BACTERIAL PATHOGENICITY

Identification of *cdtB* homologues and cytolethal distending toxin activity in enterohepatic *Helicobacter* spp.

CHIH-CHING CHIEN*, NANCY S. TAYLOR*, ZHONGMING GE*, DAVID B. SCHAUER*†, VINCENT B. YOUNG† and JAMES G. FOX*†

*Division of Comparative Medicine and †Division of Bioengineering and Environmental Health, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

A bacterial toxin that causes progressive distension and death of Chinese hamster ovary (CHO) cells and HeLa cells, termed cytolethal distending toxin (Cdt), has been identified in several diarrhoeagenic bacteria, including *Campylobacter* spp. (*C. jejuni* and *C. coli*), some pathogenic strains of *Escherichia coli* and *Shigella* spp. Genes encoding this toxin were identified as a cluster of three adjacent genes *cdtA*, *cdtB* and *cdtC*. Homologues of *cdtB* from five species of enterohepatic helicobacters (*Helicobacter hepaticus*, *H. bilis*, *H. canis* and two novel *Helicobacter* spp. isolated from mice and woodchuck, respectively) were identified by means of degenerative PCR primers, cloned and sequenced. The similarities of these partial *cdtB* nucleotide sequences from these *Helicobacter* spp. to those of *cdtB* genes known to be present in other bacteria were: *C. jejuni*, 58.3–64.8%; *E. coli*, 52.3–57.8%, *Haemophilus ducreyi*, 53.4–58.4% and *Actinobacillus actinomyces-temcomitans*, 52.7–58.1%. Bacterial lysates from four of these helicobacters caused characteristic cytolethal distension of HeLa cells. Cdt caused cell cycle arrest at G₂/M phase as measured by flow cytometry. The results demonstrated the presence of a toxin in these *Helicobacter* spp. belonging to the family of Cdt.

Introduction

An increasing number of gastrointestinal *Helicobacter* spp. has been isolated since the recognition of *H. pylori* as a causative agent of active, chronic gastritis and peptic ulcer disease [1, 2], as well as the bacterium's strong association with the development of gastric adenocarcinoma and gastric mucosa-associated lymphoma [3–5]. Several novel *Helicobacter* spp. cause persistent hepatitis and inflammatory bowel disease (IBD) in different strains of mice [6–14]. Novel enterohepatic helicobacters have also been isolated from inflamed colonic tissue of cotton-top tamarins [15] and man [16, 17], as well as from diseased livers and intestines of several other mammals [18–20]. Enterohepatic *Helicobacter* spp. have also been identified recently in bile and gall bladder tissue from patients with chronic cholecystitis [21]. However, little is known about virulence factors of these newly discovered bacteria and pathogenic mechanisms involved in inducing gastrointestinal diseases.

A bacterial toxin that causes progressive distension and death of Chinese hamster ovary (CHO) cells, termed cytolethal distending toxin (Cdt), was first described by Johnson and Lior [22] in an enteropathogenic strain of *Escherichia coli* [22]. Toxins belonging to this group were later identified in several other diarrhoeagenic bacteria, including *Campylobacter* spp. (*C. jejuni*, *C. coli* and *C. fetus*) [23, 24], and other pathogenic strains of *E. coli* [25–28] and *Shigella* spp. [29]. Cdt was also found in non-enteric pathogenic bacteria such as *Haemophilus ducreyi* [30] and *Actinobacillus actinomyces-temcomitans* [31]. Genes encoding this toxin were identified as a cluster of three adjacent genes *cdtA*, *cdtB* and *cdtC*, and produce proteins of *c.* 28, 30 and 20 kDa, respectively [26, 27]. Studies in *A. actinomyces-temcomitans* suggested that all three proteins, CdtA, CdtB and CdtC, are required for the function of the toxin [31, 32]. These genes have been cloned and sequenced from a number of bacteria including *E. coli* [26–28], *S. dysenteriae* [29], *C. jejuni* [24], *H. ducreyi* [30] and *A. actinomyces-temcomitans* [31]. In *C. jejuni*, *cdt* encodes proteins of 268, 265, and 189 amino acids (aa), respectively. Cdts from *E. coli*, *C. jejuni* and *A. actinomyces-temcomitans* are known to inhibit cell cycle progression by arresting cells in the G₂/M phase [27, 31, 33, 34].

Received 27 July 1999; revised version received 5 Nov. 1999; accepted 6 Nov. 1999.

Corresponding author: Dr J. G. Fox (e-mail: jgfox@mit.edu).

Because many *Helicobacter* spp. can cause intestinal diseases in their natural hosts, an earlier study investigated whether or not Cdt was also present in enterohepatic *Helicobacter* spp.; if present, this would help explain the adverse effect of these bacteria on host tissue. Recently, Cdt in *H. hepaticus* and *H. pullorum* was partially characterised [35]. This report confirms and extends these findings, – it describes the cloning and sequencing of partial *cdtB* homologues from five species of enterohepatic helicobacters (*H. hepaticus*, *H. bilis*, *H. canis* and two newly described *Helicobacter* spp. isolated from mice and woodchucks, respectively) by degenerative PCR primers. It also examined the cytotoxic effects of bacterial lysates on HeLa cell morphology and replication.

