bangladesh	2,173 ± 295 (1,962, 2,102, 2,528)
85-439	Human, Mexico	3,369 ± 1,691 (4,448, 1,420, 4,238)
85-448	Human, Mexico	2,122 ± 1,454 (2,572, 496, 3,298)
85-452	Human, Mexico	386 ± 143 (526, 392, 240)
79-193	Human, Colorado	2,941 ± 2,230 (490, 3,480, 4,852)
85-316	Human, Colorado	1,193 ± 46 (1,223, 1,216, 1,140)
85-360	Human, Colorado	117 ± 10 (128, 110, 112)
C31	Human, Virginia	408 ± 255 (693, 202, 329)
G13	Human, Georgia	2,755 ± 1,848 (668, 3,414, 4,184)
G14	Human, Georgia	2,897 ± 2,295 (2,544, 5,348, 796)
84-19	Human spinal fluid, England	22 ± 16 (42, 20, 3, 22)
84-49	Human blood, Colorado	1,297 ± 563 (654, 1,530, 1,706)
D133	Cow, United States	11 ± 19 (5, 0, 0, 39)
D135	Cow, Japan	1,285 ± 527 (1,891, 936, 1,028)
D1118	Cow's milk, Vermont	1,924 ± 264 (1,739, 1,806, 2,226)
D1156	Cow, Vermont	1,016 ± 221 (1,062, 776, 1,211)
D141	Goat, Canada	754 ± 144 (588, 850, 824)
D142	Lamb, United States	598 ± 22 (622, 581, 590)
<i>C. coli</i> strains		
43473	Human, Belgium	0 (0, 0, 0, 0)
43482	Human, Canada	14 ± 14 (33, 14, 4, 4)
43485	Human, Georgia	0 (0, 0, 0, 0)
43488	Human, Israel	2 ± 3 (6, 3, 0, 0)
D730	Human, Washington	0 (0, 0, 0, 0)
D2593	Human, Texas	1 ± 1 (0, 2, 0, 0)
D2598	Human, Minnesota	13 ± 11 (6, 16, 27, 3)
78-64	Human blood, Colorado	18 ± 19 (13, 32, 16, 10)
D2591	Human blood, United States	3 ± 4 (0, 2, 9, 0)
D2594	Human blood, Arizona	1 ± 2 (4, 0, 0, 0)
D115	Cow, United States	2 ± 2 (0, 1, 2, 4)
D126	Sheep, Canada	2 ± 1 (1, 4, 3, 2)

^a Isolates from humans were taken from stools unless otherwise indicated. Source information was taken from references 1, 4, 9, 11, and 27 or was kindly provided by M. J. Blaser (3) and M. Nicholson (25).

^b The titers shown are based on at least three independent HeLa cell assays performed on different days with lysates isolated on the day of assay. The numbers in parentheses are the actual titers used to calculate the means. Titers for a given assay were calculated by dividing the reciprocal of the highest lysate dilution that caused at least 50% of the HeLa cells in a given well to become distended by the A_{560} of the resuspended bacteria prior to sonication.

Calif.) and DH5 α MCR (GIBCO/BRL, Gaithersburg, Md.) competent cells were used as recipients in transformation experiments. *E. coli* strains were grown in L medium (24) and, when necessary, supplemented with ampicillin or kanamycin (to final concentrations of 50 and 30 μ g/ml, respectively).

Toxin assay. The HeLa cell assay for CDT was based on that of Johnson and Lior (12) and was done essentially as described by Pickett et al. (30), except that *Campylobacter* strains were routinely grown overnight on a single brucella agar plate. Bacterial cells were suspended from the plates with brucella broth, pelleted, and resuspended in 1 ml of Eagle's minimal essential medium prior to sonication. The A_{590} of a 10-fold dilution of the resuspended cells was measured. Titers of the sonic lysates were calculated by dividing the reciprocal of the highest dilution that affected 50 to 75% of the HeLa cells in a well by the A_{590} of the resuspended bacterial cells. Duplicate assays of a given lysate were highly reproducible, even after up to 2 weeks of storage at -20°C . Repeat assays of a given strain using lysates prepared on different days tended to vary. Consequently, each strain was assayed in at least three independent assays. Negative controls routinely included *E. coli* SURE and DH5 α MCR; the positive control was either *E. coli* 9142-88 or *C. jejuni* 81-176.

Plasmids. Plasmids pK184 (12) and pBluescript IISK⁻ (Stratagene) were used as cloning vectors. Plasmid pK184 contains a p15a origin of replication and a kanamycin resistance gene as the selectable marker. Plasmids pCP9312, pCP9417, pCP9429, pCP9441, pCP9431, pCP9451, pCP9413, pCP9514, pCP9515, and pCP9410 were derived during the course of this study (Fig. 1 and Table 2). Plasmids pCP9312, pCP9417, pCP9429, pCP9441, pCP9431, and pCP9410 have the pBluescript vector, and pCP9451, pCP9413, pCP9514, and pCP9515 have the pK184 vector. Plasmid pCP9441 was derived from pCP9429 by deletion of two *Pst*I fragments, thereby removing all but 0.65 kb of the *C. jejuni* DNA downstream of the *cdt* genes. Plasmids pCP9431 and pCP9413 contain a 3.8-kb *Ssp*I fragment from pCP9441, and plasmids pCP9410 and pCP9451 contain a 1.9-kb *Eco*RV-*Pst*I fragment from pCP9441. Plasmid pCP9514 was derived

FIG. 1. Restriction map of the *C. jejuni* 81-176 *cdt* genes. Arrows indicate the direction of transcription of the *cdt* genes. The plasmids listed at the left contain all or portions of the *cdt* genes, as indicated by the horizontal lines. The short vertical lines mark the limits of the *C. jejuni* DNA in the plasmids. Further details about the specific plasmids are given in Table 2 and Materials and Methods. Restriction endonuclease abbreviations: *Cl*a, *Cl*aI; *R*I, *Eco*RI; *R*V, *Eco*RV; *H*i, *H*i, *H*i; *N*s, *N*sI; *P*v, *P*v, *P*v; *S*s, *S*sI.

TABLE 2. Plasmids used in this study

Plasmid(s) ^a	Intact gene(s) ^b	Promoter orientation(s) ^c
pCP9312	<i>cdtBC</i>	—
pCP9417	<i>cdtA</i>	—
pCP9429	<i>cdtABC</i>	—
pCP9441	<i>cdtABC</i>	—
pCP9413, pCP9431	<i>cdtAB</i>	—, —
pCP9514	<i>cdtA</i>	—
pCP9515	<i>cdtB</i>	—
pCP9451, pCP9410	<i>cdtC</i>	+, —

^a Plasmids with identical inserts are listed together.

^b The *cdt* genes were derived from *C. jejuni* 81-176.

