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Characterization of a *Haemophilus ducreyi* Mutant Deficient in Expression of Cytolethal Distending Toxin

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Haemophilus ducreyi **expresses a soluble cytolethal distending toxin (CDT) that kills HeLa, HEp-2, and other human epithelial cells in vitro. *H. ducreyi* CDT activity is encoded by a three-gene cluster (*cdtABC*), and antibody to the *cdtC* gene product can neutralize CDT activity in vitro (L. D. Cope, S. R. Lumbley, J. L. Latimer, J. Klesney-Tait, M. K. Stevens, L. S. Johnson, M. Purven, R. S. Munson, Jr., T. Lagergard, J. D. Radolf, and E. J. Hansen, Proc. Natl. Acad. Sci. USA 94:4056–4061, 1997). Culture supernatant fluid from a recombinant *Escherichia coli* strain containing the *H. ducreyi cdtABC* gene cluster readily killed both HeLa cells and HaCaT keratinocytes and had a modest inhibitory effect on the growth of human foreskin fibroblasts. Insertional inactivation of the *cdtC* gene in this recombinant *E. coli* strain eliminated the ability of this strain to kill HeLa cells and HaCaT keratinocytes. This mutated *H. ducreyi cdtABC* gene cluster was used to construct an isogenic *H. ducreyi cdtC* mutant. Monoclonal antibodies against the *H. ducreyi* CdtA, CdtB, and CdtC proteins were used to characterize protein expression by this *cdtC* mutant. Culture supernatant fluid from this *H. ducreyi cdtC* mutant did not detectably affect any of the human cells used in this study. The presence of the wild-type *H. ducreyi cdtC* gene in *trans* in this *H. ducreyi* mutant restored its ability to express a CDT that killed both HeLa cells and HaCaT keratinocytes. The isogenic *H. ducreyi cdtC* mutant was shown to be as virulent as its wild-type parent strain in the temperature-dependent rabbit model for experimental chancroid. Lack of expression of the *H. ducreyi* CdtC protein also did not affect the ability of this *H. ducreyi* mutant to survive in the skin of rabbits.**

Haemophilus ducreyi is an extremely fastidious, gram-negative coccobacillus which causes chancroid, a sexually transmitted ulcerogenital disease that has a high degree of prevalence in some parts of Africa and Asia. In the United States, chancroid is uncommon (61), and outbreaks are often associated with prostitution, crack cocaine usage, and multiple sex partners (15, 39). Although chancroidal ulcers are usually relatively superficial, they can facilitate transmission of the human immunodeficiency virus (72).

There is a paucity of information concerning the gene products which allow *H. ducreyi* to cause genital ulcers. The organism is apparently not able to invade intact skin (64), and it is assumed that microabrasions sustained during sexual activity permit entry of the organism beneath the skin surface. The introduction of a number of new model systems for studying the interaction of *H. ducreyi* with host cells both in vitro and in vivo (8, 23, 24, 33, 68, 69, 71) has facilitated studies intended to identify virulence factors of this pathogen. In the past few years, a number of *H. ducreyi* gene products have been postulated to be involved directly or indirectly in virulence expression (7, 9–11, 17–19, 34, 37, 38, 48, 49, 59, 60, 66, 73), including at least two proteins which have cytotoxic activity.

The first of these two cytotoxins was originally described by Lagergard and colleagues (15, 31, 32, 54, 56) as being present in *H. ducreyi* culture supernatant fluid and active against several different types of human epithelial cell lines (e.g., HeLa cells) in vitro. The second cytotoxin, first described by Palmer and Munson (45), proved to be a hemolysin that was similar to the hemolysins expressed by *Proteus mirabilis* and *Serratia marcescens*, appeared to be bacterial cell-associated, and was found to kill human foreskin fibroblasts but not HeLa cells in culture (2, 43–45, 47, 70). It was recently shown that an isogenic *H. ducreyi* hemolysin-deficient mutant caused pustule formation in the human model for experimental chancroid (47).

The soluble cytotoxic activity in *H. ducreyi* culture supernatant fluid (15, 31, 32, 54, 56) was recently shown to be the result of the activity of a homolog of the cytolethal distending toxin (CDT) (13) expressed by a number of enteric pathogens, including *Escherichia coli* (26, 51, 62), *Shigella* species (27, 42), and *Campylobacter* species (28, 52). CDT activity is characterized by relatively slow morphological changes in cultured epithelial cells, including progressive cellular distention and death within 96 to 120 h (26).

The *H. ducreyi* CDT is chromosomally encoded by three genes, *cdtA*, *cdtB*, and *cdtC* (13), whose predicted protein products possess 24 to 51% identity with the CdtABC proteins from *E. coli* (51, 62). A monoclonal antibody (MAb) to the *H. ducreyi* CdtC protein neutralized CDT activity in vitro (13) and implicated at least the *H. ducreyi cdtC* gene product as being involved, directly or indirectly, in the expression of cytotoxic activity. As part of our continuing efforts to elucidate virulence mechanisms of *H. ducreyi*, we constructed an isogenic *H. ducreyi cdtC* mutant and tested this mutant in relevant in vitro and in vivo systems. Elimination of the ability to elaborate the CdtC protein caused this *H. ducreyi* mutant to be unable to kill HeLa cells and HaCaT keratinocytes in culture. In contrast, this mutation did not detectably affect the ability of *H. ducreyi* to cause skin lesions in the temperature-dependent rabbit model.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
E. coli		
DH5α	Host strain for cloning experiments	58
HB101	Host strain essential for propagating plasmids carrying mutated *H. ducreyi* DNA inserts used in electroporation	5, 58
DH5α(pJL300)	Contains *H. ducreyi* 35000 *cdtABC* gene cluster; expresses *H. ducreyi* CDT activity	This study
DH5α(pJL303)	Contains *H. ducreyi cdtABC* gene cluster with mutated *cdtC* gene; expresses no CDT activity	This study
DH5α(pJL303)(pJL300-C)	DH5α(pJL303) also containing pJL300-C with the *H. ducreyi cdtC* gene; expresses CDT activity	This study
DH5α(pJL303)(pLS88)	DH5α(pJL303) also containing pLS88; expresses no CDT activity	This study
H. ducreyi		
35000	Wild-type strain isolated in Winnipeg, Manitoba, Canada; produces CDT	21
35000.303	Isogenic *cdtC* mutant with a *cat* cartridge inserted into the *cdtC* gene; does not express CDT activity	This study
35000.303(pJL300-C)	35000.303 containing pJL300-C with the *cdtC* gene; expresses CDT activity	This study
35000.303(pLS88)	35000.303 containing pLS88; expresses no CDT activity	This study
Plasmids		
pBR322	Cloning vector; Amp^r Tet^r	58
pJL300	pBR322 with a 3-kb *H. ducreyi* 35000 DNA insert containing the *cdtABC* gene cluster	This study
pJL303	pJL300 with an *cat* cartridge inserted into the *Bse*RI site within the *cdtC* gene	This study
pLS88	Cloning vector capable of replication in *H. ducreyi*; Kan^r Sm^r Sul^r	16
pJL300-C	pLS88 with a 813-bp PCR-derived insert containing the *H. ducreyi cdtC* gene	This study

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used for the majority of the experiments in this study are listed in Table 1. All *H. ducreyi* strains were cultivated on chocolate agar (CA) plates containing 1% (vol/vol) IsoVitaleX (BBL Microbiological Systems, Cockeysville, Md.) in a humidified atmosphere of 95% air–5% CO_2 at 33°C as described previously (53). Stock cultures of all *H. ducreyi* strains were stored at −70°C in fetal bovine serum. *H. ducreyi* cultures used for cytotoxicity assays were grown in modified Columbia broth (74). *H. ducreyi* transformants were isolated on either CA containing chloramphenicol (2 μg/ml) or GC-heme agar (66) containing kanamycin (30 μg/ml). *E. coli* strains HB101 and DH5α were grown in Luria-Bertani (LB) broth or on LB agar medium at 37°C. For selection of *E. coli* recombinants, chloramphenicol, kanamycin, and tetracycline were used at final concentrations of 30, 30, and 15 μg/ml, respectively.

MAbs. Female BALB/c mice were immunized with purified fusion proteins consisting of six histidine residues (His) coupled to most or all of the putative mature forms of the *H. ducreyi* CdtA, CdtB, and CdtC proteins as described previously (13). A synthetic peptide (EPTHRSGNILDYAILHDAHLPRREQA RER) derived from the amino acid sequence of the *H. ducreyi* CdtB protein was covalently coupled to keyhole limpet hemocyanin; this peptide-protein conjugate was used to immunize mice as described previously (74). Splenocytes from the immunized mice were used in a hybridoma fusion protocol (57), and culture supernatant fluids from these fusions were screened for the presence of MAbs by means of an enzyme-linked immunosorbent assay (ELISA). The antigens used in this ELISA to identify the CdtA-reactive MAb 1G8 and the CdtC-reactive MAbs 8C9 and 9E9 were the purified, homologous His-tagged fusion proteins. The CdtC-reactive MAb 9E9 was used for colony blot radioimmunassays (RIAs); the CdtC-reactive MAb 8C9 was used in Western blot analysis. Cell envelopes from a recombinant *E. coli* strain expressing a glutathione *S*-transferase–CdtB fusion protein were used in the ELISA to identify the CdtB-reactive MAb 20B2. The oligonucleotide primers used for PCR-based amplification of the portion of the *cdtB* gene used in this fusion construct were 5′-GCGGATCCAACTCATCATC ATCCCCACC-3′ and 5′-TCCCCCGGGGCGATCACGAACAAAACTAACA G-3′; the underlined sequences denote *Bam*HI and *Sma*I restriction sites, respectively, used to insert this gene fragment into the pGEX-4T-2 vector (Pharmacia Biotech, Piscataway, N.J.).

