Cytolethal distending toxin B gene (*cdtB*) homologues in taxa 2, 3 and 8 and in six canine isolates of *Helicobacter* sp. flexispira

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The presence of the cytolethal distending toxin B gene (*cdtB*) was examined in eight *Helicobacter* sp. flexispira reference strains, *Helicobacter trogontum* ATCC 700114^T and 12 Finnish porcine *H. trogontum* strains and canine flexispira isolates. Part of the *cdtB* gene was amplified by PCR with degenerate primers VAT2 and DHF1, cloned and sequenced. The presence/absence of the *cdtB* gene as determined by PCR was confirmed by Southern hybridization and toxin production by HeLa cell-line experiments. PCR amplification resulted in approximately 700 bp fragments from *Helicobacter* sp. flexispira taxa 2 (ATCC 49314), 3 (ATCC 49320) and 8 (ATCC 43880, ATCC 49308, ATCC 43879), from six canine isolates as well as from the control strains *Helicobacter bilis* and *Helicobacter hepaticus*. The hybridization patterns of *HaeIII*-, *HindIII*- and *Asel*-digested chromosomal DNA confirmed the results of the PCR experiments. The *cdtB*-positive strains had effects ranging from weak to strong on HeLa cell cultures. PCR amplification from the reference strains *Helicobacter* sp. flexispira taxa 1 (ATCC 43968), 4 (ATCC 49310) and 5 (ATCC 43966) and *H. trogontum* (ATCC 700114^T), and also six of the Finnish strains, was unsuccessful. No toxic effect on HeLa cells was evident when bacterial suspensions of PCR-negative strains were used for toxicity assay. Our results are in accordance with previous observations that the *cdtB* gene is not present in all *Helicobacter* species. Further, the presence/absence of the *cdtB* gene in *Helicobacter* sp. flexispira strains was in accordance with recent taxonomic analysis of the same strains, which suggests that it could serve as a useful marker in *Helicobacter* taxonomy.

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INTRODUCTION

Cytolethal distending toxin (CDT) causes distension and eventually death of cultured eukaryotic cells. The mechanism of CDT activity is reported to involve G₂/M cell-cycle arrest in target cells, possibly by preventing activation of cdc2 (Comayras *et al.*, 1997). The CDT toxin is encoded by three adjacent genes, *cdtA*, *cdtB* and *cdtC*, all of which are required for the toxin's function (Mayer *et al.*, 1999; Lara-Tejero & Galán, 2001). CdtB is proposed to be the enzymically active subunit of the holotoxin (Lara-Tejero & Galán, 2001).

Flexispiras are a group of organisms with very similar phenotypic characteristics: a distinctive cigar-like cell shape and external fibrils surrounding the cell and bundles of sheathed flagella at both ends of the cell (Bryner *et al.*, 1987).

The taxonomy of this group is not valid, because of the limited number of isolates available for study. According to a recent proposal based on phylogenetic analysis of 16S rRNA sequences, these organisms are members of the genus *Helicobacter*, forming a group that includes at least ten phylogenetic groups widely interspersed among intestinal *Helicobacter* species (Dewhirst *et al.*, 2000). A provisional name, *Helicobacter* sp. flexispira, has been proposed by Dewhirst *et al.* (2000) for this group of organisms until a valid taxonomic description is provided.

The *cdtB* gene is found to be the most conserved among the three *cdt* genes (Mayer *et al.*, 1999; Pickett *et al.*, 1996; Young *et al.*, 2000a). With degenerate PCR primers, *cdtB* homologues have been identified in enteropathogenic *Campylobacter* species (Pickett *et al.*, 1996) and in several enteric and enterohepatic *Helicobacter* species (*Helicobacter pullorum*, *Helicobacter hepaticus*, *Helicobacter bilis* and *Helicobacter canis*) (Young *et al.*, 2000a, b; Chien *et al.*, 2000). Neither the *cdt* gene cluster nor CDT activity has been identified in

Abbreviation: CDT, cytolethal distending toxin.