^c Symbols: +, the *lac* promoter present on pBluescript is oriented such that it can promote transcription of the *cdt* genes; —, the *lac* promoter is not in the proper orientation to direct transcription of the *cdt* genes.

from pCP9413 by deletion of a 595-bp *EcoRI* fragment; pCP9515 was derived from pCP9413 by deletion of a 560-bp *NsiI* fragment located within the *cdtA* gene (Fig. 1).

DNA techniques and nucleotide sequencing. Chromosomal DNA was isolated from *Campylobacter* strains as described by Silhavy et al. (36). Plasmid DNA was isolated by an alkaline lysis method (22). Enzymes were used in accordance with supplier specifications (New England Biolabs, Boston, Mass.; GIBCO/BRL; and Boehringer Mannheim, Indianapolis, Ind.). DNA fragments were purified from agarose gels with QIAEX (Qiagen, Studio City, Calif.). Nick translation of DNA fragments was accomplished with a kit (Promega, Madison, Wis.) and [α -³²P]dATP (NEN Research Products, Boston, Mass.). Southern blotting and DNA-DNA hybridization were done by standard procedures (22, 34). Hybridizations were done under nonstringent conditions at 42°C, after which the blots received three 15-min washes in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS), followed by a single 15-min wash in 0.1× SSC–0.1% SDS. The blots were washed at increasing temperatures to obtain information about the relatedness of the hybridizing sequences. Under the conditions presented here, and assuming a 500-bp probe with 33% G+C content, washes performed at 55°C in 6× SSC would allow an approximately 30 to 40% base pair mismatch (34). The colony blot procedures used were those of Wahl and Berger (38). DNA sequencing was done with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) and [α -³⁵S]dATP (NEN Research Products). The nucleotide and predicted amino acid sequences were analyzed by PCgene (IntelliGenetics, Inc., Mountain View, Calif.).

PCR. PCR reagents were supplied by Perkin Elmer (Norwalk, Conn.). All primers were supplied by Integrated DNA Technologies, Inc. (Coralville, Iowa). Degenerative primer VAT2 is based on the amino acid sequence VATWNLQ (Fig. 2B, amino acids 25 to 32) and has the sequence 5'-GT(ACGT)GC(ACG T)AC(GCT)TGGA(CT)CT(AGCT)CA(AG)GG-3'. Degenerative primer WM I1, based on the amino acid sequence WMILGDFN (Fig. 2B, amino acids 187 to 194), has the sequence 5'-(GA)TT(GA)AA(GA)TC(AGCT)CC(TC)AA(TGA)AT CATCC-3'. Chromosomal DNA from *C. jejuni* 81-176, *C. fetus*, *C. lari*, *C. coli* D126, *C. coli* D2594, *C. upsaliensis*, or *C. hyointestinalis* was used as the template in reactions with these degenerative primers. All reactions contained 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, 1.5 mM MgCl₂, 1× *Taq* DNA polymerase buffer, a 0.4 μM concentration of each primer, 0.5 μg of template DNA, and 2 U of *Taq* polymerase. Parameters for all reactions were 30 cycles of 94°C for 1 min, 42°C for 2 min, and 72°C for 3 min.

Minicell experiments. *E. coli* minicell experiments were performed as described previously (30). Proteins produced in minicells were labeled with 50 μCi of [³⁵S]methionine and cysteine (American Radiolabeled Chemicals, Inc., St. Louis, Mo.) per ml and analyzed on an SDS–12% polyacrylamide gel. For each sample, approximately 200,000 cpm of labeled material was applied to the gel.

Nucleotide sequence accession number. The *C. jejuni* 81-176 *cdt* genes have been assigned GenBank accession number U51121.

RESULTS

Cloning and genetic analysis of the *C. jejuni* 81-176 *cdt* genes. Degenerative oligonucleotide primers based on various regions of the *E. coli* 9142-88 and E6468/62 *cdtB* genes (30, 35) were prepared and tested in PCR experiments with *C. jejuni* 81-176 template DNA. Two primers, VAT2 and WM1 (Fig. 2), produced a single product of approximately 475 bp. Since this was close to the expected size (494 bp), the PCR product was cloned and sequenced, revealing a sequence which indi-

A.

<i>Cj</i>	M--KQIIVFILCCFMTFFLYACSSKFENVNPLGRSFGFEFDTPDLKLGLE	48
<i>Ec</i>	MANKRTPIFIAGILIPILLNGCSSGKNKAYLDPKVPFPQVEGGP-----T	45
	* * * * *	
<i>Cj</i>	PTFTPTNQEIPSLISGADLVPIPTIPITPLTRTSNSANNNAANGINPRFKDE	98
<i>Ec</i>	VPSDEPGLPLPGPGPALPTNAPITIP-----	72
	* * * * *	
<i>Cj</i>	AFNDVLIFENRPVSDFTLILGPSGAALTVWALAQGNWIWGYTLIDSXGF	148
<i>Ec</i>	-----EPGTAPAVSLMMDGSLVLTWMSRGAGSSSLWAYIIGDSNSF	112
	* * * * *	
<i>Cj</i>	GDARVWQLLL--YPNDFAMIKNAKTNTCLNAY-----GNGIVHYPCDASNH	192
<i>Ec</i>	GELRNWQIMPGRPTNTIQFRNVVDVGTCTMSTFGPKGGVQLSTAPCKFGPE	162
	* * * * *	
<i>Cj</i>	AQMWKLIIPMSNTAVQIKNLGNGKCIQAPITNLGYDFHKVFKIFTVECAK	242
<i>Ec</i>	RDFDQPMATRNNGNYQLKSLSTGLCIRANFLGRTPSSPYATTLTMRCPSS	212
	* * * * *	
<i>Cj</i>	D--NFDQWFLTTP-----PFTAKPL----YRQGEVR	268
<i>Ec</i>	GEKNFEFMSISSEPLRPALATIAKEIRFPFPQIEPDEHSTGGEQ	258
	* * * * *	

B.

