BACTERIAL PATHOGENICITY

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

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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 actinomyctemcomitans, 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 enterohelipenic 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]. Enterohelipenic 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.

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Because many Helicobacter spp. can cause intestinal diseases in their natural hosts, an earlier study investigated whether or not Cdt was also present in enterohpatic Helicobacter spp.; if present, this would help explain the adverse effect of these bacteria on host tissue. Recently, Cdt in H. hepaticus and H. pylori 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 enterohpetic 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 examines 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 (ATCC51160) [8], H. canis (ATCC51401) [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 cholecytitis 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 H<sub>2</sub> 10%, CO<sub>2</sub> 5%, and balanced with N<sub>2</sub>) 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 (Coraville, IA, USA). Degenerative primers VAT2 (forward, 5'-GTNG CANABTTGAAAYTNCARGG-3') and WMII (reverse, 5'-RTTRARTNCYADATACGC-3') were designed as described by Pickett et al. [24]. Another degenerative primer designated DHF1 was designed (reverse, 5'-DANCNGGARATGRTC-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 MgCl<sub>2</sub>, 1 mol/L to a final concentration of 2.25 mmol/L), 0.5 µmol of each forward and reverse primer, 200 µmol 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°C 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 WMII 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<sup>-</sup>-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 sequence kit purchased from Amerham Life Science, (Cleveland, OH, USA) following the procedures provided by the supplier’s manual. These pGEM<sup>-</sup>-T recombinant plasmids with DNA inserts from Helicobacter spp. in E. coli – pHPlH1 (from H. hepaticus), pHB2 (from H. bilis), pHCl (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 (Amerham, 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<sup>TM</sup> 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 \( [\alpha-^{32}\text{P}] \) dCTP-labelled probe in the nick translation system (BRL). The membrane was pre-hybrised in a buffer (5 \( \times \) SSC, formamide 50\%, denatured salmon-sperm DNA 100 \( \mu \)g/ml, 0.001 \( \mu \)l EDTA and 1 \( \times \) Denhardt’s solution) at 37°C for 1 h. Denatured \( ^{32} \text{P} \)-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 \(-80^\circ \)C overnight.

**Analysis of nucleotide sequences and sequence accession numbers**

The partial sequences of cdT 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 (DNASTar, 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.

**Cytotoxic 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 10,000g (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-\( \mu \)m filter. Samples were stored at \(-70^\circ \)C until tested.

**Tissue-culture assay for morphological changes by Cdt**. One ml of HeLa cells at 10\(^3\) 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 CO\(_2\) 8\% in air, 10 \( \mu \)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 cm\(^2\)) were seeded with 5 ml of EMEM-fetal bovine serum 10\% at 1 \( \times \) 10\(^5\) HeLa cells (CCL-2)/ml. Flasks were inoculated with 100 \( \mu \)l of filter-sterilised bacterial sonicate and then incubated at 37°C with CO\(_2\) 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 \( \mu \)g/ml, RNAase A 9 U/ml, Triton-X 100 0.1\%, bovine serum albumin 0.0001% in 4 \( \mu \)m sodium citrate) and then incubated at 37°C for 20 min. One ml of salt solution (PEG 3\%, propidium iodide 4 \( \mu \)g/ml, Triton-X 100 0.1\%, bovine serum albumin 0.0001% in 0.4 \( \mu \)l NaCl) was added, the solution was thoroughly mixed, and then stored at 4°C for 3 h. DNA analysis was performed on a FACSscan flow cytometer (BeckmanDickenson, Franklin Lakes, NJ, USA) with Cell Quant software for data acquisition. The ModFit program was used for data analysis. For each experiment, 1 \( \times \) 10\(^5\) cells were analysed.

**Results**

Cloning and sequence determination of cdT homologues from enteroheliotic *Helicobacter* spp.

With primers VAT2 and WM1 described previously by Pickett et al. [24], PCR amplicons approximating the expected length (494 bp of the *C. jejuni* cdT) 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* cdT, it was possible to amplify products of the expected length (714 bp according to the cdT gene of *C. jejuni*) from all five enteroheliotic *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. cans*; 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 cdT 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 cdT homologues of *H.*
hepatic, 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 cdB homologues of Helicobacter sp. strain 98-6070 and Helicobacter sp. strain 96-1001 shared 69.2% similarity. The similarity matrix based on the cdB nucleotide sequences from these bacteria is shown in Table 1 [40].

Deduced amino-acid sequences of CdB 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 CdB of the five Helicobacter spp. with that of C. jejuni is shown in Fig. 1. The phylogenetic analysis indicated that CdB 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 pH11 (the *H. hepaticus* cdB homologue) hybridised with genomic DNA of *H. hepaticus* with the standard procedure provided by ECL™. It also hybridised weakly with *H. bilis* and *H. canis* genomic DNA (Fig. 3a). However, the probe prepared from the *H. hepaticus* cdB 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 cdB 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).

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

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<th>(1)</th>
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<td>57.1</td>
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<td>(5) Helicobacter 98-6070</td>
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<td>60.8</td>
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<td>(9) <em>C. jejuni</em></td>
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<td>(10) <em>A. actinomycetemcomitans</em></td>
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*Alignment done by Jotun Hein Method [40] included in Lasergene software (DNASTAR, Madison, WI, USA).

1Sequence from Scott and Kaper [27].

2Sequence from Pecket et al. [26].

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 CdB did not affect the morphology of HeLa cells. Cells treated with the Cd 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 cytostostending effects in 10–15% of the HeLa cells, whereas the supernatant fraction of sonicates of Helicobacter sp. strain 99-1001 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-
CDT of ENTEROHEPATIC HELICOBACTER SPP. 529

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 sp. 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: AseI; lanes 2, 4, 6, 8, 10, 12: HindIII. 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 AseI. (b) The same membrane with a cdtB gene probe from Helicobacter sp. strain 96–1001. The probe hybridised with genomic DNA of Helicobacter sp. 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).

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 hepatisis 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
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 cdB 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 cdB gene of *C. jejuni* failed to hybridise or only weakly hybridised to the DNA of *C. fetus*, *C. hyointestinalis*, *C. laris* and *C. coli* [24]. Because of the divergence of this gene, use of selected degenerative primers may not always amplify the desired cdB fragment from species that possess the cd 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 marine 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.

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<th>Organism</th>
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<td>G₁/G₀ phase</td>
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<tr>
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PCR primers from various conserved regions are needed to evaluate the presence or absence of the cdτ 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 cdτ genes could have originated from the same
ancestral gene [31]. Although the sequences of the Cdt in enterohpatic 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 enterohpatic 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 enterohpatic 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.

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