The GenBank/EMBL/DDBJ accession numbers for the partial *Helicobacter cdtB* sequences reported in this paper are AF467235–AF467243.

two enterohepatic *Helicobacter* species, *Helicobacter fennelliae* and *Helicobacter cinaedi* (Young *et al.*, 2000a). The aim of this study was to determine the presence or absence of the *cdtB* gene in *Helicobacter* sp. flexispira strains including reference strains, 12 Finnish porcine *Helicobacter trogontum* strains (Hänninen *et al.*, 2003) and canine flexispira isolates. Part of the *cdtB* gene was amplified by PCR using degenerate primers and was cloned and sequenced. The presence/absence of the *cdtB* gene as determined by PCR was confirmed by Southern hybridization and toxin production by cell-line experiments.

METHODS

Bacterial strains and purification of DNA. Eleven *Helicobacter* reference strains, six Finnish porcine *H. trogontum* strains and six canine flexispira isolates were included in the PCR analysis of *cdtB* gene homologues (Table 1). The isolation procedure and cultivation of

strains have been described by Hänninen *et al.* (2003). DNA was isolated by the method of Pitcher *et al.* (1989) as described previously (Hänninen *et al.*, 1996).

PCR amplification, cloning and sequencing. The degenerate primers VAT2 (forward, 5'-GTGNCACBTGGAACTNCARGG-3'), WMI1 (reverse, 5'-RTTAAARTCNCCYAADATCATCC-3') (Pickett *et al.*, 1996) and DHF1 (reverse, 5'-DACNGGRAARTGRTC-3') (Chien *et al.*, 2000) were used for PCR with genomic DNA of 23 *Helicobacter* strains (Table 1) as templates. Reactions (50 µl) were set up with 100 ng DNA, 20 pmol of each primer, 200 µM of each nucleotide and 2 U DyNzyme DNA polymerase (Finnzymes) or *Taq* DNA polymerase (Fermentas Inc.). The cycling conditions were essentially as described previously (Young *et al.*, 2000a).

A total of 11 PCR products of the expected size were cloned and sequenced. PCR fragments were extracted from 1.5% NuSieve GTG low-melting agarose gel (BMA), purified with the QIAquick gel extraction kit (Qiagen), cloned into pGEM-T Easy (Promega) and transformed into *Escherichia coli* DH5α or JM 109 cells. Plasmid DNA was extracted with the Qiagen plasmid Mini kit and sequenced by

Table 1. Strains and isolates used in this study

Helicobacter sp. flexispira reference strains, porcine *H. trogontum* strains (Hänninen *et al.*, 2003) and canine flexispira isolates, *H. bilis* and *H. hepaticus* strains included in PCR experiments and in toxicity assays in HeLa cell cultures are indicated.