<i>Cf</i>	*****	
<i>Cj</i>	GSSAITEKSWNISVRQIIS	
<i>Ec</i>	MKKIICLFLSFNLAFA--NLENFNVTWNQGSAAATESKWSVSRQLVS	48
	MKKYIISLIVFLSFYAQADLTDFRVATWNQGSAAATESKWNINVRQLIS	50
	*** * * * *	
<i>Cf</i>	CENPADILAVQAEAGNLPTALPTGRSINQGTIVTEHLWQLGSGSRPFQV	
<i>Cj</i>	GANPLDILMIQEAAGTLPTATPTGRHVQGGQTPIDEYEWNLGLTSLRPFDRV	98
<i>Ec</i>	GENAVDILAVQAEAGSPSTAVDTGTLPSPGIPVRELIWNLTNSRPFQV	100
	* * * * *	
<i>Cf</i>	YIYYAQIDTGANRVNLAIIVSRKADEIILPPLTVASRPLIGIRIGNDVF	
<i>Cj</i>	FIYYSRVVVGANRVNLAIIVSRMQAEVVLVPPPTTVSRPLIGIRIGNDVF	148
<i>Ec</i>	YEYFSAVDALGGVRNLAIIVSNRRADVEVFLSPVRQGRPLIGIRIGNDAF	150
	* * * * *	
<i>Cf</i>	FKIHALANGGVDAPIINSVDFRFRNMPN-----IT	
<i>Cj</i>	FNIALANGGTDVGAIIITAVDAHFNMPQ-----VNMIAIGDFNRDPSTI	193
<i>Ec</i>	FTAHAIAMRNNDAPALVEEVYNNFFRDSRDPVHQALNWMILGDRNRREPAKL	200
	* * * * *	
<i>Cj</i>	TSTVDRELANRIRRVFPTSATQASGTLTDYAITGNSNRQQTYPPLAAI	243
<i>Ec</i>	EMNLTVPVRRASEIISPAATQTSQRTLDYAVAGNSV---AFRPSPLQAG	247
	* * * * *	
<i>Cj</i>	LMLASLRSHIVSDHFPVNRKF	265
<i>Ec</i>	IVYGARRTQISSDHFVGVSR	247
	* * * * *	

C.

<i>Cj</i>	MKKIITLFFMFITLAFATPTGDLKDFTEMV-----SIRSLETGI-FL	41
<i>Ec</i>	MKKLAIVFTMLLIAGCSSSQDSANNQIDELGKNNSLFTFRNIQSGPMIH	50
	* * * * *	
<i>Cj</i>	SAFRDTSKDPIDQNNNIKEIVLSDELKQKDKLADLPFGVYQFTNPKESE	91
<i>Ec</i>	NGLHGHQRETIG--WEIVPVKTPEALVTDQS-----GWIMIRTP-NTD	91
	* * * * *	
<i>Cj</i>	LCLAILEDGTFGAKSCQDDLKDGKLETVFSIMPTTSAVQIRSLVLESDE	141
<i>Ec</i>	QCLGTPDGRNLLKMTNCSTAKK---TLFSLIPSTTGAVQIKSVL--SGL	135
	* * * * *	
<i>Cj</i>	CIVTFNPNIPQKRFIAPCTLD-PIFFAEVNMELIITPPLTAATPLE	189
<i>Ec</i>	CFLDSKNSGLS---PETGKCIADFKKPFVFPQSHLWMLNPLNTESPI	180
	* * * * *	

FIG. 2. Comparison of the predicted amino acid sequences of CdtA, CdtB, and CdtC from *C. jejuni* 81-176 (*Cj*) and *E. coli* 9142-88 (*Ec*) and a portion of the CdtB protein from *C. fetus* (*Cf*). A, CdtA; B, CdtB; C, CdtC. The amino acids are numbered at the right. Identical amino acids are indicated by asterisks; conserved amino acids are indicated by periods. Dashes represent gaps introduced by the PCgene alignment program. The overlined residues indicate a possible consensus cleavage site for lipoproteins; cleavage of potential leader peptides for secretion is predicted to occur after the residues in boldface type. The underlined amino acid residues mark the regions of CdtB that were used to construct degenerative oligonucleotides VAT2 and WM1.

cated that the relevant portion of a *cdtB* gene had been amplified. The cloned fragment was subsequently isolated and used as a probe in hybridizations to identify restriction fragments of *C. jejuni* 81-176 DNA that carried *cdt* genes. The