Materials and methods

Bacterial strains and cultivation

All strains were obtained from the Division of Comparative Medicine culture collection at Massachusetts Institute of Technology: *H. hepaticus* 3B1 (ATCC51449) [6], *H. bilis* (ATCC51630^T) [8], *H. canis* (ATCC51401^T) [36], *H. pylori* (NCTC11639) and *C. jejuni* 81-176 [37]. *Helicobacter* sp. strain 96-1001 was a novel urease-negative helicobacter isolated from a mouse and induced IBD and cholecystitis experimentally in mice [38]. *Helicobacter* sp. strain 98-6070 was isolated from woodchuck livers infected with hepadnavirus [20]. *Helicobacter* spp. were grown on Columbia blood agar (sheep blood 5%; Remel) plates or in Brucella broth supplemented with bovine calf serum 5% and incubated under micro-aerobic conditions in vented jars (by replacing the head space once with a gas mixture containing H₂ 10%, CO₂ 5%, and balanced with N₂) at 37°C for 3–5 days. *E. coli* strain JM109 (Promega, Madison, WI, USA) was used as a host for the plasmid harbouring the genes of interest and grown in LB broth supplemented with ampicillin 50 µg/ml when appropriate.

DNA extraction and PCR amplification

Bacterial cells were harvested from agar plates, washed twice with 1 ml of PBS and then collected by centrifugation at 8000 g. DNA was extracted from cell pellets of *H. pylori*, *H. hepaticus*, *H. canis*, *H. bilis*, *Helicobacter* sp. strain 96-1001, *Helicobacter* sp. strain 98-6070 and *C. jejuni* 81-176 with a commercial kit (High Pure PCR Template Preparation Kit, Boehringer Mannheim) according to the manufacturer's instructions.

Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Degenerative primers VAT2 (forward, 5'-GTNG CNACBTGGAAYCTNCARGG-3') and WMI1 (reverse, 5'-RTTRAARTCNCCYAADATCATCC-3') were designed as described by Pickett *et al.* [24]. Another

degenerative primer designated DHF1 was designed (reverse, 5'-DACNGGRAARTGRTC-3') which was deduced from the amino acid sequence DHFPV (amino acids 256–260 derived from the *cdtB* gene of *C. jejuni*) [24]. With these degenerative primers, the PCR assay used a 100-µl reaction mixture containing 5 µl of the DNA preparation, 1 × *Taq* polymerase buffer (provided by the manufacturer but supplemented with MgCl₂ 1 mol/L to a final concentration of 2.25 mmol/L), 0.5 µM of each forward and reverse primer, 200 µM of each deoxynucleotide (ddATP, ddGTP, ddCTP and ddTTP), bovine serum albumin 20 µg and *Taq* polymerase (Boehringer Mannheim) 2 U. The thermocycling programme consisted of 35 cycles of 94°C for 1 min, 42° for 2 min, 72°C for 3 min, followed by incubation at 72°C for 8 min for elongation.

Cloning and nucleotide sequence determinations

PCR amplicons of expected sizes (494 bp from VAT2 and WMI1 and 714 bp from VAT2 and DHF1, respectively) were purified with QIAEX II (Qiagen, Studio City, CA, USA) from low melting point agarose (1%) gel. The purified DNA fragments were subsequently cloned into a vector pGEM[®]-T Easy (Promega) and then transformed into competent *E. coli* cells provided by the same company, according to the manufacturer's instructions. Recombinant plasmid clones were selected on LB agar plates supplemented with ampicillin 50 µg/ml, Xgal 80 µg/ml and (0.5 mM) IPTG. Plasmid DNA was isolated from transformed *E. coli* cells by an alkaline lysis procedure [39]. Cloned inserts (both strands) were sequenced with a thermocycling sequenase kit purchased from Amersham Life Science, (Cleveland, OH, USA) following the procedures provided by the supplier's manual. These pGEM[®]-T recombinant plasmids with DNA inserts from *Helicobacter* spp. in *E. coli* – pHH1 (from *H. hepaticus*), pHB2 (from *H. bilis*), pHC1 (from *H. canis*), pHM2 (from *Helicobacter* sp. strain 96-1001) and pHWC4 (from *Helicobacter* sp. strain 98-6070 – were sequenced.

Southern hybridisation

Southern hybridisation was conducted to confirm the origin of the PCR-amplified *cdtB* homologues. Chromosomal DNA samples (~5 µg) of *H. hepaticus*, *H. bilis*, *H. canis*, *Helicobacter* sp. strain 96-1001 and *Helicobacter* sp. strain 98-6070 were digested with *AseI* and *HindIII*, electrophoretically separated on to an agarose 1% gel and then transferred on to a Hybond N nylon membrane according to the manufacturer's protocol (Amersham, Arlington Heights, IL, USA). Membrane-bound DNA was UV cross-linked and then hybridised with probes prepared from one of the recombinant plasmids containing *Helicobacter* spp. *cdtB* DNA. Southern hybridisation procedures were employed with ECL[™] direct nucleic acid labelling and detection systems with the standard procedure accord-

ing to the manufacturer's instructions (Amersham Life Science). Low-stringency hybridisation was performed with [α - 32 P]dCTP-labelled probe in the nick translation system (BRL). The membrane was pre-hybridised in a buffer (5 $\times$ SSC, formamide 50%, denatured salmon-sperm DNA 100 μ g/ml, 0.001 M EDTA and 1 $\times$ Denhardt's solution) at 37°C for 1 h. Denatured 32 P-labelled probe was added to the prehybridisation solution and the membrane was incubated at 37°C overnight. The membrane was washed twice in a solution of 2 $\times$ SSC and sodium dodecyl sulphate (SDS) 1% at 37°C for 1 h and exposed at -80°C overnight.