Preparation of bacterial culture supernatant fluid for cytotoxicity testing. After overnight growth (16 h), each *E. coli* or *H. ducreyi* culture was subjected to centrifugation at 7,600 × *g* for 15 min to remove bacterial cells and debris, followed by centrifugation at 140,000 × *g* for 2 h to remove membrane fragments. The resultant supernatant fluid was sterilized by filtration through a cellulose acetate filter (0.2-μm pore size) and either used immediately for cytotoxicity testing or stored at −70°C until used. *H. ducreyi* CDT activity in bacterial culture supernatant fluid was stable for 1 month at −70°C.

Cytotoxicity assays. Three different human cell lines were used in cytotoxicity testing of bacterial culture supernatant fluids. HeLa cells (ATCC CRL7923) were grown in Dulbecco's minimal essential medium (GIBCO-BRL, Gaithersburg, Md.) with 10% (vol/vol) fetal bovine serum, 1% (vol/vol) GlutaMAX I (GIBCO), and both penicillin and streptomycin. A spontaneously immortalized human keratinocyte line (HaCaT) that shows normal keratinization and differentiation in vitro (6) was grown in the same medium as the HeLa cells with the addition of 25 mM HEPES buffer. A human foreskin fibroblast line described previously (3) was grown in RPMI 1640 (Mediatech, Herndon, Va.) with 10% (vol/vol) fetal bovine serum, 1 mM sodium pyruvate, 1% (vol/vol) GlutaMAX I, and both penicillin and streptomycin. All three cell lines were incubated in an atmosphere of 95% air–5% CO_2 at 37°C.

HeLa cells were seeded at 2×10^4 cells per well in a 24-well tissue culture plate; the HaCaT keratinocytes and human foreskin fibroblasts were seeded at 6×10^4 cells per well. After overnight incubation of the cells in 1 ml of the appropriate tissue culture medium, a 1-ml portion of filter-sterilized bacterial culture supernatant fluid was added to each well and the plates were incubated for 3 h as described above. Then, the fluid in each well was aspirated and replaced with fresh tissue culture medium. The plates were incubated for 72 to 96 h, at which time each well was photographed with an inverted phase-contrast microscope.

SDS-PAGE, Western blot, and colony blot analyses. Whole-cell lysates were prepared from bacterial cells grown overnight on solidified medium as described previously (22). The bacterial cell suspension used to prepare the whole-cell lysate was adjusted to a density of 300 Klett units with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing, New York, N.Y.). A 10-μl portion of each whole-cell lysate was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins resolved by this method were transferred to nitrocellulose for Western blot analysis (30). Hybridoma culture supernatant fluids were used as the source of MAbs in all experiments. Affinity-purified and radioiodinated goat anti-mouse immunoglobulin was used as the secondary antibody in both Western blot analysis and the colony blot RIA (20).

Nucleotide sequence analysis. A model 373A automated DNA sequencer (Applied Biosystems Inc., Foster City, Calif.) was used to determine the nucleotide sequences of DNAs contained in recombinant plasmids and PCR products. DNA sequences were assembled into larger contiguous sequences and analyzed by using AssemblyLign and MacVector DNA analysis software (version 6.0; Oxford Molecular Group, Campbell, Calif.).

Cloning and mutagenesis of the *H. ducreyi cdtABC* gene cluster. The *Pfu* DNA polymerase system (Stratagene, La Jolla, Calif.) was employed to amplify DNA for use in construction of a plasmid containing the wild-type *H. ducreyi cdtABC* gene cluster. The oligonucleotide primers P1 (5′-GGAATTCCTTGTAGATTA TTCACCGTC-3′) and P2 (5′-CGGAATTCCAATTCCAGTTGATTCACC-3′) (Fig. 1) were used in a PCR to generate a 3-kb DNA product from the *H. ducreyi* 35000 chromosome. This PCR product contained approximately 500 bp of contiguous *H. ducreyi* chromosomal DNA both upstream and downstream of the *cdtABC* gene cluster, together with the restriction site for *Eco*RI at each end (underlined in the oligonucleotide primer sequences). After digestion with *Eco*RI, this PCR-derived product was ligated into the *Eco*RI site in pBR322, and the ligation reaction mixture was used to transform *E. coli* DH5α. After selection with tetracycline, the transformants were screened for reactivity with the *H. ducreyi* CdtC-reactive MAb 9E9. One of the tetracycline-resistant, MAb 9E9-reactive transformants was selected at random, and its recombinant plasmid was designated pJL300.

Plasmid pJL300 was digested with *Bse*RI, which cuts once within the *H. ducreyi cdtC* gene (Fig. 1), and the 1.4-kb chloramphenicol acetyltransferase gene (*cat*) cartridge was excised from the plasmid pUCΔECAT with the restriction enzyme

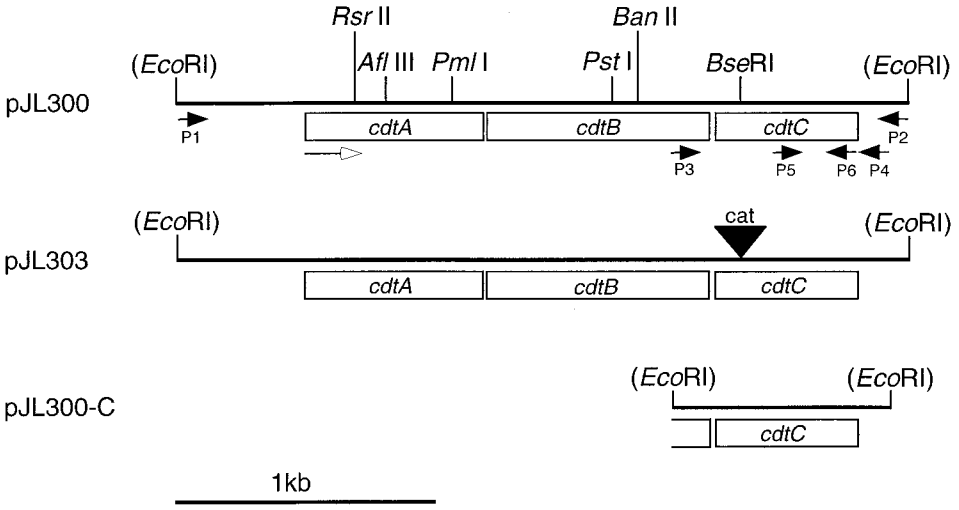

FIG. 1. Partial restriction maps of the *H. ducreyi* 35000 *cdtABC* gene cluster in pJL300 and related constructs. Plasmid pJL300 was constructed by inserting the PCR-derived 3-kb *cdtABC* gene cluster into the *Eco*RI site in pBR322. Plasmid pJL303 was constructed by inserting a *cat* cartridge into the *Bse*RI site in the *cdtC* gene within pJL300. Plasmid pJL300-C was constructed by inserting the 813-bp PCR product containing the *cdtC* open reading frame into the *Eco*RI site within pLS88. Oligonucleotide primers P1 to P6 are indicated and are described in detail in Materials and Methods. The open arrow indicates the direction of transcription. Restriction sites in parentheses are vector-based sites and are not present in *H. ducreyi* chromosomal DNA. A 1-kb size marker is indicated on the lower left.

*Bam*HI. Both of these linear DNA molecules were treated with the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, Mass.) to create blunt ends, and then the *cat* cartridge was blunt-end ligated into the *cdtC* gene. The ligation mixture was used to transform *E. coli* DH5α, and the desired recombinants were selected by growth on LB medium supplemented with chloramphenicol and then screened for lack of reactivity with the *H. ducreyi* CdtC-reactive MAb 9E9 in the colony blot RIA. One of these chloramphenicol-resistant transformants that was unreactive with MAb 9E9 was chosen at random, and its recombinant plasmid was designated pJL303 (Fig. 1).

Construction of the isogenic *H. ducreyi cdtC* mutant. Plasmid pJL303, containing the mutated *cdtC* gene, was used to transform *E. coli* HB101. This plasmid was purified from this recombinant strain by means of the Wizard *Plus* Minipreps DNA Purification System (Promega, Madison, Wis.). Approximately 10 μg of plasmid DNA was digested with *Nru*I. After a phenol-chloroform extraction followed by a chloroform extraction, the linearized plasmid was precipitated by the addition of 1/10 volume of 3.0 M sodium acetate and 2 volumes of 100% ethanol, followed by two washes in 70% ethanol. The washed DNA was dissolved in 10 μl of distilled water and used to electroporate *H. ducreyi* 35000 as described previously (22). Chloramphenicol-resistant transformants were screened in a colony blot RIA for the absence of reactivity with the *H. ducreyi* CdtC-reactive MAb 9E9; one such transformant, designated 35000.303, was selected for further characterization.