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1  TGCTAAATATAAGTGTTAAGATACATATAAATTCTACCTTTAAAAACAACAAAATAAAACATTTTAAAAAGCGGAAAATTATAATGAAATTTATGTT
    CdtA
1  M Q K I I V F I L C C F M T F F L Y A C
101 ATTATTTTCTTAAAAATTTAAATACATATCAAGGTTTTTAATGCAAAAAATTATAGTTTTTATTTATGTTGTTTTATGACTTTTTTCTTTATGCATGT
21  S S K F E N V N P L G R S F G E F E D T D P L K L G L E P T F P T N
201 TCTTCTAAATTTGAAAATGTAAATCCTTTGGGGCGTTCATTGGGAGAATTGGAAGATACTGATCCTTTAAACTAGGACTTGAACCTACTTTTCCTACCA
55  Q E I P S L I S G A D L V P I T P I T P P L T R T S N S A N N N A
301 ATCAAGAAATTCAGTTTAATTAGCGGTGCTGATTAGTACCTATTACTCCTATTACCCACCTTTAACTAGAACAAAGCAATAGTGCCAAACAATAATGC
88  A N G I N P R F K D E A F N D V L I F E N R P A V S D F L T I L G
401 AGCAAATGGGATCAATCCTCGCTTTAAAGACGAAGCTTTTAATGATGTTTTAATTTTGAATAATCGCCCTGCGGTTTCTGATTTTTTAACCATTTTAGGC
121 P S G A A L T V W A L A Q G N W I W G Y T L I D S K G F G D A R V W
501 CCTAGCGGAGCAGCTTTAACGGTTTGGGCTTTAGCACAAGGAAATTTGGATTGGGGCTATACTTTAATCGATAGCAAAGGATTTGGCGATGCTAGAGTTT
155 Q L L L Y P N D F A M I K N A K T N T C L N A Y G N G I V H Y P C
601 GGCAACTTTTGCTTTATCCTAATGATTTTGCAATGATTAATAATGCCAAACCAATACTTGCTTAATGCTTATGGTAATGGAATTGTCATTATCCTTG
188 D A S N H A Q M W K L I P M S N T A V Q I K N L G N G K C I Q A P
701 TGATGCAAGCAATCAGCACAATGTGGAACCTTATCCCTATGAGCAATACAGCGGTTCAAATTTAAATTTAGGAAATGGAAATGCATACAAGCACCT
221 I T N L Y G D F H K V F K I F T V E C A K K D N F D Q Q W F L T T P
801 ATTACAAATCTTTATGGTGATTTTCACAAGGTTTTTAAATTTTACCGTAGAGTGTGCAAAAAAAGATAATTTTGATCAACAATGGTTTTTAACTACTC
255 P F T A K P L Y R Q G E V R ---
901 CACCTTTTACCGCAAAACCTTTATATCGCCAGGAGAGGTACGATGAAAAAATTTATATGTTTATTTTATCTTTTAACTTGCTTTTGCAAAATTTAGAA
    CdtB
1  M K K I I C L F L S F N L A F A N L E
1001 AATTTTAAATGTTGGCACTTGGAAATTTGCAAGGCTCATCCGAGCCACAGAAAGCAATGGAGTGTAGTGTAAAGACAATGTAAGTGGAGCAAAACCCCT
20  N F N V G T W N L Q G S S A A T E S K W S V S V R Q L V S G A N P L
1101 TATGATATCTTAATGATACAAGAAGCAGGAACCTTTACCAAGAAGACCCACTCCAACAGGACGCCATGTGCAACAAGGTGGAACACCTATTGATGAATATGA
54  D I L M I Q E A G T L P R T A T P T G R H V Q Q G G T P I D E Y E
1201 GTGGAATTTAGGAACCTCTTTCAAGGCTGATAGGGTTTTTATTATTATCTCGCGTTGATGTAGGAGCTAATCGTGAAATTTAGCTATAGTTTCAAGA
87  W N L G T L S R P D R V F I Y Y S R V D V G A N R V N L A I V S R
1301 ATGCAAGCTGAAGAAGTGATTGTTTTACCTCCACCTACTACAGTTTCAAGACCCATTATAGGAATTCGCAATGGAAATGATGCTTTTTTCAATATCCATG
120  M Q A E E V I V L P P P T T V S R P I I G I R N G N D A F F N I H A
1401 CTTTAGCTAATGGAGGAACAGATGTAGGAGCAATTATCACAGCTGTAGATGCACATTTTGCAATATGCCTCAAGTTAACTGGATGATAGCAGGGGATTT
154  L A N G G T D V G A I I T A V D A H F A N M P Q V N W M I A G D F
1501 TAACCGTGATCCTTCTACTATAACAAGTACAGTGGATAGAGAATTAGCAATAGAATTAGAGTGGTTTTTCCAACCTAGCGCAACTCAAGCAAGCGGAGGG
187  N R D P S T I T S T R E A L A N R I R V V F P T S A T Q A S G G
1601 ACTCTTGATTATGCAATTACAGGAATTTCAATAGACAACAACCTTATACTCCACCGCTTTTAGCTGCGATTTTAATGCTTGCAAGTTAAGATCTCATA
220  T L D Y A I T G N S N R Q Q T Y T P P L L A A I L M L A A S L T R S H I
1701 TAGTTTCAGATCATTTTCCAGTAAATTTAGAAAAATTTAGGACATTTAATATGAAAAAATTTATTTACTTTGTTTTTATGTTTATACTTTAGCCTTTG
253/1 V S D H F P V N F R K F --- M K K I I T L F F M F I T L A F A
    CdtC
1801 CAACTCCTACTGGAGATTTGAAAGATTTTACCGAAATGGTTTCTATAAGAAGCTTAGAAACGGGAATTTTTTAAAGCGCTTTAGGGATACCTCAAAGA
18  T P T G D L K D F T E M V S I R S L E T G I F L S A F R D T S K D
1901 TCCTATTGATCAAAATTTGGAATATTAAAGAAATGTTTTAAGCGATGAGTTAAACAAAAAGATAAATTAGCTGATGAACCTTCCTTTTGGTTATGTGCAA
51  P I D Q N W N I K E I V L S D E L K Q K D K L A D E L P F G Y V Q
2001 TTTACAAATCCAAAGAAAGCGATCTTTGTTTAGCCATCTTAGAAGATGGAACCTTTGGAGCAAAATCTTGCAAGATGATCTAAAAGATGGTAAATTAG
84  F T N P K E S D L C L A I L E D G T F G A K S C Q D D L K D G K L E
2101 AAACGTATTTTCTATAATGCCAACAACTTCAAGTGTGCAAAATCGTTCTTTAGTTTTGGAATCTGATGAATGTATAGTAACTTTTTTAAATCCAAA
118  T V F S I M P T T T S A V Q I R S L V L E S D E C I V T F F N P N
2201 TATTCCTATACAAAACGCTTTGGAATAGCCCCCTGCACCTAGATCCTATTTTTTTGCTGAAGTAAATGAACCTAATGATTATAACCCACCTTTAACA
151  I P I Q K R F G I A P C T L D P I F F A E V N E L M I I T P P L T
2301 GCTGCTACCCCTTTAGAAATAAGATTTTTATCTTGTCTATTTTTATATTTATTTAATATTTATGATATTACTAAAAATACACAAAATAATTAATAATA
184  A A T P L E ---
2401 CAATGTAATTTACCTTGCTCTATAATTTTTTTATTTAATGTAATTTTTTGTACATAAATTTATACATAATAATTATCAAGGAGGAAAAATGGACA
    LctP
    I L T W Q Q I Y D P F S N I W L S A L V A F L P I L C F L V C L V
2501 AATTTTAAATGCAACAAATTTATGACCTTTTCAAATATTTGGCTAAGTGCTTTAGTGGCATTTTACCTATACTATGTTTTAGTTTGTGTTGGTT

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FIG. 3. Complete nucleotide sequence of the *C. jejuni* 81-176 *cdt* genes. Predicted amino acid sequences are given in the single-letter code either directly above or below the appropriate codon. The nucleotides are numbered continuously at the left; the amino acids are numbered for each open reading frame, also at the left. Probable initiation codons, or their complement, are in boldface letters. Possible ribosome-binding sites are in boldface letters and underlined. Dashes represent stop codons. The underlined bases at the end of *cdtC* (nucleotides 2314 to 2339) could form a stem-loop structure and may indicate the presence of a rho-independent transcription termination site.

initial fragment identified and cloned was a 6.0-kb *Cla*I fragment. Sequence analysis of the insert in the resultant plasmid, pCP9312, indicated that while intact *cdtB* and *cdtC* genes were present, only the carboxy-terminal two-thirds of CdtA was encoded on this 6.0-kb *Cla*I fragment. Further hybridizations identified an 8.3-kb *Pvu*II fragment that likely carried the missing *cdt* sequences. Attempts to clone this intact *Pvu*II fragment were unsuccessful, but several clones, represented here by pCP9417, did contain the remaining *cdtA* sequences, as well as various amounts of additional *C. jejuni* 81-176 DNA 5' of the *cdtA* gene. The 5' portion of the *cdtA* gene carried by four of these *Pvu*II clones was sequenced; all of the sequences were identical, indicating that any deletion event that had occurred subsequent to delivery of the plasmids to *E. coli* likely did not involve the *cdtA* gene. A clone containing all three *C. jejuni* 81-176 *cdt* genes was constructed by inserting a 5.9-kb *Eco*RV fragment from pCP9312, which contained all of the insert in pCP9312 except for the 0.54-kb *Cla*I-*Eco*RV fragment, into the unique *Eco*RV site in pCP9417 (Fig. 1). The resulting clone, pCP9429, contained intact *cdtA*, *cdtB*, and *cdtC* genes and produced active CDT in *E. coli* (Table 3). Expression of the *C. jejuni* 81-176 *cdt* genes on pCP9429 may be directed by *C. jejuni* promoters, since the *lac* promoter on pBluescript is in the orientation opposite to that required to drive expression of the *cdt* genes.