Analysis of nucleotide sequences and sequence accession numbers

The partial sequences of *cdtB* genes of *Helicobacter* spp. obtained from this study were compared with the DNA and amino-acid sequences available in the public databases. Nucleotide and protein sequences were analysed with the DNA Lasergene software package (DNA Star, Madison, WI, USA). The Genbank accession numbers for the nucleotide sequence data of the *cdt* genes used and presented in the study are: *E. coli*, U04208 [26]; *E. coli*, U03293 [27]; *C. jejuni*, U51121 [24]; *A. actinomycetemcomitans*, AB011405; *H. ducreyi*, 53215; *H. bilis*, AF243077; *H. hepaticus*, AF243076; *H. canis*, AF243078; *Helicobacter* sp. strain 96-1001, AF243080; *Helicobacter* sp. strain 98-6070, AF243079.

Cytolethal distending toxin assay

Preparation of cell sonicates. Bacteria were grown in Brucella broth containing fetal calf serum 5% with shaking at 100 rpm, or on Columbia blood agar sheep blood 5%, Remel) plates, under micro-aerobic conditions and incubated for 4 days. Cells were harvested at 4°C by centrifugation at 10 000 *g* (Sorval RC5B). The pellet was washed once with PBS and then resuspended to 1 ml in PBS. The pellet was sonicated on ice with four 30-s pulses at a setting of 20 with an Artek sonic dismembrator Model 150. The sonicate was centrifuged at 8000 rpm and the supernatant fraction was filter-sterilised with a 0.22- μ m filter. Samples were stored at -70°C until tested.

Tissue-culture assay for morphological changes by Cdt. One ml of HeLa cells at 10³ cells/ml of EMEM (Minimum Essential Medium Eagle with Earle's salts; Sigma, fetal calf serum (Summit Biotechnology, Fort Collins, CO, USA) 10% were seeded into wells on 24-well plates. After incubation for 3 h at 37°C in an atmosphere of CO₂ 8% in air, 10 μ l of filter-sterilised supernate of cell sonicate were added to each well. Plates were re-incubated and observed daily for 3 days. After 72 h, the wells were washed with phosphate-buffered saline, stained with Diff-Quick Stain (Dade

International, Miami, FL, USA), and observed microscopically.

Flow cytometry methods

Tissue-culture flasks (25 cm²) were seeded with 5 ml of EMEM-fetal bovine serum 10% at 1 $\times$ 10⁵ HeLa cells (CCL-2)/ml. Flasks were inoculated with 100 μ l of filter-sterilised bacterial sonicate and then incubated at 37°C with CO₂ 8% for 72 h. The cells were detached by treatment with 0.5 ml of trypsin 0.5%-EDTA 0.2% for 3–5 min. Cells were transferred to a 1.5-ml microfuge tube and pelleted at 3000 rpm for 3 min in a Heraeus Instruments Biofuge (VWR). The pellets were gently resuspended in 1 ml of stain solution (PEG-8000 3%, propidium iodide 4 μ g/ml, RNAase A 9 U/ml, Triton-X 100 0.1%, bovine serum albumin 0.0001% in 4 mM sodium citrate) and then incubated at 37°C for 20 min. One ml of salt solution (PEG 3%, propidium iodide 4 μ g/ml, Triton-X 100 0.1%, bovine serum albumin 0.0001% in 0.4 M NaCl) was added, the solution was thoroughly mixed, and then stored at 4°C for 3 h. DNA analysis was performed on a FACScan flow cytometer (Beckman Dickinson, Franklin Lakes, NJ, USA) with Cell Quant software for data acquisition. The ModFit program was used for data analysis. For each experiment, 1 $\times$ 10⁴ cells were analysed.

Results

Cloning and sequence determination of cdtB homologues from enterohepatic Helicobacter spp.

With primers VAT2 and WMI1 described previously by Pickett *et al.* [24], PCR amplicons approximating the expected length (494 bp of the *C. jejuni cdtB*) were produced from the novel urease-negative *Helicobacter* sp. strain 96-1001 (479 bp) and *H. canis* (506 bp), but not from *H. hepaticus*, *H. bilis*, *Helicobacter* sp. strain 98-6070 and *H. pylori*. With degenerative primers VAT2 and DHF1 based on sequences in the conserved region of *C. jejuni* and *E. coli cdtB*, it was possible to amplify products of the expected length (714 bp according to the *cdtB* gene of *C. jejuni*) from all five enterohepatic *Helicobacter* spp. examined but not from *H. pylori*. These amplicons were cloned into vector pGEM[®]-T Easy. The sizes of the inserts from respective recombinant plasmids were as follows: 738 bp (246 aa) in pHH1 for *H. hepaticus*; 738 bp (246 aa) in pHB2 for *H. bilis*; 738 bp (246 aa) in pHCl for *H. canis*; 711 bp (237 aa) in pHWC4 for *Helicobacter* sp. strain 98-6070 and 693 bp (231 aa) in pHM2 for *Helicobacter* sp. strain 96-1001. The similarities of the nucleotide sequences (without primer regions) from these *Helicobacter* spp. to those known *cdtB* genes present in other bacteria were in the ranges: *C. jejuni*, 58.3–64.8%; *E. coli*, 53.5–57.8% [26]; *E. coli*, 52.3–55.2% [27]; *H. ducreyi*, 53.4–58.4% and *A. actinomycetemcomitans*, 52.7–58.1%. The nucleotide sequences of the partial *cdtB* homologues of *H.*

hepaticus, *H. bilis* and *H. canis* showed more similarity to each other (between 68.9 and 75.3%) than to *Helicobacter* sp. strain 98-6070 (58.1–59.3%) and the novel urease-negative *Helicobacter* sp. strain 96-1001 (56.7–59.4%). Interestingly, the partial *cdtB* homologues of *Helicobacter* sp. strain 98-6070 and *Helicobacter* sp. strain 96-1001 shared 69.2% similarity. The similarity matrix based on the *cdtB* nucleotide sequences from these bacteria is shown in Table 1 [40].