Strain/isolate designation	Original designation	Source of isolation	<i>cdtB</i> *	Toxicity test†	Reference(s)
<i>H. trogontum</i> HU 1 SU		Pig, faecal sample	—	ND	Hänninen <i>et al.</i> (2003)
<i>H. trogontum</i> HU 2 SU		Pig, faecal sample	—	ND	Hänninen <i>et al.</i> (2003)
<i>H. trogontum</i> HU 3 SU		Pig, faecal sample	—	ND	Hänninen <i>et al.</i> (2003)
<i>H. trogontum</i> HU A4		Pig, faecal sample	—	ND	Hänninen <i>et al.</i> (2003)
<i>H. trogontum</i> HU H95S		Pig, stomach	—	ND	Hänninen <i>et al.</i> (2003)
<i>H. trogontum</i> HU H158S		Pig, stomach	—	ND	Hänninen <i>et al.</i> (2003)
<i>H. trogontum</i> ATCC 700114 ^T	8581	Rat, colon	—	ND	Hänninen <i>et al.</i> (2003)
<i>Helicobacter</i> sp. taxon 1 ATCC 43968	86-13625	Pig	—	—	Dewhirst <i>et al.</i> (2000)
<i>Helicobacter</i> sp. taxon 4 ATCC 49310	86-1775	Sheep	—	—	Dewhirst <i>et al.</i> (2000)
<i>Helicobacter</i> sp. taxon 5 CCUG 28992 (=ATCC 43966)	84-3345	Aborted sheep fetus	—	—	Bryner <i>et al.</i> (1987); Kirkbride <i>et al.</i> (1985, 1986)
<i>Helicobacter</i> sp. taxon 2 ATCC 49314	86-2279	Sheep	+	(+)	Dewhirst <i>et al.</i> (2000)
<i>Helicobacter</i> sp. taxon 3 ATCC 49320	88-2491	Pig, stomach	+	(+)	Dewhirst <i>et al.</i> (2000)
Isolate KO534B		Dog, faecal sample	+	ND	
Isolate KO214		Dog, faecal sample	+	ND	
Isolate KO114 (Alma)		Dog, faecal sample	+	+++	
Isolate KO115 (Eppu)		Dog, faecal sample	+	ND	
Isolate Hilli		Dog, faecal sample	+	ND	
Isolate KO220		Dog, faecal sample	+	+++	
<i>Helicobacter</i> sp. taxon 8 CCUG 38944 (=ATCC 43880)	14020	Human stool	+	ND	Romero <i>et al.</i> (1988)
<i>Helicobacter</i> sp. taxon 8 CCUG 41386 (=ATCC 49308)	39051	Dog stool	+	ND	Romero <i>et al.</i> (1988)
<i>Helicobacter</i> sp. taxon 8 CCUG 223435 (=ATCC 43879)	38264	Human stool	+	+	Romero <i>et al.</i> (1988)
<i>H. bilis</i> (taxon 9) ATCC 51630 ^T		Mouse faeces	+	++	Fox <i>et al.</i> (1995)
<i>H. hepaticus</i> CCUG 33637 ^T (=ATCC 51448 ^T)		Mouse liver	+	ND	Fox <i>et al.</i> (1994)

* *cdtB* positivity as determined by PCR.

† Scored as: —, no effect; (+), weak effect with undiluted sonicate; +, effect; ++, strong effect; +++, very strong effect. ND, Not done.

automated cycle sequencing with Big Dye terminators (ABI 377, PE Biosystems). To exclude artefacts, at least three replicate clones were sequenced for each cloned PCR product.

Southern hybridization. Approximately 4 µg chromosomal DNA was digested with *Asel*, *HaeIII* and *HindIII*, separated by 1% agarose gel electrophoresis and transferred to a Hybond-N nylon membrane according to the manufacturer's protocol (Amersham). Procedures for pre-hybridization, hybridization, probe labelling and membrane washing have been described previously (Jalava *et al.*, 1998, 1999). The probe was PCR-amplified from one of the recombinant clones (taxon 8, CCUG 38944/ATCC 43880). Membranes were incubated overnight at either 58 °C (*HaeIII*- and *HindIII*-digested DNA) or 37 °C (*Asel*-digested DNA) for low-stringency hybridization.

Toxicity assay in HeLa cell cultures. Nine strains were included in a toxicity assay (Table 1). For preparation of cell sonicates, bacteria were grown on brucella blood agar plates with 7% bovine blood under micro-aerobic conditions and incubated for 2–3 days. Cells were harvested by centrifugation and the pellet was resuspended in 1 ml PBS and sonicated on ice with four 30-s pulses (30 s interval). The sonicate was centrifuged at 6000 g and the supernatant fraction was filter-sterilized with a 0.22 µm filter and used for toxicity studies.

Toxicity testing was performed by a modification of the assay of Chien *et al.* (2000). A HeLa cell culture (20 ml) was trypsinized and diluted 1:5 in minimum essential medium with Earle's salts supplemented with 10% fetal calf serum, 200 IU penicillin ml⁻¹ and 200 µg streptomycin ml⁻¹. One ml cell suspension per well (approx. 20 000 cells ml⁻¹) was pipetted into wells of 24-well plates (Nunc). The filter-sterilized supernatant fractions to be tested were diluted in PBS from 1:1 to 1:16. Aliquots of 10 and 20 µl of each dilution were added onto HeLa cells in tissue culture-plate wells and the plate was incubated at 37 °C in 5% CO₂ for 4 days. Wells for control of cell growth were included. The cells were observed daily microscopically for toxic effects (cell growth, changes and death).

