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Involvement of a Plasmid in Virulence of *Campylobacter jejuni* 81-176

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Campylobacter jejuni strain 81-176 contains two, previously undescribed plasmids, each of which is approximately 35 kb in size. Although one of the plasmids, termed pTet, carries a *tetO* gene, conjugative transfer of tetracycline resistance to another strain of *C. jejuni* could not be demonstrated. Partial sequence analysis of the second plasmid, pVir, revealed the presence of four open reading frames which encode proteins with significant sequence similarity to *Helicobacter pylori* proteins, including one encoded by the *cag* pathogenicity island. All four of these plasmid-encoded proteins show some level of homology to components of type IV secretion systems. Mutation of one of these plasmid genes, *comB3*, reduced both adherence to and invasion of INT407 cells to approximately one-third that seen with wild-type strain 81-176. Mutation of *comB3* also reduced the natural transformation frequency. A mutation in a second plasmid gene, a *virB11* homolog, resulted in a 6-fold reduction in adherence and an 11-fold reduction in invasion compared to the wild type. The isogenic *virB11* mutant of strain 81-176 also demonstrated significantly reduced virulence in the ferret diarrheal disease model. The *virB11* homolog was detected on plasmids in 6 out of 58 fresh clinical isolates of *C. jejuni*, suggesting that plasmids are involved in the virulence of a subset of *C. jejuni* pathogens.

Although *Campylobacter jejuni* is one of the major causes of bacterial diarrhea worldwide (51, 59), the details of its molecular pathogenesis are not well understood. The clinical symptoms of campylobacter infection can range from a mild, watery diarrhea to a dysentery-like illness with fecal blood and leukocytes (2). Although there are reports of numerous cytotoxins, only the cytolethal distending toxin, which arrests eukaryotic cells at the G_2 phase of the cell cycle (64), has been characterized in detail. There are numerous reports that *C. jejuni* strains can invade intestinal epithelial cells in vitro (20, 21, 24, 29, 30, 38), although levels of invasion by different strains vary considerably (20, 28, 38, 52). Strain 81-176, originally isolated from a diarrheal outbreak associated with raw-milk consumption (31), is one of the best-characterized strains of *C. jejuni*. This strain has been shown to cause an inflammatory diarrhea in two human feeding studies (8; D. Tribble, unpublished data) and to cause disease in experimental models using primates (40) and ferrets (19, 67). Further, *C. jejuni* strain 81-176 invades INT407 cells at levels higher than those of most other *C. jejuni* strains (28, 38).

Plasmids have been found in between 19 and 53% of *C. jejuni* strains (5, 9–11, 41, 53–58), and many of these have been reported to be R plasmids that are transmissible among *Campylobacter* spp. but not to *Escherichia coli* (53–58, 60). Despite the importance of plasmids to virulence in numerous other pathogens, it is generally believed that plasmids play no role in *Campylobacter* pathogenicity. This paradigm is based on the rather low level at which plasmids have been reported and on an early study which compared the plasmid content and relative virulence of different *C. jejuni* strains in a guinea pig

model of disease (55). In previous studies with 81-176, we have observed the presence of two cryptic plasmids (R. Yao and P. Guerry, unpublished data). Herein, we show that one of these plasmids is an R factor encoding tetracycline resistance and that partial sequence analysis of the second plasmid revealed open reading frames (ORFs) encoding predicted proteins which display strong similarity to *Helicobacter pylori* proteins, one of which is encoded by the *cag* pathogenicity island. Moreover, we report that mutation of two of these genes, which encode homologs of type IV secretion systems, affect the virulence of *C. jejuni* 81-176.

MATERIALS AND METHODS

Strains and plasmids. *C. jejuni* 81-176 has been described previously (8, 31). The isolate used in the present studies was isolated from a patient in the human feeding studies described by Black et al. (8). *E. coli* DH5α was used as the host for cloning experiments, and pBluescript was used as the cloning vector. *C. jejuni* VC83 Str[r] Nal[r] was made by selection of the previously described VC83 Str[r] mutant (26) onto Mueller-Hinton (MH) agar supplemented with 50 μg of nalidixic acid per ml. Fresh clinical isolates from patients with diarrhea were obtained through the Armed Forces Research Institute in Bangkok, Thailand. Isolates were picked from campylobacter selective media directly onto blood agar plates for hybridization analysis (see below) without additional in vitro laboratory passage.