Deduced amino-acid sequences of CdtB from *H. hepaticus*, *H. canis* and *H. bilis* shared more similarity with each other (72.2–83.8%) than with those of *Helicobacter* sp. strain 96-1001 (53.5–54.4%) and *Helicobacter* sp. strain 98-6070 (54.7–57.4%). Comparison of the predicted amino-acid sequences of CdtB of the five *Helicobacter* spp. with that of *C. jejuni* is shown in Fig. 1. The phylogenetic analysis indicated that CdtB proteins of *H. hepaticus*, *H. bilis* and *H. canis* were clustered in a group, whereas *Helicobacter* sp. strain 96-1001 and *Helicobacter* sp. strain 98-6070 were in another cluster (Fig. 2).

Southern blot hybridisation

A probe prepared from the insert DNA of pHH1 (the *H. hepaticus cdtB* homologue) hybridised with genomic DNA of *H. hepaticus* with the standard procedure provided by ECLTM. It also hybridised weakly with *H. bilis* and *H. canis* genomic DNA (Fig. 3a). However, the probe prepared from the *H. hepaticus cdtB* homologue did not hybridise with DNA of *Helicobacter* sp. strain 96-1001 and *Helicobacter* sp. strain 98-6070. In contrast, the probe prepared from the partial sequence of the *cdtB* gene from *Helicobacter* sp. strain 96-1001 hybridised with DNA of *Helicobacter* sp. strain 96-1001 and *Helicobacter* sp. strain 98-6070, but not with genomic DNA of *H. hepaticus*, *H. canis* and *H. bilis* (Fig. 3b). Low-stringency hybridisation with [³²P]dCTP labelled probe gave the same results (results not shown).

Cytotoxic effects of crude cell extracts of *Helicobacter* spp. on HeLa cells

The response of HeLa cells exposed for 72 h to PBS and filter-sterilised supernatant fractions of bacterial cell sonicates of various *Helicobacter* spp. varied according to which *Helicobacter* spp. fraction was used (Fig. 4A–D). Supernatant fractions of sonicates from both *H. pylori* strains that lacked the gene for CdtB did not affect the morphology of HeLa cells. Cells treated with the Cdt toxin of the positive control strain *C. jejuni* 81-176 became distended and the nuclei were larger than those in control cells (Fig. 4B). Many cells were multinucleate and some cells appeared to be undergoing nuclear fragmentation. The effect of supernatant fractions of cell sonicates from *H. hepaticus* (Fig. 4C) and *Helicobacter* sp. strain 98-6070 (data not shown) caused morphological changes in >80% of HeLa cells; these results were similar to those obtained with *C. jejuni* strain 81-176, the positive control (Fig. 4B). *H. canis* caused changes in ~40% of the HeLa cells (Fig. 4D). The preparation from *H. bilis* showed similar cytodistending effects in 10–15% of the HeLa cells, whereas the supernatant fraction of sonicates of *Helicobacter* sp. strain 96-1001 had no effect on the HeLa cells.

Cell cycle arrest in G₂/M

Filter-sterilised supernatant fractions of cell sonicates of six species of *Helicobacter* were examined for their ability to block cell cycle progression by analysing HeLa cell DNA content by flow cytometry (Fig. 5). Sub-confluent HeLa cells were treated with the supernatant fractions of cell sonicates for 72 h. The cytolethal distending toxin in the positive control strain of *C. jejuni* (81-176) caused 54.8% of the cells to become arrested in G₂/M compared with only 6.9% of control cells treated with PBS. The preparations from *H. pylori* and the novel urease-negative *Helicobacter* sp. strain 96-1001 gave results similar to those of the control. The fractions from *H. hepaticus* and *Helico-*

Table 1. Similarity matrix based on the nucleotide sequences of partial *cdtB**

Organism	Percentage similarity with									
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
(1) <i>H. hepaticus</i>	...	69.6	68.9	59.4	59.3	57.0	53.9	55.4	59.5	57.3
(2) <i>H. bilis</i>		...	75.3	56.7	58.1	53.6	54.5	53.5	60.4	54.7
(3) <i>H. canis</i>			...	57.1	58.9	53.6	52.3	53.6	58.3	54.4
(4) <i>Helicobacter</i> 96-1001				...	69.2	58.4	55.2	57.1	64.0	58.1
(5) <i>Helicobacter</i> 98-6070					...	53.4	54.9	57.8	64.8	52.7
(6) <i>H. ducreyi</i>						...	51.1	54.3	58.0	95.8
(7) <i>E. coli</i> [†]							...	56.2	54.1	52.0
(8) <i>E. coli</i> [‡]								...	60.8	54.2
(9) <i>C. jejuni</i>									...	57.0
(10) <i>A. actinomycetemcomitans</i>										...

*Alignment done by Jotun Hein Method [40] included in Lasergene software (DNASTAR, Madison, WI, USA).

[†]Sequence from Scott and Kaper [27].

[‡]Sequence from Pickett *et al.* [26].

Fig. 1. Comparison of the predicted amino-acid sequences of CdtB proteins from *C. jejuni*, *H. hepaticus*, *H. bilis*, *H. canis*, *Helicobacter* spp. strain 96–1001 and strain 98–6070. The sequences of the primer regions are underlined with open arrows. Regions of similarity are in the enclosed boxes. In *Helicobacter* sp. strain 96–1001, there was a single base deletion in the codon for amino acid 157 that created a frameshift which resulted in a premature termination. A nucleotide N was inserted in the third position of the amino acid 157 (*X) of *Helicobacter* sp. strain 96–1001 to correct for the frameshift mutation, allowing completion of the sequence.