Complementation analysis of *E. coli* and *H. ducreyi* strains. DNA containing the *H. ducreyi cdtC* gene was amplified from the *H. ducreyi* 35000 chromosome by PCR with the *Pfu* DNA polymerase system (Stratagene) together with the oligonucleotide primers P3 (5′-GGAATTCTGCACATTTACCACGTAG-3′) and P4 (5′-GGAATTCATCTTACTGCGTCTGCCTGG-3′) (Fig. 1). Both primers contained *Eco*RI sites (underlined). This 813-bp fragment was ligated into the *Eco*RI site in pLS88 (16), and the ligation reaction mixture was used to transform *E. coli* DH5α. Kanamycin-resistant *E. coli* recombinants expressing the *H. ducreyi* CdtC protein were identified by their reactivity with the *H. ducreyi* CdtC-reactive MAb 9E9 in the colony blot RIA. The recombinant plasmid from one of these MAb 9E9-reactive transformants was designated pJL300-C and was used to electroporate both *E. coli* DH5α(pJL303) and the *H. ducreyi cdtC* mutant 35000.303; in both instances, the desired transformants were selected with kanamycin.

Southern blot analysis. Purified chromosomal DNAs from *H. ducreyi* strains were digested to completion with *Pst*I and used in Southern blot analysis as described previously (74). The probe for the *cdtC* gene was obtained by PCR with the oligonucleotide primers P5 (5′-ATGTTTTGCTTTCCTGGG-3′) and P6 (5′-ACCCTGATTTCTTCGCAC-3′) (Fig. 1) to amplify a 269-bp fragment from *H. ducreyi* 35000 chromosomal DNA. The probe for the *cat* cartridge was obtained by using the oligonucleotide primers 5′-CCAGGTTTTCACCGTAACACGC-3′ and 5′-TCCCAATGGCATCGTAAAGAAC-3′ to amplify a 331-bp fragment from this antibiotic resistance gene. Both probes were radiolabeled with [α-^{32}P] dCTP by use of the Random-Primed DNA Labeling Kit (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) and purified on Quick Spin columns (Boehringer).

Virulence testing. The temperature-dependent rabbit model for experimental chancroid was used to determine the relative virulences of the *H. ducreyi* wild-type and mutant strains used in this study (53). These studies were approved by the Institutional Animal Care and Research Advisory Committee. Briefly, eight New Zealand White adult male rabbits were used in this experiment. These animals were housed at a temperature of between 15 and 17°C. All other housing procedures and animal care procedures were performed in accordance with the standards of the U.S. Department of Agriculture and the Association for the Assessment and Accreditation of Laboratory Animal Care International. Ten-fold serial dilutions of the *H. ducreyi* strains were injected intradermally. Each animal was injected with both the wild-type strain and the *cdtC* mutant in this experiment, with one injection of each dilution of the inoculum. Inocula were coded prior to injection to prevent bias in scoring of the resultant lesions. Lesion characteristics were scored as described previously (53), using the following numeric values: 0, no change; 1, erythema; 2, induration; 3, nodule; and 4, pustule or necrosis. Lesion scores were recorded on days 2, 4, and 7 postinfection. On day 7 postinfection, material excised from lesions caused by injection of 10^5 CFU was cultured on CA plates. Statistical analyses were performed as described previously (65, 66).

RESULTS

Production of MAbs reactive with the protein products of the *H. ducreyi cdtABC* gene cluster. To provide reagents necessary to confirm the isogenic nature of the desired *H. ducreyi cdtC* mutant, MAbs to the *H. ducreyi* CdtA, CdtB, and CdtC proteins were produced as described in Materials and Methods. It should be noted that the predicted sizes of the putative mature forms of the *H. ducreyi* CdtA, CdtB, and CdtC proteins are 23, 29, and 19 kDa, respectively (13).

In Western blot analysis, MAb 1G8, raised against the His-CdtA fusion protein, bound a 24-kDa antigen in whole-cell lysates of the recombinant *E. coli* strain DH5α(pJL300) (Fig. 2A, lane 2), which contained the entire *H. ducreyi* 35000 *cdtABC* gene cluster. Repeated attempts to generate an *H. ducreyi* CdtB-reactive MAb by immunizing with a His-CdtB fusion protein (13) were unsuccessful. The use of an immunogen consisting of a CdtB-derived peptide coupled to keyhole limpet hemocyanin eventually yielded MAb 20B2, which bound a 30-kDa antigen expressed by the recombinant *E. coli* strain DH5α(pJL300) (Fig. 2B, lane 2). MAb 8C9, raised against the His-CdtC fusion protein, bound to a 20-kDa antigen expressed by *E. coli* DH5α(pJL300) (Fig. 2C, lane 2). None of these MAbs bound any antigens in the *E. coli* strain containing only the vector pBR322 (Fig. 2, lanes 1).

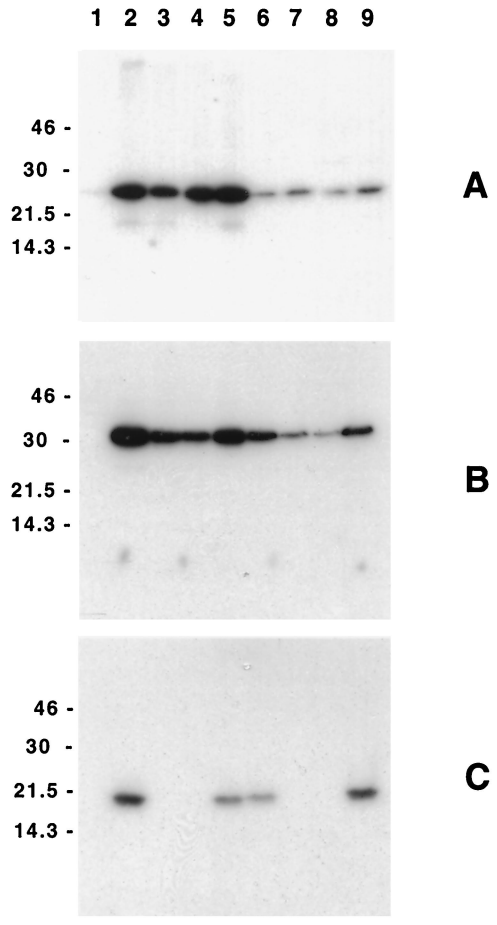

FIG. 2. Western blot analysis of the *H. ducreyi* CdtABC proteins expressed by selected *E. coli* and *H. ducreyi* strains. Whole-cell lysates of these strains were probed with the *H. ducreyi* CdtA-reactive MAb 1G8 (A), the *H. ducreyi* CdtB-reactive MAb 20B2 (B), and the *H. ducreyi* CdtC-reactive MAb 8C9 (C). Lanes: 1, *E. coli* DH5α(pBR322); 2, *E. coli* DH5α(pJL300); 3, *E. coli* DH5α(pJL303); 4, *E. coli* DH5α(pJL303)(pLS88); 5, *E. coli* DH5α(pJL303)(pJL300-C); 6, wild-type *H. ducreyi* 35000; 7, *H. ducreyi cdtC* mutant 35000.303; 8, *H. ducreyi* 35000.303 (pLS88); 9, *H. ducreyi* 35000.303(pJL300-C). Molecular size markers (in kilodaltons) are listed on the left of each panel.

The CdtA-directed MAb 1G8, the CdtB-directed MAb 20B2, and the CdtC-directed MAb 8C9 were shown to be reactive with the homologous proteins produced by seven different *H. ducreyi* strains (35000, Hd12, Cha-1, 541, 041, 135, and Hd13) (66) isolated in six different geographic locations (data not shown). This finding reinforced the likelihood that the CdtABC proteins are fairly well conserved among strains of *H. ducreyi* (13). These three MAbs did not bind to any proteins expressed by *H. ducreyi* 512, which has previously been shown to lack the *cdtABC* gene cluster (13).

***H. ducreyi* CDT readily kills HeLa cells and HaCaT keratinocytes.** Recombinant *H. ducreyi* CDT was previously shown to kill HeLa cells in culture (13), and therefore these epithelial cells were included in these experiments as a positive control for cytotoxic activity. Culture supernatant fluid from *E. coli* DH5α(pJL300) was used as the source of *H. ducreyi* CDT for cytotoxicity testing that involved two different types of human cells potentially relevant to the infectious process in chancroid: keratinocytes (i.e., the HaCaT cell line) and human foreskin fibroblasts. The recombinant form of *H. ducreyi* CDT readily killed both HeLa cells (Fig. 3B) and HaCaT keratinocytes (Fig. 3G). Recombinant *H. ducreyi* CDT had only a modest effect on human foreskin fibroblasts as evidenced by an inhibition of growth (Fig. 3L). Culture supernatant fluid from a recombinant *E. coli* strain containing only the plasmid vector pBR322 had no effect on any of these three cell lines (Fig. 3A, F, and K).