Sequence analysis. The DNA sequences of the partial *cdtB* genes from 11 flexispira strains obtained from this study were compared with homologous sequences available in public databases. Partial *cdtB* sequences from *H. canis* (AF243078), *H. hepaticus* (AF243076), *H. pullorum* (AF220065) and *H. bilis* (AF243077), *Campylobacter jejuni* (U51121) and two novel *Helicobacter* spp. isolates 96-1001 (AF243080) and 98-6070 (AF243079) (Chien *et al.*, 2000) were included in the analysis. Nucleotide sequences were translated to amino acid sequences by the TRANSEQ program included in the EMBOSS software analysis package. Percentage similarity values were calculated by BioNumerics version 2.50 (Applied Maths). The most parsimonious phylogenetic trees were constructed and bootstrap support (Felsenstein, 1985), performing 1000 replicates with 10 random additions for each node, was assessed using PAUP 4.0 (Swofford, 1996).

RESULTS

PCR amplification with degenerate primers, cloning and sequencing

PCR amplification with primer pair VAT2+WMI1 was not successful from any of the 22 templates. Another pair of degenerate primers, VAT2 and DHF1, amplified approximately 700 bp fragments from *Helicobacter* sp. flexispira taxa 2, 3 and 8 and from six canine isolates as well as from *H. bilis* and *H. hepaticus* (Table 1). PCR amplification with this pair of primers from six porcine *H. trogontum* strains and the reference taxa 1, 4 and 5 was unsuccessful (Table 1).

The cloned PCR fragments were sequenced from at least three recombinant plasmids. The inserts were 708 (taxa 2 and 3), 702 (Hilli) and 699 bp [taxon 8 and canine isolates 534b, KO214, KO220, KO114 (Alma) and KO115 (Eppu)] in length (excluding primer sequences). The lengths of the respective amino acid sequences were 236, 234 and 233 residues. The partial *cdtB* sequences of taxa 2 and 3 included an insertion of 9 bp, of which the third codon was the translation termination codon UAA/TAA in the coding frame (Fig. 1). The *cdtB* sequence of isolate Hilli had an insertion of 3 bp (Fig. 1).

Three taxon 8 reference strains were included in the PCR and sequence analysis (Table 1). The partial *cdtB* sequences of these strains were identical, and only one DNA sequence, PCR-amplified from strain CCUG 38944/ATCC 43880, has been entered in the GenBank database under the accession number AF467241. The GenBank accession numbers for the

VAT 2 ⇒

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1. SSASTEN---KNNVSVRQLITGDNPDILMVQEAGSVPASARPTGRMIQPGGTPIQEVVW
2. SSASTEN---KNNVSVRQLITGDNPDILMVQEAGSVPASARPTGRMIQPGGTPIQEVVW
3. SSASTEN---KNNVSVRQLITGDNPDILMVQEAGSVPASARPTGRMIQPGGTPIQEVVW
4. SSASTEN---KNNVSVRQLITGDNPDILMVQEAGSVPASARPTGRMIQPGGTPIQEVVW
5. SSASTEN---KNNVSVRQLITGDNPDILMVQEAGSVPTSARPTGRMIQPGGTPIQEVVW
6. SSASTEN---KNNVSVRQLITGDNPDILMVQEAGSVPTSARPTGRMIQPGGTPIQEVVW
7. SSASTEN---KNNVSVRQLITGDNPDILMVQEAGSVPTSARPTGRMIQPGGTPIQEVVW
8. SSASTEN---KNNVSVRQLITGDNPDILMVQEAGSVPTSARPTGRMIQPGGTPIQEVVW
9. SSASTENKNNKNNVSVRQLITGDNPDILMVQEAGSVPTSARPTGRMIQPGGTPIQEVVW
10. SSASTENKNNKNNVSVRQLITGDNPDILMVQEAGSVPTSARPTGRMIQPGGTPIQEVVW
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1. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
2. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
3. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
4. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
5. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
6. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
7. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
8. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
9. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
10. DLGTSRSPRSVFIIYANIDVGARRVNLAIIVGRQADEVFVISQRTIAPEVSRPVIGIRLG
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1. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
2. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
3. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
4. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
5. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
6. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
7. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
8. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
9. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
10. NDVFFNIHALARGGGDAAALVTAVHDHFVQPSINWLIAGDFNRDPANLLSGLDTRITN
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1. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
2. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
3. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
4. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
5. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
6. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
7. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
8. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
9. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
10. HTRIVTQNSATHFSMGAANRILDYAIVGRSSND-RRLALPTITALLMAASVRSLSLS
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⇐ DHF1