Fig. 2. Phylogenetic relationship of CdtB proteins between *C. jejuni*, *E. coli*, *H. ducreyi*, *A. actinomycetemcomitans*, *H. hepaticus*, *H. bilis*, *H. canis*, *Helicobacter* spp. strain 96-1001 and strain 98-6070. The alignment was done by the method described by Hein [39] included in Lasergene software (DNASTAR). The length of each pair of branches represents the distance between the respective CdtB sequence pairs. The scale beneath the tree measures the distance between the CdtB sequences. Units indicate the number of substitution events.

Fig. 3. Southern hybridisation with probes prepared from *cdtB* genes of *Helicobacter* spp. Restriction endonucleases used to digest DNA in each lane: lanes 1, 3, 5, 7, 9 and 11: *Ase*I; lanes 2, 4, 6, 8, 10, 12: *Hind*III. Lanes 1 and 2, *H. hepaticus*; 3 and 4, *Helicobacter* sp. strain 96-1001; 5 and 6, *H. bilis*; 7 and 8, *H. canis*; 9 and 10, *Helicobacter* sp. strain 98-6070; 11 and 12, *H. pylori*. (a) Results with the *H. hepaticus* *cdtB* gene probe. The probe hybridised with genomic DNA of *H. hepaticus* (lanes 1 and 2) and weakly hybridised with *H. bilis* (5 and 6) and *H. canis* (8). There is no hybridisation signal of the lane 7, probably because *H. canis* DNA could not be completely digested by *Ase*I. (b) The same membrane with a *cdtB* gene probe from *Helicobacter* sp. strain 96-1001. The probe hybridised with genomic DNA of *Helicobacter* spp. strain 96-1001 (lanes 3 and 4) and strain 98-6070 (9 and 10), but not with *H. hepaticus*, *H. bilis* and *H. canis*. Neither probe hybridised with *H. pylori* DNA (11 and 12).

bacter sp. strain 98-6070 resulted in 61.6% and 45.4% of the HeLa cells being arrested in G₂/M, respectively. *H. bilis* and *H. canis* supernatant fractions also blocked cell cycle progression in G₂/M phase, although their effects were lower than those obtained with the Cdt proteins of *C. jejuni*, *H. hepaticus* and *Helicobacter* sp. 98-6070. Table 2 summarises the ability of supernatant fractions of sonicates of different *Helicobacter* spp. to block HeLa cell cycle in G₂/M as measured by flow cytometry.

Discussion

There is accumulating evidence that many enterohepatic *Helicobacter* spp. play an important, pathogenic role in gastrointestinal and hepatic diseases in man and animals [17, 19, 21]. This includes the ability of *H. hepaticus* to cause hepatitis and hepatocellular carcinoma in susceptible strains of mice [6, 7, 41, 42]. The increased apoptosis noted in male B₆C₃F₁ mice with chronic hepatitis in part may be due to the presence of persistent hepatic infection with *H. hepaticus* and chronic exposure to Cdt [43]. *H. hepaticus* is also directly linked with naturally occurring as well as experimentally produced IBD in a variety of immune dysregulated mice [9-11, 44]. Interestingly, the other helicobacters thus far shown to produce Cdt, i.e., *H. bilis*, *H. canis*, *H. pullorum*, also cause hepatitis and have been associated with diarrhoea in their respective hosts [35].

The demonstration of the *cdt* homologues in many novel enterohepatic *Helicobacter* spp. confirms and extends the preliminary finding of *cdt* in *H. hepaticus* and *H. pullorum* [35]. The discovery of Cdt in enteropathogenic helicobacters may help clarify the pathogenic potential of different *Helicobacter* spp. Cdt

Fig. 4. Giemsa-stained HeLa cells exposed for 72 h to filter-sterilised supernatant fractions of cell sonicates of *H. pylori* (a), *C. jejuni* strain 81-176 (b), *H. hepaticus* (c) and *H. canis* (d). *C. jejuni*, *H. hepaticus*, *H. canis* and *Helicobacter* sp. strain 98-6070 (not shown) induced the formation of giant, mono- and multi-nucleated cells with large nuclei. Bar = 75 μ m.

is believed to play a role in producing diarrhoea in human patients infected with enteric pathogens such as *C. jejuni*, *E. coli* and *S. dysenteriae*. Bacteria that produce Cdt, like many other bacterial pathogens, produce more than one toxin that may contribute to their pathogenesis. For example, heat-labile (LT) enterotoxin and cytotoxic necrotising factors may also be present in *E. coli* which has Cdt [22, 28]. *H. ducreyi* elaborates other toxins in addition to Cdt, including haemolysin and a cytotoxin [45, 46]. *H. hepaticus* also has another toxin that causes granulation in a mouse hepatic cell line that is distinct from the cytopathogenic effect of Cdt [47]. The two toxins differ in at least two ways: first, the granulating cytotoxin is stable at 56°C for 30 min, whereas Cdt is heat-labile [22, 23, 47]; and second, *H. hepaticus* polyclonal antibody is able to neutralise the *H. hepaticus* Cdt toxin activity on HeLa cells, but does not neutralise the granulating cytotoxin activity on mouse hepatic cells (data not shown). Thus,

Cdt in *H. hepaticus*, like Cdt in other bacterial pathogens, may function in concert with other purported virulence factors [30, 31].