Inactivation of the *H. ducreyi cdtC* gene in *E. coli*. Antibody against the *H. ducreyi* CdtC protein was previously shown to neutralize the activity of *H. ducreyi* CDT (13). To confirm the involvement of the *H. ducreyi* CdtC protein in CDT activity, the *cdtC* gene within the cloned *H. ducreyi cdtABC* gene cluster was inactivated by insertional mutagenesis. Specifically, the *cdtC* gene in pJL300 was disrupted by insertion of a *cat* cartridge into the *Bse*RI site within this open reading frame, yielding the mutated plasmid pJL303 (Fig. 1). Western blot analysis of whole-cell lysates of the recombinant *E. coli* strain DH5α(pJL303) indicated that the mutated *H. ducreyi cdtABC* gene cluster did not express any CdtC protein detectable by Western blot analysis (Fig. 2C, lane 3). *E. coli* DH5α(pJL303) still expressed both the *cdtA* gene product (Fig. 2A, lane 3) and the *cdtB* gene product (Fig. 2B, lane 3), although the level of CdtB appeared to be somewhat reduced.

This insertion of the *cat* cartridge into the *H. ducreyi cdtC* gene eliminated the ability of culture supernatant fluid from the recombinant *E. coli* strain to kill HeLa cells (Fig. 3C) and HaCaT keratinocytes (Fig. 3H). This mutation also eliminated the modest inhibitory effect of the recombinant *H. ducreyi* CDT on human foreskin fibroblasts (Fig. 3M).

Construction of an isogenic *H. ducreyi cdtC* mutant. Plasmid pJL303, containing the mutated *cdtC* gene, was linearized and used to electroporate the wild-type *H. ducreyi* strain 35000. A chloramphenicol-resistant transformant, 35000.303, was shown to be unable to express the CdtC protein as determined by Western blot analysis (Fig. 2C, lane 7). In contrast, the CdtC protein expressed by the wild-type parent strain (Fig. 2C, lane 6) was readily detected in this system. This mutant strain also expressed CdtA (Fig. 2A, lane 7) and CdtB (Fig. 2B, lane 7); again, the level of CdtB appeared to be somewhat less than that expressed by the wild-type parent strain (Fig. 2B, lane 6). To eliminate the possibility that an undetected secondary mutation had occurred in or near the *cdtABC* gene cluster during insertion of the *cat* cartridge or during allelic exchange, the entire *cdtABC* gene cluster containing the *cat* cartridge and immediately flanking DNA was amplified from this mutant strain by PCR and subjected to nucleotide sequence analysis to confirm that there were no other mutations in this DNA region.

Southern blot analysis was used to confirm that *H. ducreyi* 35000.303 had only a single *cat* cartridge insertion in its chromosome. When chromosomal DNA from the wild-type parent strain 35000 was probed with a 269-bp fragment derived by PCR from the *H. ducreyi cdtC* gene (Fig. 1), a 4.5-kb *Pst*I fragment hybridized to this probe (Fig. 4A, lane 1). This same probe hybridized to a 6-kb *Pst*I fragment of chromosomal DNA from 35000.303 (Fig. 4A, lane 2). This result is consistent with the replacement, in strain 35000.303, of the wild-type *cdtC* gene with the mutated allele containing the 1.4-kb *cat* cartridge. When a 331-bp fragment derived from the *cat* cartridge was used as the probe, a 6-kb *Pst*I fragment from 35000.303 also hybridized to this *cat* probe (Fig. 4B, lane 2). Chromosomal DNA from the wild-type parent strain 35000 did not hybridize to the *cat* probe (Fig. 4B, lane 1).

Culture supernatant fluid from the wild-type *H. ducreyi* strain 35000 readily killed both HeLa cells (Fig. 5A) and HaCaT keratinocytes (Fig. 5E) and had a modest inhibitory effect on human foreskin fibroblasts (Fig. 5I). In contrast, culture

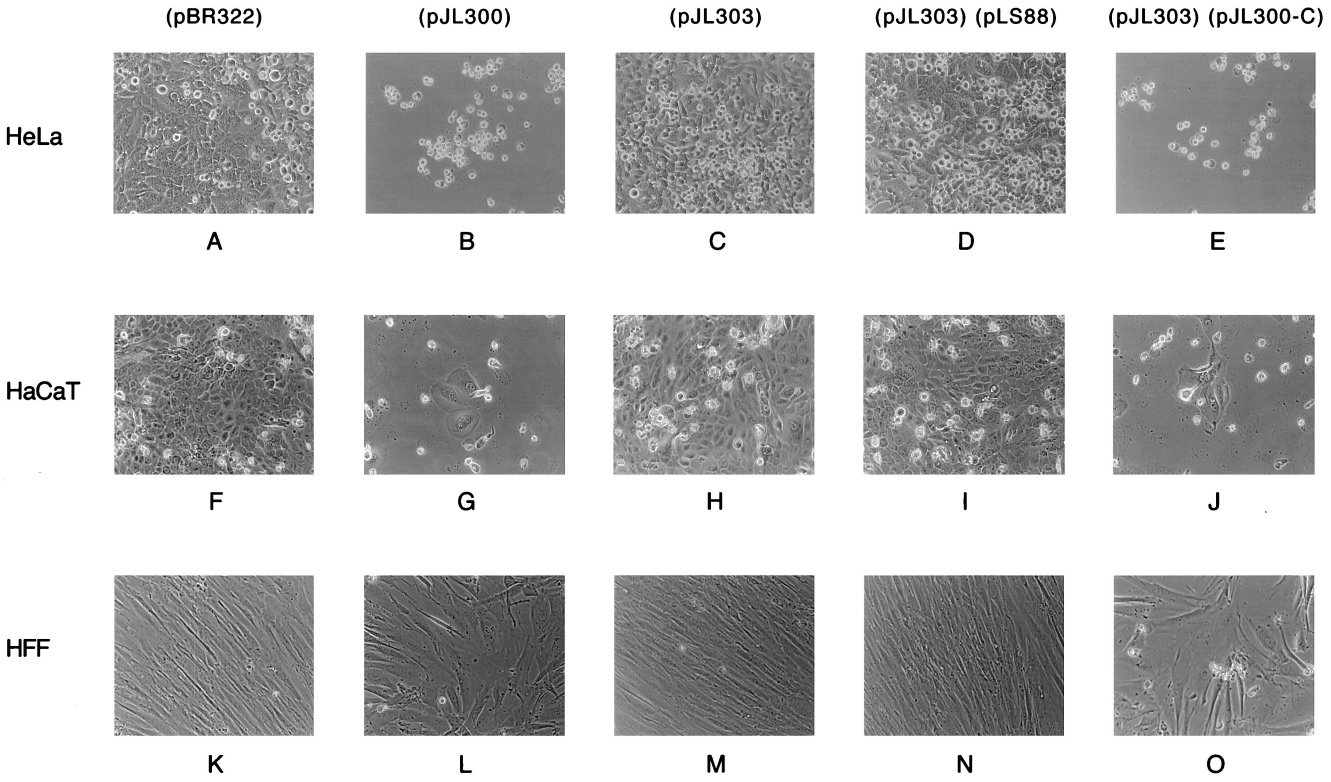

FIG. 3. CDT activity in culture supernatant fluids from recombinant strains of *E. coli*. HeLa cells (A to E), HaCaT keratinocytes (F to J), and human foreskin fibroblasts (HFF) (K to O) were exposed to filter-sterilized *E. coli* culture supernatant fluids as described in Materials and Methods and were photographed 72 h later (HeLa cells) or 96 h later (HaCaT cells and human foreskin fibroblasts). (A, F, and K) *E. coli* DH5α(pBR322); (B, G, and L) *E. coli* DH5α(pJL300); (C, H, and M) *E. coli* DH5α(pJL303); (D, I, and N) *E. coli* DH5α(pJL303)(pLS88); (E, J, and O) *E. coli* DH5α(pJL303)(pJL300-C). Magnification, ×37.

supernatant fluid from the *H. ducreyi cdtC* mutant 35000.303 did not kill either HeLa cells (Fig. 5B) or HaCaT keratinocytes (Fig. 5F) and also did not inhibit growth of human foreskin fibroblasts (Fig. 5J).

Complementation of the *cdtC* mutant. Complementation analysis was used to eliminate the possibility that an unlinked secondary mutation was responsible for the lack of cytotoxicity observed with the *H. ducreyi cdtC* mutant 35000.303. The *cdtC* open reading frame together with a small amount of flanking DNA was amplified by PCR from the chromosome of *H. ducreyi* 35000 and cloned into the shuttle vector pLS88 to obtain pJL300-C (Fig. 1). When introduced into the recombinant *E. coli* strain DH5α(pJL303) containing the mutated *cdtC* gene, pJL300-C restored both expression of the CdtC protein (Fig. 2C, lane 5) and cytotoxicity of this strain for HeLa cells (Fig. 3E) and HaCaT keratinocytes (Fig. 3J). Culture supernatant fluid from *E. coli* DH5α(pJL303)(pJL300-C) also inhibited the growth of human foreskin fibroblasts (Fig. 3O). The presence of only the pLS88 vector in this same recombinant *E. coli* strain [DH5α(pJL303)] (Fig. 2, lanes 4, and Fig. 3D, I, and N) did not affect the phenotype of this strain in the relevant test systems.