Fig. 1. Alignment of predicted amino acid sequences of partial CdtB proteins of the *Helicobacter* sp. flexispira canine isolates and *H. bilis*. Amino acid sites that differ between strains are in bold. Sequences were derived from: 1, *H. bilis*; 2, KO115 (Eppu); 3, KO220; 4, KO534B; 5, taxon 8; 6, KO214; 7, KO114 (Alma); 8, Hilli; 9, taxon 2; 10, taxon 3.

other new partial *cdtB* sequences are AF467235 (isolate Hilli), AF467236 (isolate KO214), AF467237 (isolate KO534B), AF467238 (isolate KO220), AF467239 [isolate KO114 (Alma)], AF467240 [isolate KO115 (Eppu)], AF467242 (taxon 2) and AF467243 (taxon 3).

Sequence comparisons

The partial *cdtB* DNA sequences of the *Helicobacter* sp. flexispira reference strains of taxa 2, 3 and 8 and six canine isolates (Hilli, KO114, KO115, KO214, KO534B and KO220) showed approximately 70 % similarity to homologues from *H. pullorum* (AF220065) and *H. hepaticus* (AF243076) and 76 % similarity to the *H. canis* homologue (AF243078). Comparison of amino acid sequences of the same *Helicobacter* strains resulted in 80–84 % identity. Comparisons of flexispira *cdtB* DNA sequences to *C. jejuni* (U51121) and to *Helicobacter* spp. isolates 96–1001 (AF243080) and 98–6070 (AF243079) (Chien *et al.*, 2000) revealed 56–61 % similarity. The amino acid sequences of *C. jejuni* and isolate 98–6070 revealed 66–70 % identity and that of isolate 96–1001 less than 40 % identity to flexispira sequences.

Pairwise comparisons of partial *cdtB* DNA sequences of taxa 2, 3 and 8 and the six Finnish canine isolates revealed 90–99 % similarity. Among the amino acid sequences of the same strains, identity was 94–100 %. Comparison of the sequences of taxa 2 and 3 revealed one synonymous substitution at the DNA level, i.e. the predicted amino acid sequences were identical. The *cdtB* DNA sequences of two isolates, KO115 and KO220, differed at only six sites and by only one amino acid. The DNA sequence of another dog isolate, 534B, differed from KO115 and KO220 at 14 sites and by 4 and 5 amino acids, respectively. These three sequences, as well as KO214, differed from the DNA sequence of ATCC 43880 (taxon 8) at 17–19 sites and by 2–6 amino acids. Isolate KO114 showed most similarity to isolate KO214 (respectively 27 and 6 different sites in the DNA and amino acid sequences) and isolate Hilli to isolate KO114 (Alma) and KO214 (in addition to a 3 bp insertion, respectively 36 and 12 different sites in the DNA and amino acid sequences). Parsimony analysis was done for both DNA and amino acid sequences of *cdtB*. The consensus tree of the two most-parsimonious trees of the *cdtB* DNA sequences and the bootstrap values are presented in Fig. 2.