The partial *cdtB* sequences of several enterohepatic *Helicobacter* spp. indicate that the gene, similar to that described for *Campylobacter* spp., has a distinct divergence among species. In the genus *Campylobacter*, the probe prepared from the *cdtB* gene of *C. jejuni* failed to hybridise or only weakly hybridised to the DNA of *C. fetus*, *C. hyointestinalis*, *C. lari* and *C. coli* [24]. Because of the divergence of this gene, use of selected degenerative primers may not always amplify the desired *cdtB* fragment from species that possess the *cdt* gene. For example, PCR did not produce the expected amplicon from *H. bilis* with the primers described by Pickett *et al.* [24], but it was possible to obtain the amplicon with the primers designed in the present study. Additional degenerative

Fig. 5. DNA content of HeLa cells analysed by flow cytometry and propidium iodide fluorescence. HeLa cells were exposed for 72 h to (a) phosphate-buffered saline (control) or bacterial cell sonicates. Compared with control cells, HeLa cells exposed to (b) *C. jejuni*, (c) *H. hepaticus* and (d) *H. canis* cell sonicates showed an increase in the number of cells with 4 N DNA content. The novel *Helicobacter* sp. strain 98-6070 from the woodchuck had a response similar to *H. hepaticus*; and *H. pylori* and the murine isolate (*Helicobacter* sp. strain 96-1001) were identical to control cells (data not shown).

Table 2. DNA content analyses of HeLa cell cultures treated with filter-sterilised supernatant fractions of bacterial cell sonicates of *C. jejuni* and different *Helicobacter* spp.

Organism	Percentage of sample that were in		
	G ₀ /G ₁ phase	G ₂ /M phase	S phase
Control (PBS)	72.5	6.9	20.6
<i>C. jejuni</i>	34	54.8	11.2
<i>H. hepaticus</i>	28.3	61.6	10.1
<i>Helicobacter</i> sp. strain 98-6070	36.1	45.4	18.5
<i>H. canis</i>	55.8	28.3	15.9
<i>H. bilis</i>	65.7	18.9	15.4
<i>Helicobacter</i> sp. strain 96-1001	72.7	7.2	20.1
<i>H. pylori</i>	71.5	7.8	20.7

PCR primers from various conserved regions are needed to evaluate the presence or absence of the *cdt* homologue in the growing number of novel *Helicobacter* spp. as well as other bacterial pathogens.

An unusually high percentage amino-acid sequence identity (99%) between *S. dysenteriae* Cdt and *E. coli*

Cdt-I has been reported by Okuda *et al.* [27, 48]. Also, the amino-acid sequences of the Cdt produced by *H. ducreyi* and *A. actinomycetemcomitans* share a high degree of similarity (91%, 97% and 94% in CdtA, CdtB and CdtC, respectively) [31]. Evolutionarily, these two bacteria are closely related, suggesting that their *cdt* genes could have originated from the same

ancestral gene [31]. Although the sequences of the Cdt in enterohepatic *Helicobacter* spp. do not share this high degree of similarity, the results of the present study still suggest that CdtB proteins of *H. hepaticus*, *H. bilis* and *H. canis* are more closely related to each other than to the CdtB homologues of other bacteria (Fig. 2). 16S rRNA phylogenetic analysis also indicates that these three species of *Helicobacter* are closely related [21].

The complete genomic DNA sequences from two different strains of *H. pylori* indicate that the *cdt* gene is absent from this gastric pathogen [49, 50]. Attempts to amplify a *cdt* homologue with degenerative PCR primers from *H. pylori* and another gastric helicobacter, *H. mustelae*, were also unsuccessful (unpublished data). Whether the absence of the *cdt* gene is also a feature of other gastric *Helicobacter* spp. will require further investigation. The primers used in this study also failed to produce the expected size of amplicon from another intestinal helicobacter '*H. rappini*' (ATCC 43879) (results not shown), which supports the recent finding that the CdtB homologue is not present in all *Helicobacter* species [35].

Filter-sterilised supernatant fractions from whole-cell lysates of four of five *Helicobacter* spp. caused morphological alterations in HeLa cells and blocked the cell cycle in G₂/M. These data further confirm the presence of *cdt* homologues in these enterohepatic *Helicobacter* spp. *H. bilis* possessed significantly lower activity despite the presence of the toxin genes. Other authors have also noted a wide variability in the amount of toxin produced in different strains of *C. jejuni* as well as *C. coli* [24]. A recent study of 70 strains of *C. jejuni* isolated from poultry indicated that 100% of the strains had *cdt* genes; however, one strain failed to produce a Cdt-induced cytopathology on HeLa cells. The authors suggested that this strain might have a mutation in the *cdtB* gene that rendered it incapable of producing the toxin [51]. There was a single base deletion in the *cdtB* gene of *Helicobacter* sp. strain 96-1001 that may have caused the protein to be truncated (Fig. 1), accounting for the lack of detectable toxin activity in this bacterium. Further investigation, including determination of the full sequences of *cdtA* and *cdtC* genes and direct cloning of the toxin gene from genomic DNA instead of determining the sequence by PCR, will be necessary to fully explore this hypothesis.

S. dysenteriae Cdt is capable of causing diarrhoea with colonic erosions and reparative enterocyte hyperplasia in suckling mice [48]; the presence of Cdt in enterohepatic *Helicobacter* spp. suggests that this toxin may also be associated with the ability of these bacteria to interact with host enterocytes and intestinal inflammatory cells and play a role in intestinal disease. This laboratory is continuing to screen other novel helicobacters for the presence of *cdt* homologues to help determine if this toxin might play a role in the

pathogenesis of hepatobiliary as well as gastrointestinal disease produced by this genus. Studies also are underway in this laboratory to purify the *Helicobacter* Cdt toxin, to clone and identify the genes that encode the toxin and to construct isogenic *Helicobacter* mutants that lack Cdt. These isogenic mutants could then be used for pathogenesis studies *in vivo*. It is hoped that characterisation of Cdt in *Helicobacter* species as well as targeted development of in-vivo pathogenesis models will help to elucidate the host-pathogen relationships of helicobacter infection in a variety of animal hosts.