When pJL300-C was introduced into the isogenic *H. ducreyi cdtC* mutant 35000.303 (Fig. 2C, lane 9), the presence of the wild-type *H. ducreyi cdtC* gene in *trans* restored expression of the CdtC protein. The presence of the pLS88 vector alone in this mutant had no detectable effect on its inability to express the CdtC protein (Fig. 2C, lane 8). Complementation with pJL300-C restored the ability of this *H. ducreyi cdtC* mutant to kill both HeLa cells (Fig. 5D) and HaCaT keratinocytes (Fig. 5H) and to inhibit the growth of human foreskin fibroblasts (Fig. 5L). The presence of only the pLS88 vector in this *H. ducreyi* mutant did not restore cytotoxicity for HeLa cells (Fig. 5C) and HaCaT keratinocytes (Fig. 5G).

Southern blot analysis confirmed that the wild-type *cdtC* gene in the complemented *cdtC* mutant was still present in the plasmid (Fig. 4). A chromosomal DNA preparation from 35000.303(pJL300-C) was digested with *Pst*I, which cuts

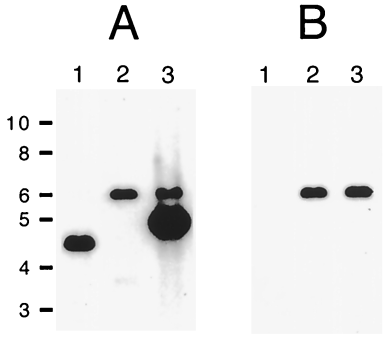

FIG. 4. Southern blot analysis of chromosomal DNA preparations from *H. ducreyi* wild-type, mutant, and recombinant strains. Chromosomal DNAs were digested with *Pst*I, resolved by agarose gel electrophoresis, and probed with either a 269-bp PCR product derived from the *H. ducreyi cdtC* gene (A) or a 331-bp PCR product derived from the *cat* cartridge (B). Lanes 1, strain 35000; lanes 2, *cdtC* mutant 35000.303; lanes 3, 35000.303(pJL300-C). Size markers (in kilobases) are on the left.

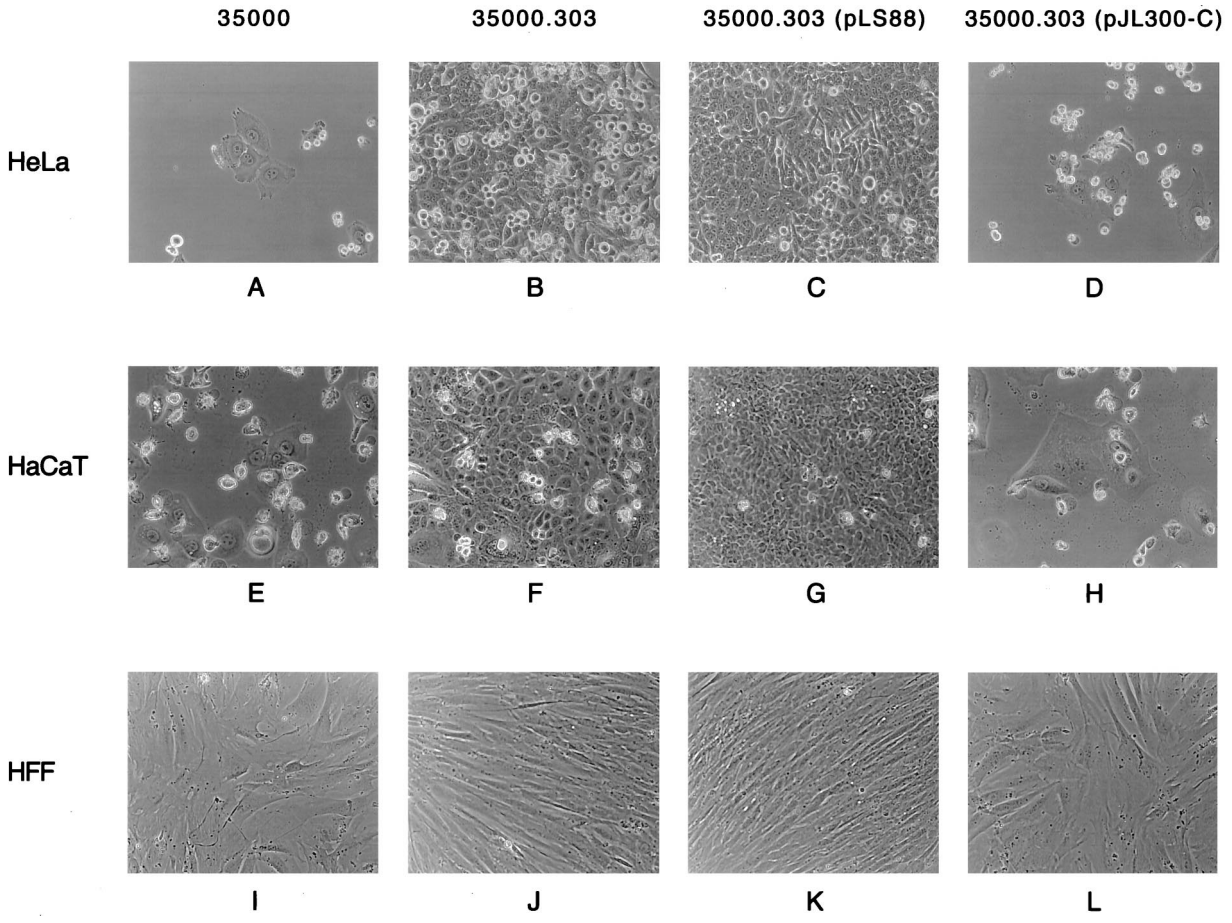

FIG. 5. CDT activity in culture supernatant fluids from wild-type, mutant, and recombinant strains of *H. ducreyi*. HeLa cells (A to D), HaCaT keratinocytes (E to H), and human foreskin fibroblasts (HFF) (I to L) were exposed to filter-sterilized *H. ducreyi* culture supernatant fluids as described in Materials and Methods and were photographed 72 h later (HeLa cells) or 96 h later (HaCaT cells and human foreskin fibroblasts). (A, E, and I) wild-type *H. ducreyi* 35000; (B, F, and J) *H. ducreyi cdtC* mutant 35000.303; (C, G, and K) *H. ducreyi* 35000.303(pLS88); (D, H, and L) *H. ducreyi* 35000.303(pJL300-C). Magnification, ×42.

pJL300-C twice, yielding a 4.8-kb fragment containing the cloned *H. ducreyi cdtC* gene and a 0.8-kb fragment (data not shown). This 4.8-kb plasmid fragment bound the *cdtC* probe, as did the 6-kb *Pst*I chromosomal fragment containing the mutated *cdtC* gene (Fig. 4A, lane 3). The *cat* probe bound only to the 6-kb *Pst*I chromosomal fragment from this complemented mutant strain (Fig. 4B, lane 3).

Effect of the *cdtC* mutation on virulence of *H. ducreyi*. The isogenic *cdtC* mutant strain 35000.303 was compared to the wild-type parent strain for its ability to produce lesions in the temperature-dependent rabbit model (53). In an experiment involving eight animals, the *cdtC* mutant proved to be as virulent as the wild-type parent strain with regard to lesion production (Table 2). In addition, in seven of eight animals, viable *H. ducreyi* cells were isolated from the lesions resulting from the injection of 10^5 CFU for both of the strains (data not shown). The numbers of CFU of the wild-type strain and the *cdtC* mutant recovered from the infected animals were equivalent (data not shown). Expression of *H. ducreyi* CDT in vivo was confirmed by Western blot-based analysis of serum obtained from rabbits that had been infected several times with viable *H. ducreyi* 35000; this serum contained antibodies directed against the *H. ducreyi* CdtC protein (data not shown).

DISCUSSION

CDT was originally identified by Johnson and Lior (26, 28, 29), in studies of enteric pathogens, as the soluble factor in bacterial culture supernatant fluids which caused the appearance of giant elongated Chinese hamster ovary (CHO) cells after prolonged incubation. Subsequent studies from their and

TABLE 2. Lesion formation by the wild-type *H. ducreyi* strain 35000 and the *cdtC* mutant strain 35000.303 in the temperature-dependent rabbit model[a]

Strain	Inoculum size (CFU)	Mean lesion score (SD) on day:			*P* value[b]
		2	4	7	
35000	10^5	3.75 (0.46)	3.88 (0.35)	3.88 (0.35)	
35000.303	10^5	4.00 (0)	3.88 (0.35)	3.88 (0.35)	0.104
35000	10^4	3.00 (0)	3.63 (0.52)	3.75 (0.46)	
35000.303	10^4	3.13 (0.35)	3.63 (0.52)	3.88 (0.35)	

[a] Eight rabbits were used in this experiment.
[b] *P* value calculated for the difference between wild-type and test strain lesion scores. *P* values were calculated by using the lesion scores from both inoculum sizes and from all 3 days.

other laboratories revealed that CDT is a novel toxic activity released by some *E. coli* strains (26, 51, 62) and *Shigella* isolates (27, 42) and by many *Campylobacter* species (28, 40, 52). It has now been shown that CDT causes elongation followed by progressive cellular distention and cytotoxicity with certain mammalian cell lines (i.e., CHO, HeLa, HEp-2, and Vero) in vitro (29). Moreover, recent studies using HeLa cells have shown that *E. coli* CDT blocks the cell cycle at the G_2/M transition, apparently by preventing Cdc2 protein kinase dephosphorylation and activation (12, 50, 75). In addition, *E. coli* CDT affects F-actin assembly by CHO cells in culture (4).