The complete nucleotide sequence of the *C. jejuni* 81-176 *cdt* genes is shown in Fig. 3. The *cdt* genes of *C. jejuni* 81-176 are arranged in a manner similar to that of the *E. coli* 9142-88 and E6468/62 *cdt* genes (30, 35). There are three adjacent genes which encode putative proteins CdtA, CdtB, and CdtC. The *C. jejuni* 81-176 *cdtA* and *cdtB* genes have a 4-nucleotide overlap, and the *cdtB* and *cdtC* genes are separated by 10 nucleotides; this is very similar to the arrangement of the genes in *E. coli* 9142-88 (30), the only difference being that the *cdtB* and *cdtC* genes in *E. coli* 9142-88 are separated by 14 nucleotides. The *C. jejuni* 81-176 and *E. coli* 9142-88 *cdt* genes are related but not highly homologous; the highest homology, 59%, exists in the 5' half of the *cdtB* genes. The percent G+C contents of the *C. jejuni* 81-176 *cdtA*, *cdtB*, and *cdtC* genes are 34, 36, and 31%, respectively, which is typical for this species. Each of the three *cdt* genes is preceded by an appropriately spaced consensus ribosome-binding site (Fig. 3). Examination of the noncoding region upstream of *cdtA* did not reveal any close homology to known promoter sequences. At the 3' end of *cdtC*, and slightly overlapping the gene, is an inverted repeat which could serve as a rho-independent transcription termination site (Fig. 3); similar potential structures were not seen following the *cdtA* and *cdtB* genes.

An apparent open reading frame upstream of, and on the opposite strand from, the *cdt* genes in *C. jejuni* 81-176 starts at either of the two alternative sites shown in Fig. 3. The initial 150 amino acids of the predicted protein from this upstream open reading frame did not appear to have any significant homology to other known proteins (data not shown).

The 180 bp following *cdtC* appear to be noncoding, but at nucleotide 2502 of Fig. 3, another gene appears to start. The predicted product of this gene possesses significant homology to the predicted product of the L-lactate permease gene of *E. coli* (7). The designation of the somewhat unusual initiation codon for this *C. jejuni* 81-176 gene is based on the start of the *E. coli* gene. Of the first 170 amino acids of the *C. jejuni* permease, 110 are identical to those of the *E. coli* permease, indicating that there has been a high level of conservation in the amino-terminal membrane-spanning regions of these two proteins (data not shown).

The predicted amino acid sequences of the *C. jejuni* 81-176

TABLE 3. Expression of *C. jejuni cdt* genes in *E. coli*

Plasmid(s)	<i>cdt</i> gene(s) present	CDT titer ^a
pCP9514	<i>cdtA</i>	0
pCP9515	<i>cdtB</i>	0
pCP9413 or pCP9431	<i>cdtAB</i>	0
pCP9410 or pCP9451	<i>cdtC</i>	0
pCP9514 and pCP9410	<i>cdtA</i> , <i>cdtC</i>	0
pCP9515 and pCP9410	<i>cdtB</i> , <i>cdtC</i>	0
pCP9413 and pCP9410	<i>cdtAB</i> , <i>cdtC</i>	40
pCP9429	<i>cdtABC</i>	60

^a CDT titers were calculated from HeLa cell assay results as described in Materials and Methods. Each clone was tested in at least three independent assays.

Cdt proteins are compared to those of *E. coli* 9142-88 in Fig. 2. The *C. jejuni* CdtA protein has a predicted molecular weight of 29,919 and a pI of 6.98. The overall level of amino acid sequence homology to the *E. coli* CdtA protein is modest at 34%. (This represents the total of 21% identical amino acids and 13% conservative amino acid substitutions.) Both the *C. jejuni* and *E. coli* CdtA proteins have four Cys residues at conserved locations, and both have a possible cleavage site for leader sequences of lipoproteins (Fig. 2A) (31). The CdtB proteins of *C. jejuni* and *E. coli* are more conserved, with 61% amino acid sequence similarity (46% identity; Fig. 2B). This homology exists throughout the entire CdtB protein, and no particular regions with known functions are apparent, with the exception of a potential leader sequence for secretion. The unprocessed *C. jejuni* CdtB protein is predicted to have a molecular mass of 28,989 kDa and a pI of 8.22. Removal of a potential leader peptide would create a mature CdtB protein with a predicted molecular weight of 27,131 (Fig. 2B). The CdtC proteins of *C. jejuni* and *E. coli*, in common with the CdtA proteins, have only a modest level of amino acid sequence homology. These proteins share 21% identical and 17% conserved amino acids (Fig. 2C). Much of the homology seen between these two CdtC proteins is concentrated in a small region between amino acid residues 118 and 136, but whether this region represents a functional site of some type is unknown. The unprocessed *C. jejuni* CdtC protein has a predicted molecular weight of 21,157 and a pI of 4.13. However, like CdtA and CdtB, CdtC has an apparent leader peptide for secretion which, if removed, would produce a mature CdtC protein with a molecular weight of 19,140 (Fig. 2C).

All three *C. jejuni* 81-176 *cdt* genes are required for the production of CDT activity in *E. coli*. Only when clones encoding all three genes were present in *E. coli* was CDT detected (Table 3). These results are in agreement with the findings of Scott and Kaper (35) and Pickett et al. (30) on the *E. coli cdt* genes.

Minicell results illustrating the production of the *C. jejuni* 81-176 Cdt proteins by selected pK184 clones in *E. coli* are shown in Fig. 4. (Minicell results from pBluescript clones were not helpful for confirming CdtA and CdtB production in *E. coli*; the mature β -lactamase completely obscured CdtB, and CdtA was partially obscured by the unprocessed form of β -lactamase [data not shown].) Plasmid pCP9413 contains intact *cdtA* and *cdtB* genes and produced two proteins with apparent molecular weights of 32,500 and 30,500 (arrowheads in Fig. 4, lane 2). To confirm the identities of these two proteins, two derivatives of pCP9413 were made. Plasmid pCP9514 contains an intact *cdtA* gene, but a portion of the *cdtB* gene was deleted (Fig. 1). Minicells containing pCP9514 produced the upper, less prominent of these two bands but not the lower band (Fig.

FIG. 4. Expression of the *C. jejuni* 81-176 *cdtA*, *cdtB*, and *cdtC* genes in *E. coli* minicells. The locations of molecular weight standards (sizes are in kilodaltons) are indicated at the left. The arrowheads indicate the locations of CdtA, CdtB, and CdtC (see text). Minicells contained different plasmids: lane 1, pK184; lane 2, pCP9413; lane 3, pCP9514; lane 4, pCP9515; lane 5, pCP9451.

4, lane 3). Plasmid pCP9515 contains a deletion within the *cdtA* gene, and minicells containing this plasmid no longer produced the upper band yet still produced the 30.5-kDa band (Fig. 4, lane 4). A protein with an apparent molecular weight of 26,500 was produced by minicells containing pCP9451, which contains an intact *cdtC* gene (Fig. 4, lane 5).