Southern hybridization

Chromosomal DNA of flexispira strains was digested with three different restriction enzymes. No hybridization signal was observed in *Helicobacter* sp. flexispira taxon 1, 4 or 5, nor in any of the porcine isolates. A clear hybridization signal was apparent in digested DNA of taxa 2, 3 and 8, as well as in the canine isolates, *H. bilis* and *H. hepaticus* in all three hybridization experiments. The hybridization pattern of *Hind*III-digested DNA revealed a fragment of over 20 kb from all the strains studied. Hybridization of *Hae*III-digested DNA resulted in a fragment of > 20 kb from taxon 8 and from the canine isolates, *H. bilis* and *H. hepaticus* and an

Fig. 2. Consensus tree of the two most-parsimonious trees of partial *cdtB* DNA sequences of *Helicobacter* sp. flexispira taxa 2, 3 and 8 reference strains, Finnish canine flexispira isolates, *H. canis*, *H. bilis* and *H. hepaticus*. Bootstrap support values appear above branches.

approximately 15 kb fragment from the reference taxa 2 and 3. The hybridization pattern of *Ase*I-digested DNA revealed a fragment of approximately 2 kb from the canine isolates, taxa 2, 3 and 8 and *H. bilis* and an approximately 1.2 kb fragment from *H. hepaticus*.

Toxicity assay

The reference strain for the CDT-producing bacterium *H. bilis* caused a clear reduction in cell growth at dilutions of 1 : 1 to 1 : 4. Long, fibroblast-like cells and, finally, cell death occurred (Table 1). The cell layer was only 40 % confluent, compared with 90 % of the cells of control wells. Three strains, taxa 2, 3 and 8, had a weak toxic effect, with specific cell alteration in 1 : 1 or 1 : 2 dilutions (Table 1). Bacterial suspensions of taxa 1, 4 and 5 were negative; the cells multiplied, with no toxic effect detected on cells. Two isolates, KO114 and KO220, showed a clear, strong effect in all dilutions between 1 : 1 and 1 : 16. During the first day of observation, fibroblast-like cells and multinucleate cells were evident. From day 2 to day 4, the cells became rounded and they detached from the bottoms of wells.

DISCUSSION

For six reference strains of *Helicobacter* sp. flexispira taxa 2, 3 and 8 and six canine flexispira isolates, a PCR amplification product of approximately 700 bp suggests that these organisms have the *cdtB* gene identified in earlier studies from several Gram-negative pathogens including *H. pullorum*, *H. hepaticus* and *H. bilis* (Young *et al.*, 2000a, b; Chien *et al.*, 2000). That the amplification was unsuccessful for three of the reference strains of taxa 1, 4 and 5 and *H. trogonum* and for Finnish porcine strains suggests that these organisms lack a *cdtB* gene. All these strains are, according to our recent

results, members of *H. trogontum* (Hänninen *et al.*, 2003). Our results are in accordance with previous observations that the *cdtB* gene is not present in all *Helicobacter* species (Young *et al.*, 2000a, b). Southern hybridization of the digested chromosomal DNA with a 700 bp partial *cdtB* gene amplified from *Helicobacter* sp. flexispira reference strain ATCC 43880 (taxon 8) as a probe confirmed the results of the PCR experiments. No hybridization signal appeared in taxon 1, 4 or 5, in *H. trogontum* ATCC 700114^T or in porcine *H. trogontum* strains, whereas a clear hybridization signal appeared in taxa 2, 3 and 8, the canine isolates and *H. bilis* and *H. hepaticus*. Chien *et al.* (2000) have reported that the primers VAT2, WMI1 and DHF1 failed to produce an amplicon of the expected size from the reference strain ATCC 43879 (referred to as '*Helicobacter rappini*' by Chien *et al.*, 2000). We succeeded in amplifying a *cdtB* gene fragment from this strain (taxon 8; see Table 1) with the primer pair VAT2 and DHF1. In accordance with Chien *et al.* (2000), we were also unsuccessful in amplification of the expected PCR product from *H. bilis* with primers VAT2 and WMI1. Primers VAT2 and WMI1 are designed for the *cdtB* gene of *C. jejuni* and, due to divergence of the gene, use of these degenerate primers may not amplify the *cdtB* fragment from species that possess the gene.