To date, the *cdtABC* gene clusters from a number of enteric pathogens have been sequenced. These include three *E. coli* strains (50, 51, 62), a *Shigella dysenteriae* strain (42), and one strain of *Campylobacter jejuni* (52, 75). The exact in vivo function of the enteric CDT proteins has not been determined, and a primary role for CDT in the pathogenesis of disease caused by these enteric pathogens has not been established (1). However, in one recent study using a suckling mouse model, recombinant CDT from *S. dysenteriae* was reported to be diarrheagenic (41).

Despite the recent identification of CDT homologs in numerous different organisms, including, most recently, *Actinobacillus actinomycetemcomitans* (36, 67), information on the exact nature of the CDT cytotoxic moiety remains limited. It has been reported that both *A. actinomycetemcomitans* CDT and *H. ducreyi* CDT, similar to that of *E. coli*, induce cell cycle arrest in the G_2 phase (14, 67). At least in *H. ducreyi*, CDT activity in culture supernatant fluid was shown to involve a soluble protein with an apparent molecular mass in SDS-PAGE of approximately 20 kDa and an N-terminal amino acid sequence identical to that contained in the proposed mature form of the *H. ducreyi* CdtC protein (13, 55). Antibody to the *H. ducreyi* CdtC protein was shown to neutralize CDT activity in *H. ducreyi* culture supernatant fluid (13), and a similar result was reported recently for polyclonal antibody reactive with the CdtC protein expressed by *A. actinomycetemcomitans* (67). The last study also indicated that polyclonal antibody to the CdtA protein had a modest blocking effect on CDT activity (67).

It can be inferred from the accumulated data that at least the CdtC protein is involved, directly or indirectly, in the ability of *H. ducreyi* CDT to kill certain types of human cells. The functions of the CdtA and CdtB proteins remain to be determined, although it appears that all three gene products (i.e., CdtA, CdtB, and CdtC) must be expressed by the pathogen for CDT to be active in culture supernatant fluid (51, 52, 62, 67). All three proteins encoded by the *H. ducreyi cdtABC* gene cluster appear to have signal peptides, although the existence of a signal peptide has been confirmed only for the CdtC protein of *H. ducreyi* (13, 55).

Construction of an isogenic *H. ducreyi cdtC* mutant in the present study allowed us to confirm that CDT was responsible for the observed killing of HeLa cells by cell-free *H. ducreyi* culture supernatant fluids (13, 56). It had been previously shown that rapid killing of human foreskin fibroblasts by *H. ducreyi* required contact between the human cells and the bacterium (25). This contact-dependent killing of human foreskin fibroblasts was later shown to be the result of the activity of the HhdA hemolysin of *H. ducreyi*. Isogenic hemolysin-deficient mutants were unable to kill human foreskin fibroblasts in vitro when cocultivated with these human cells (2, 43) and had a reduced ability to kill human keratinocytes (i.e., HaCaT cells) (43). However, the isogenic hemolysin-deficient mutant still killed HeLa cells (43), a finding which indicated that this hemolysin was not the cytotoxic factor responsible for the killing of HeLa cells by *H. ducreyi*.

Culture supernatant fluid from the recombinant *E. coli* strain DH5α(pJL300) containing the intact *H. ducreyi cdtABC* gene cluster readily killed HaCaT keratinocytes (Fig. 3G). More importantly, culture supernatant fluid from the wild-type *H. ducreyi* strain killed these keratinocytes (Fig. 5E), whereas culture supernatant fluid from the isogenic *H. ducreyi cdtC* mutant had no apparent effect on these human cells (Fig. 5F). These results indicate that the soluble *H. ducreyi* CDT can kill keratinocytes, or at least the HaCaT keratinocyte cell line. It should be noted that the HhdA hemolysin of *H. ducreyi* also can affect keratinocytes as evidenced by the fact that a mutation which abolished hemolysin expression reduced but did not eliminate the ability of *H. ducreyi* cells to kill HaCaT cells during cocultivation (43). Moreover, the results of the present study indicate that the reduced killing of keratinocytes obtained with the hemolysin-deficient *H. ducreyi* mutant (43) was likely the result of CDT activity.

The cumulative results of cytotoxicity testing with culture supernatant fluids from the recombinant *E. coli* strain containing the *H. ducreyi cdtABC* gene cluster (Fig. 3L), the recombinant *E. coli* strain containing the *cat* cartridge insertion (Fig. 3M), and the wild-type *H. ducreyi* strain (Fig. 5I) and its isogenic *cdtC* mutant (Fig. 5J) indicated that *H. ducreyi* CDT can exert a negative albeit modest effect on human foreskin fibroblasts in culture. This effect was evidenced by an apparent inhibition of growth caused by CDT (Fig. 3L and 5I). Additional experiments in which decreasing numbers of human foreskin fibroblasts were exposed to culture supernatant fluid containing recombinant *H. ducreyi* CDT reinforced the impression that CDT exerted an inhibitory rather than a cytotoxic effect on these cells (35). These data are not in conflict with the reported inability of hemolysin-deficient *H. ducreyi* mutants to kill human foreskin fibroblasts in culture (2, 43). The cytotoxicity assays in the latter studies were terminated after only 24 to 30 h of incubation; this period of time is much too short to allow the CDT activity expressed by the hemolysin-deficient *H. ducreyi* mutant to be detected with human foreskin fibroblasts as the target cell line.

Interestingly, when the *H. ducreyi cdtC* mutant was tested in the temperature-dependent rabbit model for its ability to produce dermal lesions, this mutant proved to have a level of virulence expression that was not distinguishable from that of its wild-type parent strain. Other isogenic *H. ducreyi* mutants tested previously in this model, including both a *hupA* (*hgbA*) mutant unable to utilize hemoglobin (66) and a *gmhA* mutant expressing a drastically truncated lipooligosaccharide molecule (5), exhibited a reduced ability to form lesions. In these two instances, a reduction in virulence was correlated with a reduced ability to survive in vivo, as evidenced by the fact that viable mutant *H. ducreyi* could not be recovered from the lesions of these animals (5, 66). Attempts to recover viable *H. ducreyi* from lesions resulting from inoculation with the *cdtC* mutant revealed that this mutant apparently was able to survive in vivo as well as the wild-type parent strain (data not shown). These results suggest that, at least in this rabbit model, the ability of an *H. ducreyi* mutant to form dermal lesions may be an indication of its ability to survive and replicate in vivo. If this is the case, then elaboration of CDT, which could be involved in the development of ulcers, is not essential to survival of *H. ducreyi* in this rabbit model.

One limitation of the rabbit model, in contrast to naturally acquired *H. ducreyi* infection, is that the intradermal method of inoculation bypasses the keratinocytes (against which *H. ducreyi* CDT is relatively active) and introduces the bacteria into the dermis, where they initially interact with fibroblasts (against which *H. ducreyi* CDT is not very active). This artificial

method of inoculation may protect keratinocytes from the action of CDT. However, *H. ducreyi* CDT is apparently soluble in vitro, which should allow this diffusible toxin access to the nearby epidermal keratinocytes. This exposure of keratinocytes to *H. ducreyi* CDT could also be facilitated by the breakdown in the basement membrane that occurs with lesion formation.

It is also possible that lesion development caused by *H. ducreyi* in rabbits simply may not involve CDT. Efforts to determine the sensitivities of various rabbit cells to *H. ducreyi* CDT have been initiated in this laboratory. In view of the demonstrated cytotoxic effect of *H. ducreyi* CDT on several different human cell lines, the possible involvement of this cytotoxin in the development of lesions in the experimental human challenge model will have to be addressed in the future (46, 47, 63, 64).