Unlike the *E. coli* 9142-88 Cdt proteins expressed in minicells (30), the *C. jejuni* 81-176 Cdt proteins did not appear as doublets. Proteins with leader sequences for secretion often appear as doublets in minicell experiments because of incomplete processing of the proteins. Since the *C. jejuni* 81-176 *cdt* genes appear to encode leader sequences, doublets were expected. We compared our results obtained with minicells to expression of the *C. jejuni* 81-176 *cdt* genes in an in vitro transcription-translation system, where little or no processing is expected. The mobility of the Cdt proteins produced in this system corresponded exactly to the Cdt proteins indicated in Fig. 4 (data not shown). This suggests that the *C. jejuni* 81-176 Cdt proteins were not significantly processed in *E. coli* minicells.

Production of CDT by *C. jejuni* and *C. coli* strains. We wanted to screen a variety of *C. jejuni* and *C. coli* isolates for production of CDT and for the presence and relatedness of their *cdt* genes to discover the prevalence of CDT production and of *cdt* genes in these species. Our initial experiments, in which we simply tested whether CDT was produced without determining the amount of CDT made, surprised us, since it was apparent that most, if not all, of our *C. jejuni* strains made CDT. Consequently, we decided to broaden our experiment to quantitate the relative amount of CDT made by each strain. The results of these assays are shown in Table 1. These results show that all of these *C. jejuni* strains make CDT and that most of the strains produced titers of greater than 10^2 . Only two strains consistently produced significantly less toxin, D133, a cow isolate, and 84-19, which was isolated from the spinal fluid of a hypogammaglobulinemic individual (4). In contrast, none of the 12 *C. coli* strains, regardless of source, produced lysates with CDT titers of greater than 33; most *C. coli* lysates had titers of less than 10. We also tested several representative *C.*

FIG. 5. Hybridization of *Campylobacter cdtB* fragments to *C. jejuni* and *C. coli* strains. DNA fragment size standards are indicated at the right in kilobases. (A) Hybridizations with a *C. jejuni* 81-176 *cdtB* probe. (B) Hybridizations with a *C. coli* D2594 *cdtB* probe. The autoradiograms pictured were made from blots washed at 55°C in 0.1× SSC. Lanes contained chromosomal DNAs cut to completion with *Cla*I and separated on a 0.7% agarose gel. Lanes: 1, *C. coli* D126; 2, *C. coli* D115; 3, *C. coli* D2594; 4, *C. coli* D2593; 5, *C. coli* D730; 6, *C. jejuni* D133; 7, *C. jejuni* 84-49; 8, *C. jejuni* 84-19; 9, *C. jejuni* G13; 10, *C. jejuni* 85-452; 11, *C. jejuni* 84-142; 12, *C. jejuni* 81-176.

jejuni and *C. coli* strains for the presence of CDT in culture supernatants as described by Johnson and Lior (16). None of the *C. coli* strains had detectable CDT in their unconcentrated culture supernatants; only *C. jejuni* strains that had titers of greater than 100 in our assays of cell lysates had detectable CDT in their culture supernatants.

Overall, these results indicated that production of CDT by *C. jejuni* isolates can be divided into two general classes. One group, that we have called high toxin producers, had CDT titers of 10^2 or greater, while a second group, which we have called low toxin producers, had CDT titers that were significantly lower. All of the *C. coli* isolates we have screened to date appear to be low toxin producers.

Hybridization and PCR studies. Our DNA sequence results indicated that although *E. coli* 9142-88 and *C. jejuni* 81-176 both made CDT, the *cdt* genes in these species were sufficiently dissimilar that the *cdt* genes from one species would, at best, hybridize only weakly to the *cdt* genes of the other species. We therefore decided to use a *C. jejuni* 81-176 fragment of the most closely conserved of the *cdt* genes, *cdtB*, in hybridizations to DNAs from other *C. jejuni* strains or from other *Campylobacter* species. The probe consisted of the insert from the initial PCR clone and represents the DNA encoding the portion of CdtB between the two underlined regions in Fig. 2B. This probe hybridized to a 6.0-kb *Cla*I fragment from 10 of the 11 *C. jejuni* strains tested. The results obtained with seven strains are shown in lanes 6 through 12 of Fig. 5A; the only variation was obtained with strain 84-19 (lane 8), which had a

FIG. 6. PCR products obtained with VAT2 and WMI1 primers and *Campylobacter* sp. template DNA. The arrowhead points to the principal product shown to be an amplified portion of *cdtB* for *C. fetus* and *C. jejuni*. Lanes: 1 and 8, *Hae*III-digested Φ X174 size standards; 2 to 7 PCR products from reactions with different template DNAs; 2, *C. jejuni*; 3, *C. lari*; 4, *C. "upsaliensis"*; 5, *C. hyointestinalis*; 6, *C. coli*; 7, *C. fetus*.

6.9-kb *Cla*I fragment that hybridized to the *C. jejuni* 81-176 *cdtB* probe. Five *C. coli* strains were included in this experiment; each contained one *Cla*I fragment that hybridized weakly to the *C. jejuni* 81-176 *cdtB* probe (Fig. 5A, lanes 1 to 5). In these strains, two sizes of *Cla*I fragments, 6.5 (lane 1) and 5.0 (lanes 2 to 5) kb, hybridized to the *C. jejuni* *cdtB* probe. A *cdtB* probe from *C. coli* D2594 was prepared by PCR and used to probe the same blot shown in Fig. 5A. The result, shown in Fig. 5B, indicated that the *C. coli* D2594 probe hybridized relatively well to putative *C. coli* *cdtB* genes but only poorly to the *C. jejuni* DNA. The same-size fragment in each strain hybridized to both probes. These results indicate that the *C. jejuni* *cdtB* genes may not be widely divergent. The *C. coli* *cdtB* genes formed a distinct group of *cdtB* sequences that can be estimated to be about 10 to 20% dissimilar from the *C. jejuni* 81-176 *cdtB* sequence. Among themselves, the *C. coli* sequences appeared to be slightly more heterogeneous than the *C. jejuni* sequences, since only D730 DNA hybridized as well to the D2594 *cdtB* probe as did D2594 DNA (lanes 3 and 5 of Fig. 5B).