This work was supported in part by NIH grants R01CA67529, R01CA26731, RR010146 and R01DK52413.

References

- Graham DY. *Campylobacter pylori* and peptic ulcer disease. *Gastroenterology* 1989; **96**: 615–625.
- Lee A, Fox J, Hazell S. Pathogenicity of *Helicobacter pylori*: a perspective. *Infect Immun* 1993; **61**: 1601–1610.
- Nomura A, Stemmermann GN, Chyou P-H, Kato I, Perez-Perez GI, Blaser MJ. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. *N Engl J Med* 1991; **325**: 1132–1136.
- Parsonnet J, Friedman GD, Vandersteen DP *et al.* *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127–1131.
- Parsonnet J, Hanson S, Rodriguez L *et al.* *Helicobacter pylori* infection and gastric lymphoma. *Engl J Med* 1994; **330**: 1267–1271.
- Fox JG, Dewhirst FE, Tully JG *et al.* *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. *J Clin Microbiol* 1994; **32**: 1238–1245.
- Ward JM, Fox JG, Anver MR *et al.* Chronic active hepatitis and associated liver tumors in mice caused by persistent bacterial infection with a novel *Helicobacter* species. *J Natl Cancer Inst* 1994; **86**: 1222–1227.
- Fox JG, Yan LL, Dewhirst FE *et al.* *Helicobacter bilis* sp. nov., a novel *Helicobacter* species isolated from bile, livers, and intestines of aged, inbred mice. *J Clin Microbiol* 1995; **33**: 445–454.
- Ward JM, Anver MR, Haines DC *et al.* Inflammatory large bowel disease in immunodeficient mice naturally infected with *Helicobacter hepaticus*. *Lab Anim Sci* 1996; **46**: 15–20.
- Cahill RJ, Foltz CJ, Fox JG, Dangler CA, Powrie F, Schauer DB. Inflammatory bowel disease: an immunity mediated condition triggered by bacterial infection with *Helicobacter hepaticus*. *Infect Immun* 1997; **65**: 3126–3131.
- Foltz CJ, Fox JG, Cahill RJ *et al.* Spontaneous inflammatory bowel disease in multiple mutant mouse lines: association with colonization by *Helicobacter hepaticus*. *Helicobacter* 1998; **3**: 69–78.
- Shomer NH, Dangler CA, Marini R, Fox JG. *Helicobacter bilis*/*Helicobacter rodentium* co-infection associated with diarrhoea in a colony of scid mice. *Lab Anim Sci* 1998; **48**: 455–459.
- Whary MT, Morgan TJ, Dangler CA, Gaudes KJ, Taylor NS, Fox JG. Chronic active hepatitis induced by *Helicobacter hepaticus* in the A/JCr mouse is associated with a Th1 cell-mediated immune response. *Infect Immun* 1998; **66**: 3142–3148.
- Fox JG, Gorelick PL, Kullberg MC, Ge Z, Dewhirst FE, Ward JM. A novel urease-negative *Helicobacter* species associated with colitis and typhlitis in IL-10-deficient mice. *Infect Immun* 1999; **67**: 1757–1762.
- Saunders KE, Shen Z, Dewhirst FE, Paster BJ, Dangler CA, Fox JG. Novel intestinal *Helicobacter* species isolated from cotton-top tamarins (*Saguinus oedipus*) with chronic colitis. *J Clin Microbiol* 1999; **37**: 146–151.
- Totten PA, Fennell CL, Tenover FC. *Campylobacter cinaed* (sp.