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REFERENCES

1. **Albert, M. J., S. M. Faruque, A. S. G. Faruque, K. A. Bettelheim, P. K. B. Neogi, N. A. Bhuiyan, and J. B. Kaper.** 1996. Controlled study of cytolethal distending toxin-producing *Escherichia coli* infections in Bangladeshi children. J. Clin. Microbiol. **34:**717–719.
2. **Alfa, M. J., P. Degagne, and P. A. Totten.** 1996. *Haemophilus ducreyi* hemolysin acts as a contact cytotoxin and damages human foreskin fibroblasts in cell culture. Infect. Immun. **64:**2349–2352.
3. **Alfa, M. J., M. K. Stevens, P. Degagne, J. Klesney-Tait, J. D. Radolf, and E. J. Hansen.** 1995. Use of tissue culture and animal models to identify virulence-associated traits of *Haemophilus ducreyi*. Infect. Immun. **63:**1754–1761.
4. **Aragon, V., K. Chao, and L. A. Dreyfus.** 1997. Effect of cytolethal distending toxin on F-actin assembly and cell division in Chinese hamster ovary cells. Infect. Immun. **65:**3774–3780.
5. **Bauer, B. A., M. K. Stevens, and E. J. Hansen.** 1998. Involvement of the *Haemophilus ducreyi gmhA* gene product in lipooligosaccharide expression and virulence. Infect. Immun. **66:**4290–4298.
6. **Boukamp, P., R. T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, and N. E. Fusenig.** 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. **106:**761–771.
7. **Brentjens, R. J., M. Ketterer, M. A. Apicella, and S. M. Spinola.** 1996. Fine tangled pili expressed by *Haemophilus ducreyi* are a novel class of pili. J. Bacteriol. **178:**808–816.
8. **Brentjens, R. J., S. M. Spinola, and A. A. Campagnari.** 1994. *Haemophilus ducreyi* adheres to human keratinocytes. Microb. Pathog. **16:**243–247.
9. **Campagnari, A. A., R. Karalus, M. A. Apicella, W. Melaugh, A. J. Lesse, and B. W. Gibson.** 1994. Use of pyocin to select a *Haemophilus ducreyi* variant defective in lipooligosaccharide biosynthesis. Infect. Immun. **62:**2379–2386.
10. **Campagnari, A. A., L. M. Wild, G. E. Griffiths, R. J. Karalus, M. A. Wirth, and S. M. Spinola.** 1991. Role of lipopolysaccharides in experimental dermal lesions caused by *Haemophilus ducreyi*. Infect. Immun. **59:**2601–2608.
11. **Carson, S. D. B., C. E. Thomas, and C. Elkins.** 1996. Cloning and sequencing of a *Haemophilus ducreyi fur* homolog. Gene **176:**125–129.
12. **Comayras, C., C. Tasca, S. Y. Peres, B. Ducommun, E. Oswald, and J. De Rycke.** 1997. *Escherichia coli* cytolethal distending toxin blocks HeLa cell cycle at the G_2/M transition by preventing Cdc2 protein kinase dephosphorylation and activation. Infect. Immun. **65:**5088–5095.
13. **Cope, L. D., S. Lumbley, J. L. Latimer, J. Klesney-Tait, M. K. Stevens, L. S. Johnson, M. Purven, R. S. Munson, Jr., T. Lagergard, J. D. Radolf, and E. J. Hansen.** 1997. A diffusible cytotoxin of *Haemophilus ducreyi*. Proc. Natl. Acad. Sci. USA **94:**4056–4061.
14. **Cortes-Bratti, X., E. Chaves-Olarte, T. Lagergard, and M. Thelestam.** 1999. The cytolethal distending toxin from chancroid bacterium *Haemophilus ducreyi* induces cell-cycle arrest in the G2 phase. J. Clin. Invest. **103:**107–115.
15. **DiCarlo, R. P., B. S. Armentor, and D. H. Martin.** 1995. Chancroid epidemiology in New Orleans men. J. Infect. Dis. **172:**446–452.
16. **Dixon, L. G., W. L. Albritton, and P. J. Willson.** 1994. An analysis of the complete nucleotide sequence of the *Haemophilus ducreyi* broad-host-range plasmid pLS88. Plasmid **32:**228–232.
17. **Elkins, C., C.-J. Chen, and C. E. Thomas.** 1995. Characterization of the *hgbA* locus encoding a hemoglobin receptor from *Haemophilus ducreyi*. Infect. Immun. **63:**2194–2200.
18. **Frisk, A., C. A. Ison, and T. Lagergard.** 1998. GroEL heat shock protein of *Haemophilus ducreyi*: association with cell surface and capacity to bind to eukaryotic cells. Infect. Immun. **66:**1252–1257.
19. **Gibson, B. W., A. A. Campagnari, W. Melaugh, N. J. Phillips, M. A. Apicella, S. Grass, J. Wang, K. L. Palmer, and R. S. Munson, Jr.** 1997. Characterization of a transposon Tn*916*-generated mutant of *Haemophilus ducreyi* 35000 defective in lipooligosaccharide biosynthesis. J. Bacteriol. **179:**5062–5071.
20. **Gulig, P. A., C. C. Patrick, L. Hermanstorfer, G. H. McCracken, Jr., and E. J. Hansen.** 1987. Conservation of epitopes in the oligosaccharide portion of the lipooligosaccharide of *Haemophilus influenzae* type b. Infect. Immun. **55:**513–520.
21. **Hammond, G. W., C. J. Lian, J. C. Wilt, and A. R. Ronald.** 1978. Antimicrobial susceptibility of *Haemophilus ducreyi*. Antimicrob. Agents Chemother. **13:**608–612.
22. **Hansen, E. J., J. L. Latimer, S. E. Thomas, M. E. Helminen, W. L. Albritton, and J. D. Radolf.** 1992. Use of electroporation to construct isogenic mutants of *Haemophilus ducreyi*. J. Bacteriol. **174:**5442–5449.
23. **Hobbs, M. M., T. R. Paul, P. B. Wyrick, and T. H. Kawula.** 1998. *Haemophilus ducreyi* infection causes basal keratinocyte cytotoxicity and elicits a unique cytokine induction pattern in an in vitro human skin model. Infect. Immun. **66:**2914–2921.
24. **Hobbs, M. M., L. R. San Mateo, P. E. Orndorff, G. Almond, and T. H. Kawula.** 1995. Swine model of *Haemophilus ducreyi* infection. Infect. Immun. **63:**3094–3100.
25. **Hollyer, T. T., P. A. DeGagne, and M. J. Alfa.** 1994. Characterization of the cytopathic effect of *Haemophilus ducreyi*. Sex. Transm. Infect. **21:**247–257.
26. **Johnson, W. M., and H. Lior.** 1987. Response of Chinese hamster ovary cells to a cytolethal toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat-labile (LT) enterotoxin. FEMS Microbiol. Lett. **43:**19–23.
27. **Johnson, W. M., and H. Lior.** 1987. Production of Shiga toxin and a cytolethal distending toxin (CLDT) by serogroups of *Shigella* ssp. FEMS Microbiol. Lett. **48:**235–238.
28. **Johnson, W. M., and H. Lior.** 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. Microb. Pathog. **4:**115–126.
29. **Johnson, W. M., and H. Lior.** 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by *Escherichia coli* isolates from clinical material. Microb. Pathog. **4:**103–113.
30. **Kimura, A., P. A. Gulig, G. H. McCracken, Jr., T. A. Loftus, and E. J. Hansen.** 1985. A minor high-molecular-weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. Infect. Immun. **47:**253–259.
31. **Lagergard, T.** 1992. The role of *Haemophilus ducreyi* bacteria, cytotoxin, endotoxin and antibodies in animal models for study of chancroid. Microb. Pathog. **13:**203–217.
32. **Lagergard, T., and M. Purven.** 1993. Neutralizing antibodies to *Haemophilus ducreyi* cytotoxin. Infect. Immun. **61:**1589–1592.
33. **Lammel, C. J., N. P. Dekker, J. Palefsky, and G. F. Brooks.** 1993. In vitro model of *Haemophilus ducreyi* adherence to and entry into eukaryotic cells of genital origin. J. Infect. Dis. **167:**642–650.
34. **Langford, P. R., and J. S. Kroll.** 1997. Distribution, cloning, characterization and mutagenesis of *sodC*, the gene encoding copper/zinc superoxide dismutase, a potential determinant of virulence in *Haemophilus ducreyi*. FEMS Immunol. Med. Microbiol. **17:**235–242.
35. **Lumbley, S. R., J. L. Latimer, and E. J. Hansen.** 1998. *Haemophilus ducreyi* cytolethal distending toxin kills human keratinocytes but not human foreskin fibroblasts, abstr. B-288, p. 104. *In* Abstracts of the 98th General Meeting of the American Society for Microbiology 1998. American Society for Microbiology, Washington, D.C.
36. **Mayer, M. P. A., L. C. Bueno, E. J. Hansen, and J. M. DiRienzo.** 1999. Identification of a cytolethal distending toxin gene locus and features of a virulence-associated region in *Actinobacillus actinomycetemcomitans*. Infect. Immun. **67:**1227–1237.
37. **Melaugh, W., A. A. Campagnari, and B. W. Gibson.** 1996. The lipooligosaccharides of *Haemophilus ducreyi* are highly sialylated. J. Bacteriol. **178:**564–570.
38. **Melaugh, W., N. J. Phillips, A. A. Campagnari, M. V. Tullius, and B. W. Gibson.** 1994. Structure of the major oligosaccharide from the lipooligosaccharide of *Haemophilus ducreyi* strain 35000 and evidence of additional glycoforms. Biochemistry **33:**13070–13078.
39. **Mertz, K. J., J. B. Weiss, R. M. Webb, W. C. Levine, J. S. Lewis, K. A. Orle, P. A. Totten, J. Overbaugh, S. A. Morse, M. M. Currier, M. Fishbein, and M. E. St. Louis.** 1998. An investigation of genital ulcers in Jackson, Mississippi, with use of a multiplex polymerase chain reaction assay: high prevalence of chancroid and human immunodeficiency virus infection. J. Infect. Dis. **178:**1060–1066.
40. **Ohya, T., K. Tominaga, and M. Nakazawa.** 1993. Production of cytolethal distending toxin (CLDT) by *Campylobacter fetus* subsp. *fetus* isolated from