The *C. jejuni* 81-176 *cdtB* probe was also used in a hybridization experiment with DNA isolated from a single representative each of *C. fetus*, *C. hyointestinalis*, *C. lari*, and "*C. upsaliensis*." In experiments that would allow an up to 20% base pair mismatch, no fragments from these species hybridized to the *C. jejuni* 81-176 *cdtB* probe, although lysates from each of these species produced detectable CDT in our HeLa cell assay (Titers: *C. fetus*, $1,223 \pm 599$; *C. hyointestinalis*, 809 ± 187 ; *C. lari*, 37 ± 10 ; *C. "upsaliensis"*, $1,428 \pm 823$). We next tested whether degenerative PCR primers VAT2 and WMI1, which successfully amplified a portion of the *C. jejuni* 81-176 *cdtB* gene, would produce an appropriate product when DNA from the representative *C. fetus*, *C. lari*, *C. hyointestinalis*, or *C. "upsaliensis"* strain was used as the template. The results from this experiment are shown in Fig. 6. For each of these species and for representative *C. jejuni* and *C. coli* strains included for comparison, a predominant fragment of approximately 475 bp was produced. This is close to the size expected if the appropriate portion of a *cdtB* gene was amplified. For three species, additional, minor products were seen; this was most noticeable for *C. hyointestinalis* (lane 5). *C. jejuni*, *C. "upsaliensis"*, and *C. fetus* produced no visible minor products (lanes 2, 4, and 7,

respectively). The PCR product produced by *C. fetus* 27374 was isolated from an agarose gel and cloned into pBluescript. The nucleotide sequence of this cloned PCR product indicated that it represented an amplified *cdtB* gene. The level of DNA identity between the *C. fetus* 27374 *cdtB* fragment and the corresponding portion of *C. jejuni* 81-176 *cdtB* was 63%. The translation of the *C. fetus* 27374 *cdtB* fragment is shown in Fig. 2B. Comparison of the amino acid sequences of the *C. jejuni* 81-176 and *C. fetus* CdtB regions shown revealed 65% identical amino acids and 12% conservative amino acid substitutions. The *C. fetus* *cdtB* gene fragment was subsequently used as a probe in a hybridization experiment to determine if this gene fragment would hybridize to *C. fetus*, *C. coli*, *C. jejuni*, *C. lari*, *C. hyointestinalis*, or *C. "upsaliensis"* DNA. In hybridization experiments that would allow an up to 20% base pair mismatch, the *C. fetus* *cdtB* gene fragment hybridized only to the *C. fetus* DNA (data not shown).

DISCUSSION

This report indicates that the CDT produced by *C. jejuni* 81-176 is similar to the CDT produced by some *E. coli* strains (30, 35). The CDT activity from both species is encoded in three adjacent or slightly overlapping genes. The CdtA, CdtB, and CdtC proteins produced by these two species are similar in size and presumably carry out identical functions, although their amino acid sequences have diverged considerably.

The initial report of *C. jejuni* CDT by Johnson and Lior (16) suggested that a substantial proportion of *C. jejuni* isolates may not make CDT. Our results suggest that most *C. jejuni* strains make CDT, although the amounts made by different isolates can vary over a 3-log range. Our method of CDT production differs from that used by Johnson and Lior (16); we used sonic lysates of agar-grown cells, while they used unconcentrated supernatant fractions of broth-grown cells. It is possible that low levels of CDT production would not be detected in unconcentrated supernatants. We have done initial CDT assays of 60 additional *C. jejuni* isolates from a variety of sources, and our results are in agreement with those presented here. Only three of these additional isolates had CDT titers of less than 10; all of the others had CDT titers of greater than 100. We have not found a low-CDT-producing *C. jejuni* strain that was isolated from a healthy human with diarrheal disease. However, the total number of low toxin producers is very low and many more isolates would need to be screened to justify the conclusion that high toxin production is necessarily associated with *C. jejuni* strains that cause diarrheal disease in humans.

In contrast, none of the *C. coli* strains, regardless of source, produced much CDT in our assay. This observation caused us to check the identities of our low-CDT-producing *C. jejuni* strains; all of the *C. jejuni* low toxin producers mentioned here were hippurate hydrolysis positive, indicating that they were *C. jejuni* strains and not misidentified *C. coli* strains. The difference in CDT production between these two closely related species seems to be a previously unnoticed difference between them and may point to potential significant differences in their disease-causing abilities. It also suggests that it may be important to identify *Campylobacter* isolates to the species level, since absence of species identification may inadvertently mask information of relevance to people studying the pathogenicity and epidemiology of this genus.

The hybridization studies indicated that most *C. jejuni* strains have *cdtB* sequences that are relatively similar. In experiments that would allow an only 5 to 10% base pair mismatch, a probe consisting of a portion of the *C. jejuni* 81-176

cdtB gene still hybridized well to DNAs from all but one of the *C. jejuni* strains tested. The only exception was *C. jejuni* 84-19, one of the *C. jejuni* low toxin producers. A second low toxin producer, D133, was also examined in the hybridization experiment, but the results obtained with this strain were similar to those obtained with the high toxin producers. While the *C. jejuni* 81-176 *cdtB* probe hybridized well to other *C. jejuni* strains, it hybridized only weakly to five *C. coli* strains, indicating a distinct species divergence in this toxin gene.

PCR experiments were performed to verify the presence of a *cdtB* gene in *Campylobacter* species for which we did not demonstrate hybridization with *cdtB* probes. These experiments used two degenerative primers based on *E. coli* *cdtB* sequences and template DNAs from several *Campylobacter* species. We were able to show that all of the species tested, *C. fetus*, *C. hyointestinalis*, *C. lari*, and *C. "upsaliensis"*, apparently contain *cdtB* genes. These experiments also illustrated these primers' ability to amplify *cdtB* sequences from a variety of sources.

The minicell experiments were unexpectedly difficult. All of the larger plasmids, i.e., pCP9312, pCP9417, and pCP9429, were unstable in the minicell strain, and no expression of the *C. jejuni* 81-176 *cdt* genes could be demonstrated with these plasmids. When most of the DNA downstream of the *cdt* genes was deleted, as in pCP9441, we were able to see some expression from a *C. jejuni* *cdt* gene. Plasmids with DNA both upstream and downstream of the *cdt* genes deleted did even better. An additional problem was the, at best, faintly discernible CdtA band. The faintness of this band cannot be attributed to low numbers of methionine or cysteine residues; it has more than either CdtB or CdtC. The *E. coli* *cdtA* gene did not appear to be similarly poorly expressed in *E. coli* minicells (30), suggesting that the poor production of *C. jejuni* CdtA in *E. coli* minicells is a reflection of expression problems in *E. coli* and not an indication of actual levels of expression of *cdtA* in *C. jejuni*.

While certain capabilities of *C. jejuni* have been implicated as virulence factors, e.g., motility (39) and invasiveness (17), there still remains a general absence of knowledge about specific proteins involved in the pathogenesis of *Campylobacter* diarrheal disease. The isolation of the *cdt* genes from *C. jejuni* should facilitate studies of the possible contribution of CDT to diarrheal disease.