- nov.) and *Campylobacter fennelliae* (sp. nov.): two new *Campylobacter* species associated with enteric disease in homosexual men. *J Infect Dis* 1985; **151**: 131–139.
17. Burnens AP, Stanley J, Schaad UB, Nicolet J. Novel *Campylobacter*-like organism resembling *Helicobacter fennelliae* isolated from a boy with gastroenteritis and from dogs. *J Clin Microbiol* 1993; **31**: 1916–1917.
18. Fox JG, Drolet R, Higgins R et al. *Helicobacter canis* isolated from a dog liver with multifocal necrotizing hepatitis. *J Clin Microbiol* 1996; **34**: 2479–2482.
19. Fox JG, Lee A. The role of *Helicobacter* species in newly recognized gastrointestinal tract diseases of animals. *Lab Anim Sci* 1997; **47**: 222–255.
20. Fox JG, Yu S, Dangler CA, Cullen JM. A novel *Helicobacter* sp. isolated from woodchuck livers infected with woodchuck hepatitis virus (WHV). *Gastroenterology* 1999; **116**: A718 (Abstract G3116).
21. Fox JG, Dewhirst FE, Shen Z et al. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology* 1998; **114**: 755–763.
22. Johnson WM, Lior H. Response of Chinese hamster ovary cells to a cytolethal distending toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat-labile (LB) enterotoxin. *FEMS Microbiol Lett* 1987; **43**: 19–23.
23. Johnson WM, Lior H. A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. *Microb Pathog* 1988; **4**: 115–126.
24. Pickett CL, Pesci EC, Cottle DL, Russell G, Erdem AN, Zeytin H. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* genes. *Infect Immun* 1996; **64**: 2070–2078.
25. Bouzari S, Varghese A. Cytolethal distending toxin (CLDT) production by enteropathogenic *Escherichia coli* (EPEC). *FEMS Microbiol Lett* 1990; **71**: 193–198.
26. Pickett CL, Cottle DL, Pesci EC, Bikah G. Cloning, sequencing, and expression of the *Escherichia coli* cytolethal distending toxin genes. *Infect Immun* 1994; **62**: 1046–1051.
27. Scott DA, Kaper JB. Cloning and sequencing of the genes encoding *Escherichia coli* cytolethal distending toxin. *Infect Immun* 1994; **62**: 244–251.
28. Pérès SY, Marchès O, Daigle F et al. A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G2/M phase. *Mol Microbiol* 1997; **24**: 1095–1107.
29. Okuda J, Kurazono H, Takeda Y. Distribution of the cytolethal distending toxin A gene (*cdtA*) among species of *Shigella* and *Vibrio*, and cloning and sequencing of the *cdt* gene from *Shigella dysenteriae*. *Microb Pathog* 1995; **18**: 167–172.
30. Cope LD, Lumbley S, Latimer JL et al. A diffusible cytotoxin of *Haemophilus ducreyi*. *Proc Natl Acad Sci USA* 1997; **94**: 4056–4061.
31. Sugai M, Kawamoto T, Pérès SY et al. A cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. *Infect Immun* 1998; **66**: 5008–5019.
32. Mayer MPA, Bueno LC, Hansen EJ, DiRienzo JM. Identification of a cytolethal distending toxin gene locus and features of a virulence-associated region in *Actinobacillus actinomycetemcomitans*. *Infect Immun* 1999; **67**: 1227–1237.
33. Comayras C, Tasca C, Pérès SY, Ducommun B, Oswald E, De Rycke J. *Escherichia coli* cytolethal distending toxin blocks the HeLa cell cycle at the G₂/M transition by preventing cdc2 protein kinase dephosphorylation and activation. *Infect Immun* 1997; **65**: 5088–5095.
34. Whitehouse CA, Balbo PB, Pesci EC, Cottle DL, Mirabito PM, Pickett CL. *Campylobacter jejuni* cytolethal distending toxin causes a G₂-phase cell cycle block. *Infect Immun* 1998; **66**: 1934–1940.
35. Young VB, Schauer DB. Identification and characterization of cytolethal distending toxin (CDT) activity in enterohepatic *Helicobacter* species (abstract). American Society for Microbiology General Meeting 1999; **208/D/B/**: D/B-184.
36. Stanley J, Linton D, Burrens AP et al. *Helicobacter canis* sp. nov., a new species from dogs: an integrated study of phenotype and genotype. *J Gen Microbiol* 1993; **139**: 2495–2504.
37. Black RE, Levine MM, Clements ML, Hughes TP, Blaser MJ. Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis* 1988; **157**: 472–479.
38. Shomer NH, Dangler CA, Schrenzel MD, Fox JG. A novel urease-negative intestinal *Helicobacter* species causes severe proliferative typhlocolitis in *scid* mice with defined flora (abstract). *Lab Anim Sci* 1998; **48**: 408.
39. Birnboim HC, Doly JA. Rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 1979; **7**: 1513–1523.
40. Hein J. Unified approach to alignment and phylogenies. In: Doolittle RF (ed) *Molecular evolution: computer analysis of protein and nucleic acid sequences*. Methods in enzymology 183. San Diego, Academic Press. 1990: 626–645.
41. Fox JG, Li X, Yan L et al. Chronic proliferative hepatitis in A/JCr mice associated with persistent *Helicobacter hepaticus* infection: a model of helicobacter-induced carcinogenesis. *Infect Immun* 1996; **64**: 1548–1558.
42. Hailey JR, Haseman JK, Bucher JR et al. Impact of *Helicobacter hepaticus* infection in B6C3F1 mice from twelve National Toxicology Program two-year carcinogenesis studies. *Toxicol Pathol* 1998; **26**: 602–611.
43. Nyska A, Maronpot RR, Eldridge SR, Haseman JK, Hailey JR. Alteration in cell kinetics in control B6C3F1 mice infected with *Helicobacter hepaticus*. *Toxicol Pathol* 1997; **25**: 591–596.
44. Kullberg MC, Ward JM, Gorelick PL et al. *Helicobacter hepaticus* triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12 – and gamma interferon-dependent mechanism. *Infect Immun* 1998; **66**: 5157–5166.
45. Palmer KL, Grass S, Munson RS. Identification of a hemolytic activity elaborated by *Haemophilus ducreyi*. *Infect Immun* 1994; **62**: 3041–3043.
46. Purvén M, Frisk A, Lönnroth I, Lagergård T. Purification and identification of *Haemophilus ducreyi* cytotoxin by use of a neutralizing monoclonal antibody. *Infect Immun* 1997; **65**: 3496–3499.
47. Taylor NS, Fox JG, Yan L. In-vitro hepatotoxic factor in *Helicobacter hepaticus*, *H. pylori* and other *Helicobacter* species. *J Med Microbiol* 1995; **42**: 48–52.
48. Okuda J, Fukumoto M, Takeda Y, Nishibuchi M. Examination of diarrheagenicity of cytolethal distending toxin: suckling mouse response to the products of the *cdtABC* genes of *Shigella dysenteriae*. *Infect Immun* 1997; **65**: 428–433.
49. Tomb JF, White O, Kerlavage AR et al. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 1997; **388**: 539–547.
50. Alm RA, Ling L-SL, Moir DT et al. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* 1999; **397**: 176–180.
51. Eyigor A, Dawson KA, Langlois BE, Pickett CL. Detection of cytolethal distending toxin activity and *cdt* genes in *Campylobacter* spp. isolated from chicken carcasses. *Appl Environ Microbiol* 1999; **65**: 1501–1505.