calves. J. Vet. Med. Sci. **55:**507–509.
41. **Okuda, J., M. Fukumoto, Y. Takeda, and M. Nishibuchi.** 1997. Examination of diarrheagenicity of cytolethal distending toxin: suckling mouse response to the products of the *cdtABC* genes of *Shigella dysenteriae*. Infect. Immun. **65:**428–433.
42. **Okuda, J., H. Kurazono, and Y. Takeda.** 1995. 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. **18:**167–172.
43. **Palmer, K. L., W. E. Goldman, and R. S. Munson, Jr.** 1996. An isogenic haemolysin-deficient mutant of *Haemophilus ducreyi* lacks the ability to produce cytopathic effects on human foreskin fibroblasts. Mol. Microbiol. **21:** 13–19.
44. **Palmer, K. L., S. Grass, and R. S. Munson, Jr.** 1994. Identification of a hemolytic activity elaborated by *Haemophilus ducreyi*. Infect. Immun. **62:** 3041–3043.
45. **Palmer, K. L., and R. S. Munson, Jr.** 1995. Cloning and characterization of the genes encoding the haemolysin of *Haemophilus ducreyi*. Mol. Microbiol. **18:**821–830.
46. **Palmer, K. L., C. T. Schnizlein-Bick, A. Orazi, K. John, C. Y. Chen, A. F. Hood, and S. M. Spinola.** 1998. The immune response to *Haemophilus ducreyi* resembles a delayed-type hypersensitivity reaction throughout experimental infection of human subjects. J. Infect. Dis. **178:**1688–1697.
47. **Palmer, K. L., A. C. Thornton, K. R. Fortney, R. S. Munson, Jr., and S. M. Spinola.** 1998. Evaluation of an isogenic hemolysin-deficient mutant in the human model of *Haemophilus ducreyi* infection. J. Infect. Dis. **178:**191–199.
48. **Parsons, L. M., R. J. Limberger, and M. Shayegani.** 1997. Alterations in levels of DnaK and GroEL result in diminished survival and adherence of stressed *Haemophilus ducreyi*. Infect. Immun. **65:**2413–2419.
49. **Parsons, L. M., A. L. Waring, and M. Shayegani.** 1992. Molecular analysis of the *Haemophilus ducreyi groE* heat shock operon. Infect. Immun. **60:**4111–4118.
50. **Peres, S. Y., O. Marches, F. Daigle, J.-P. Nougayrede, F. Herault, C. Tasca, J. De Rycke, and E. Oswald.** 1997. A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G2/M phase. Mol. Microbiol. **24:**1095–1107.
51. **Pickett, C. L., D. L. Cottle, E. C. Pesci, and G. Bikah.** 1994. Cloning, sequencing, and expression of the *Escherichia coli* cytolethal distending toxin genes. Infect. Immun. **62:**1046–1051.
52. **Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russell, A. N. Erdem, and H. Zeytin.** 1996. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* genes. Infect. Immun. **64:**2070–2078.
53. **Purcell, B. K., J. A. Richardson, J. D. Radolf, and E. J. Hansen.** 1991. A temperature-dependent rabbit model for production of dermal lesions by *Haemophilus ducreyi*. J. Infect. Dis. **164:**359–367.
54. **Purven, M., E. Falsen, and T. Lagergard.** 1995. Cytotoxin production in 100 strains of *Haemophilus ducreyi* from different geographic locations. FEMS Microbiol. Lett. **129:**221–224.
55. **Purven, M., A. Frisk, I. Lonnroth, and T. Lagergard.** 1997. Purification and identification of *Haemophilus ducreyi* cytotoxin by use of a neutralizing monoclonal antibody. Infect. Immun. **65:**3496–3499.
56. **Purven, M., and T. Lagergard.** 1992. *Haemophilus ducreyi*, a cytotoxin-producing bacterium. Infect. Immun. **60:**1156–1162.
57. **Robertson, S. M., C. F. Frisch, P. A. Gulig, J. R. Kettman, K. H. Johnston, and E. J. Hansen.** 1982. Monoclonal antibodies directed against a cell surface-exposed outer membrane protein of *Haemophilus influenzae* type b. Infect. Immun. **36:**80–88.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
59. **San Mateo, L. R., M. M. Hobbs, and T. H. Kawula.** 1998. Periplasmic copper-zinc superoxide dismutase protects *Haemophilus ducreyi* from exogenous superoxide. Mol. Microbiol. **27:**391–404.
60. **San Mateo, L. R., K. L. Toffer, and T. H. Kawula.** 1998. The *sodA* gene of *Haemophilus ducreyi* expresses a hydrogen peroxide-inhibitable superoxide dismutase. Gene **207:**251–257.
61. **Schmid, G. P., L. L. Sanders, J. H. Blount, and E. R. Alexander.** 1987. Chancroid in the United States: reestablishment of an old disease. JAMA **258:**3265–3268.
62. **Scott, D. A., and J. B. Kaper.** 1994. Cloning and sequencing of the genes encoding *Escherichia coli* cytolethal distending toxin. Infect. Immun. **62:**244–251.
63. **Spinola, S. M., A. Orazi, J. N. Arno, K. R. Fortney, P. Kotylo, C.-Y. Chen, A. A. Campagnari, and A. F. Hood.** 1996. *Haemophilus ducreyi* elicits a cutaneous infiltrate of CD4 cells during experimental human infection. J. Infect. Dis. **173:**394–402.
64. **Spinola, S. M., L. M. Wild, M. A. Apicella, A. A. Gaspari, and A. A. Campagnari.** 1994. Experimental human infection with *Haemophilus ducreyi*. J. Infect. Dis. **169:**1146–1150.
65. **Stevens, M. K., J. Klesney-Tait, S. R. Lumbley, K. A. Walters, A. M. Joffe, J. D. Radolf, and E. J. Hansen.** 1997. Identification of tandem genes involved in lipooligosaccharide expression by *Haemophilus ducreyi*. Infect. Immun. **65:**651–660.
66. **Stevens, M. K., S. Porcella, J. Klesney-Tait, S. Lumbley, S. E. Thomas, M. V. Norgard, J. D. Radolf, and E. J. Hansen.** 1996. A hemoglobin-binding outer membrane protein is involved in virulence expression by *Haemophilus ducreyi* in an animal model. Infect. Immun. **64:**1724–1735.
67. **Sugai, M., T. Kawamoto, S. Peres, Y. Ueno, H. Komatsuzawa, T. Fujiwara, H. Kurihara, H. Suginaka, and E. Oswald.** 1998. The cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. Infect. Immun. **66:**5008–5019.
68. **Totten, P. A., J. C. Lara, D. V. Norn, and W. E. Stamm.** 1994. *Haemophilus ducreyi* attaches to and invades human epithelial cells in vitro. Infect. Immun. **62:**5632–5640.
69. **Totten, P. A., W. R. Morton, G. H. Knitter, A. M. Clark, N. B. Kiviat, and W. E. Stamm.** 1994. A primate model for chancroid. J. Infect. Dis. **169:**1284–1290.
70. **Totten, P. A., D. V. Norn, and W. E. Stamm.** 1995. Characterization of the hemolytic activity of *Haemophilus ducreyi*. Infect. Immun. **63:**4409–4416.
71. **Trees, D. L., R. J. Arko, and S. A. Morse.** 1991. Mouse subcutaneous chamber model for *in vivo* growth of *Haemophilus ducreyi*. Microb. Pathog. **11:** 387–390.
72. **Trees, D. L., and S. A. Morse.** 1995. Chancroid and *Haemophilus ducreyi*: an update. Clin. Microbiol. Rev. **8:**357–375.
73. **Tullius, M. V., R. S. Munson, Jr., J. Wang, and B. W. Gibson.** 1996. Purification, cloning, and expression of a cytidine-5′-monophosphate *N*-acetylneuraminic acid synthetase from *Haemophilus ducreyi*. J. Biol. Chem. **271:** 15373–15380.
74. **Ward, C. K., S. R. Lumbley, J. L. Latimer, L. D. Cope, and E. J. Hansen.** 1998. *Haemophilus ducreyi* secretes a filamentous hemagglutinin-like protein. J. Bacteriol. **180:**6013–6022.
75. **Whitehouse, C. A., P. B. Balbo, E. C. Pesci, D. L. Cottle, P. M. Mirabito, and C. L. Pickett.** 1998. *Campylobacter jejuni* cytolethal distending toxin causes a G_2-phase cell cycle block. Infect. Immun. **66:**1934–1940.

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