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REFERENCES

- Baig, B. H. 1986. Ph.D. thesis. Emory University, Atlanta, Ga.
- Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser. 1988. Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Dis.* 157:472-480.
- Blaser, M. J. 1995. Personal communication.
- Blaser, M. J., G. Perez-Perez, P. F. Smith, C. Patton, F. C. Tenover, A. J. Lastovica, and W. L. Wang. 1986. Extraintestinal *Campylobacter jejuni* and *Campylobacter coli* infections: host factors and strain characteristics. *J. Infect. Dis.* 153:552-559.
- Blaser, M. J., L. B. Reller, N. W. Luechtefeld, and W.-L. Wang. 1982. *Campylobacter* enteritis in Denver. *West. J. Med.* 136:287-290.
- Butzler, J. P., and M. B. Skirrow. 1979. *Campylobacter* enteritis. *Clin. Gastroenterol.* 8:737-765.
- Dong, J. M., J. S. Taylor, D. J. Latour, S. Iuchi, and E. C. C. Lin. 1993. Three overlapping *lct* genes involved in L-lactate utilization by *Escherichia coli*. *J. Bacteriol.* 175:6671-6678.
- Edmonds, P., C. M. Patton, P. M. Griffin, T. J. Barrett, G. P. Schmid, C. N. Baker, M. A. Lambert, and D. J. Brenner. 1987. *Campylobacter hyointestinalis* associated with human gastrointestinal disease in the United States. *J. Clin. Microbiol.* 25:685-691.
- Gherna, R., P. Pienta, and R. Cote (ed.). 1992. American Type Culture Collection catalogue of bacteria and bacteriophages, 18th edition, 1992. American Type Culture Collection, Rockville, Md.
- Goossens, H., B. Pot, L. Vlaes, C. Van den Borre, R. Van den Abbeele, C. Van Naelten, J. Levy, H. Cogniau, P. Marbehan, J. Verhoef, K. Kersters, J.-P. Butzler, and P. Vandamme. 1990. Characterization and description of "*Campylobacter upsaliensis*" isolated from human feces. *J. Clin. Microbiol.* 28:1039-1046.
- Guerrant, R. L., C. A. Wanke, R. A. Pennie, L. J. Barrett, A. A. M. Lima, and A. D. O'Brien. 1987. Production of a unique cytotoxin by *Campylobacter jejuni*. *Infect. Immun.* 55:2526-2530.
- Jobling, M. G., and R. K. Holmes. 1990. Construction of vectors with the p15a replicon, kanamycin resistance, inducible *lacAα* and pUC18 or pUC19 multiple cloning sites. *Nucleic Acids Res.* 18:5315-5316.
- Johnson, W. M., and H. Lior. 1987. Response of Chinese hamster ovary cells to a cytolethal distending toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat-labile (LT) enterotoxin. *FEMS Microbiol. Lett.* 43:19-23.
- Johnson, W. M., and H. Lior. 1987. Production of Shiga toxin and a cytolethal distending toxin (CLDT) by serogroups of *Shigella* spp. *FEMS Microbiol. Lett.* 48:235-238.
- Johnson, W. M., and H. Lior. 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by *Escherichia coli* isolates from clinical material. *Microb. Pathog.* 4:103-113.
- Johnson, W. M., and H. Lior. 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. *Microb. Pathog.* 4:115-126.
- Konkel, M. E., and L. A. Joens. 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter jejuni*. *J. Infect. Dis.* 162:888-895.
- Konkel, M. E., Y. Lobet, and W. Cieplak, Jr. 1992. Examination of multiple isolates of *Campylobacter jejuni* for evidence of cholera toxin-like activity, p. 193-198. In I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
- Korlath, J. A., M. T. Osterholm, L. A. Judy, J. C. Forfang, and R. A. Robinson. 1985. A point-source outbreak of campylobacteriosis associated with consumption of raw milk. *J. Infect. Dis.* 152:592-596.
- Luechtefeld, N. W., W.-L. Wang, M. J. Blaser, and L. B. Reller. 1981. *Campylobacter fetus* subsp. *jejuni*: background and laboratory diagnosis. *Lab. Med.* 12:481-487.
- Mahajan, S., and F. G. Rodgers. 1990. Isolation, characterization, and host-cell-binding properties of a cytotoxin from *Campylobacter jejuni*. *J. Clin. Microbiol.* 28:1314-1320.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Moore, M. A., M. J. Blaser, G. I. Perez-Perez, and A. D. O'Brien. 1988. Production of a Shiga-like cytotoxin by *Campylobacter*. *Microb. Pathog.* 4:455-462.
- Nagel de Zwaig, R., and S. E. Luria. 1967. Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. *J. Bacteriol.* 94:1112-1123.
- Nicholson, M. 1995. Personal communication.
- Patton, C. M., N. Shaffer, P. Edmonds, T. J. Barrett, M. A. Lambert, C. Baker, D. M. Perlman, and D. J. Brenner. 1989. Human disease associated with "*Campylobacter upsaliensis*" (catalase-negative or weakly positive *Campylobacter* species) in the United States. *J. Clin. Microbiol.* 27:66-73.
- Perez-Perez, G. L., and M. J. Blaser. 1985. Lipopolysaccharide characteristics of pathogenic campylobacters. *Infect. Immun.* 47:353-359.
- Perez-Perez, G. L., D. L. Cohn, R. L. Guerrant, C. M. Patton, L. B. Reller, and M. J. Blaser. 1989. Clinical and immunologic significance of cholera-like toxin and cytotoxin production by *Campylobacter* species in patients with acute inflammatory diarrhea in the USA. *J. Infect. Dis.* 160:460-468.
- Pickett, C. L., T. Auffenberg, E. C. Pesci, V. L. Sheen, and S. S. D. Jusuf. 1992. Iron acquisition and hemolysin production by *Campylobacter jejuni*. *Infect. Immun.* 60:3872-3877.
- Pickett, C. L., D. L. Cottle, E. C. Pesci, and G. Bikah. 1994. Cloning, sequencing, and expression of the *Escherichia coli* cytolethal distending toxin genes. *Infect. Immun.* 62:1046-1051.
- Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* 57:50-108.
- Rennie, R. P., D. Strong, D. E. Taylor, S. M. Salama, C. Davidson, and H. Tabor. 1994. *Campylobacter fetus* diarrhea in a Hutterite colony: epidemiological observations and typing of the causative organism. *J. Clin. Microbiol.* 32:721-724.
- Riley, L. W., and M. J. Finch. 1985. Results of the first year of national surveillance of *Campylobacter* infections in the United States. *J. Infect. Dis.* 151:956-959.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

35. **Scott, D. A., and J. B. Kaper.** 1994. Cloning and sequencing of the genes encoding *Escherichia coli* cytolethal distending toxin. *Infect. Immun.* **62**:244–251.
36. **Silhavy, R. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions, p. 137. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Tauze, R. V., C. M. Patton, P. Edmonds, T. J. Barrett, D. J. Brenner, and P. A. Blake.** 1985. Illness associated with *Campylobacter laridis*, a newly recognized *Campylobacter* species. *J. Clin. Microbiol.* **21**:222–225.
38. **Wahl, G. M., and S. L. Berger.** 1987. Screening colonies or plaques with radioactive nucleotide probes, p. 415–422. *In* S. L. Berger and A. R. Kimmel (ed.), *Methods in enzymology: guide to molecular cloning techniques*. Academic Press, San Diego, Calif.
39. **Wassenaar, T. M., N. M. C. Bleumink-Pluym, and B. A. M. van der Zeijst.** 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. *EMBO J.* **10**:2055–2061.

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