In the toxicity test with bacterial sonicates from taxa 1, 4 and 5, no indication of CDT production was evident in the cultured HeLa cells, which was expected and further confirmed the results from the DNA analysis. An insertion with a translation termination codon was detected in sequences from taxa 2 and 3. The presumed ribosome-binding sites for the *cdtB* ORF, as well as the probable initiation codon, are approximately 70 bp upstream from the binding site for VAT2. It is quite obvious that most of the *cdtB* gene is not translated in taxa 2 and 3. The possibility that this nonsense mutation represents a PCR artefact is unlikely, since the same change was present in both templates (taxa 2 and 3) and, altogether, in six sequenced replicate clones. A frameshift mutation in the *cdtB* gene, resulting in premature termination of protein synthesis, has also been found, for example in *Helicobacter* spp. isolate 96-1001 (Chien *et al.*, 2000). The supernatant fraction of this strain had no effect on the HeLa cell cycle (Chien *et al.*, 2000). In our experiments, a weak effect was apparent when undiluted bacterial sonicates of taxa 2 and 3 were added to the HeLa cell culture. This may indicate that some other toxin is produced in these strains. For example, another cytotoxic activity, called granulating cytotoxin, has been described in *H. hepaticus* (Taylor *et al.*, 1995). Confirmation that the CdtB protein is produced in taxa 2 and 3 requires further analysis at either the mRNA or protein level. The fact that sonicates of the two isolates KO114 and KO220 had such a strong effect in HeLa cell culture even when used in a 1:16 dilution suggests that this effect could be a result of two different toxins. These findings need to be studied further.

Our results suggest that the presence/absence of the *cdtB* gene can offer a useful tool in *Helicobacter* taxonomy. Based on phenotypic characterization, dot-blot hybridization with

DNA of the reference strain, analysis of RFLPs of 16S and 23S rDNAs and SDS-PAGE protein profiles, we have found that all the porcine isolates and the reference strains of flexispira taxa 1, 4 and 5 are actually members of *H. trogontum* and differ from the strains of taxa 2, 3 and 8, as well as from *H. bilis* (Hänninen *et al.*, 2003). Taxa 2, 3 and 8 are presently unnamed and their taxonomic characterization awaits further study (Dewhirst *et al.*, 2000). Young *et al.* (2000b) have also proposed that the presence/absence of the *cdtB* gene may prove useful in the classification of micro-aerobic spiral bacteria. Their suggestion was based on experiments that included eight clinical isolates and three reference strains of *H. pullorum*. The two isolates that were *cdtB*-negative actually represented a novel *Helicobacter* species closely related to, but distinct from, *H. pullorum*; the name *Helicobacter canadensis* has been proposed for these *cdtB*-negative strains (Fox *et al.*, 2000).

The *cdt*-positive *Helicobacter* sp. flexispira strains were analysed further by phylogenetic methods. According to phylogenetic analysis of 16S rRNA sequences, *Helicobacter* sp. flexispira taxa 2 and 3 cluster together and may represent different species (Dewhirst *et al.*, 2000). The partial *cdtB* genes of these two strains were almost identical and probably represent a pseudogene. The *cdtB* DNA sequences of taxa 2 and 3 showed 91–93 % similarity to the canine isolates, taxon 8 strains and *H. bilis* and they clustered together in the phylogenetic tree, with strong bootstrap support (Fig. 2). DNA sequences of the *cdtB* gene of the three reference strains for *Helicobacter* sp. flexispira taxon 8 were identical. These strains were isolated from stool samples of a human patient, his daughter and the family dog during a diarrhoeal episode in the family (Romero *et al.*, 1988). Our *cdtB* sequence findings further support the hypothesis that the flexispira strain was transmitted to the human patient and his daughter from the dog. The six canine flexispira isolates included in this study all cluster with *Helicobacter* sp. flexispira taxon 8, as shown by 16S rRNA sequence analysis (F. E. Dewhirst, personal communication). Partial DNA sequences of the *cdtB* gene of isolates KO115, KO220 and KO534B differed from each other and from sequences of reference strains of *Helicobacter* sp. flexispira taxon 8 by only 1–3 %. Phylogenetic analysis supports the idea that isolates KO115, KO220 and KO534B may represent taxon 8 strains, but support for isolates KO214, KO114 and Hilli is weak (Fig. 2). Because *cdtB* is not present in all *Helicobacter* species, or can represent a pseudogene in some species, the sequence diversity of this gene is not necessarily a good phylogenetic marker for taxonomic purposes.

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