Bacterial growth conditions. Bacterial strains were maintained at −80°C in brucella broth (Difco) supplemented with 40% glycerol. *C. jejuni* was grown routinely on MH agar under microaerobic conditions at 37°C. Cultures for adherence and invasion assays were grown in MH biphasic cultures, consisting of MH agar overlaid with MH broth in tissue culture flasks. Antibiotics were added when appropriate to the following concentrations: 20 μg of trimethoprim per ml, 50 μg of kanamycin per ml, and 20 μg of tetracycline per ml.

DNA manipulations. Plasmids were purified from *C. jejuni* 81-176 by using mini-Qiagen columns (Qiagen, Chatsworth, Calif.) as directed by the manufacturer. Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, Mass.). Double-stranded plasmid clones were sequenced using AmpliTaq DNA polymerase FS (Perkin-Elmer–Applied Biosystems, Foster City, Calif.) on an ABI 373XL DNA sequencer, and sequences were analyzed using Sequencer 3.1.1 (Gene Codes Corp., Ann Arbor, Mich.) and MacVector (Oxford Molecular Systems, Oxford, United Kingdom).

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Conjugative transfer of *C. jejuni* plasmids. Attempts at conjugative transfer of plasmids from 81-176 into *C. jejuni* VC83 Str^r Nal^r were made by the method of Taylor et al. (57). Essentially, overnight cultures of donor and recipient cells were mixed in ratios ranging from 1:1 to 1:10, spotted on MH agar, and incubated for 24 h at 37°C. Cultures were subsequently resuspended in 1 ml of MH broth, diluted, and plated onto the appropriate selective medium. For crosses with wild-type 81-176 into VC83 Str^r Nal^r, the selective medium contained 20 μg of tetracycline per ml, 20 μg of streptomycin per ml, and 50 μg of nalidixic acid per ml; for crosses in which we attempted to mobilize pRY111 from 81-176 into VC83 Str^r Nal^r, the selective medium was MH agar with 20 μg of chloramphenicol per ml, 20 μg of streptomycin per ml, and 50 μg of nalidixic acid per ml.

Site-specific mutagenesis. A 6-kb *Bgl*II fragment (indicated by the arrow in Fig. 1) was cloned from the 81-176 pVir plasmid into pBluescript, generating pDB100. The kanamycin resistance (Km^r) cassette from pILL600 (32) was used for all mutations. The orientation of the Km^r cassette within the target gene was confirmed by either PCR or sequence analysis to be in the same orientation as the genes are transcribed, an orientation which is nonpolar (43; A. L. McVeigh and P. Guerry, unpublished data). In the case of the *virB11* mutation, the Km^r cassette was inserted into a unique *Eco*RV site within an *Eco*RI subclone of pDB100, generating pDB102. An *Eco*RI fragment containing the *oriT* from the campylobacter suicide vector, pGK2003 (25), was treated with Klenow polymerase (New England Biolabs) to generate a blunt-ended DNA fragment, which was subsequently cloned into the unique *Sma*I site in the multiple-cloning site of pDB102 to generate pDB103. This plasmid was conjugatively mobilized by RK212.2 (22) into *C. jejuni* 81-176, and transconjugants were selected on MH agar supplemented with 10 μg of trimethoprim per ml and 50 μg of kanamycin per ml. In a similar fashion, the Km^r cassette was inserted into a unique *Bcl*I site in the *comB3* gene of pDB100 to generate pDB106, which was used to naturally transform 81-176 (26, 62). All putative *C. jejuni* mutants were characterized by PCR using primers within the target gene to confirm that the plasmids had integrated via a double crossover.

Motility assays. The motility of *C. jejuni* strains was determined by stabbing the culture onto MH broth supplemented with 0.4% agar and observing the zone of motility after a 24-h incubation at 37°C (25).

Adherence and invasion assays. Adherence and invasion assays were done essentially as previously described (28, 38, 67). Briefly, mid-log-phase bacteria were added to a semiconfluent monolayer of approximately 2×10^5 INT407 cells at a multiplicity of infection of ca. 10 in 1 ml of culture medium per well. Infected monolayers were incubated for 2 h at 37°C in a 5% CO$_2$–95% air atmosphere. For determination of adherence, the cells were washed four times in Hanks' balanced salt solution with strong agitation for 2 min, the monolayer was lysed with 0.01% Triton X-100 for 30 min at room temperature on an orbital shaker, and total bacteria were enumerated by the plate count method. For determination of invasion, the monolayer was washed twice with Hanks' balanced salt solution, and fresh prewarmed medium containing gentamicin at 100 μg/ml was added to kill extracellular bacteria. After a 2-h incubation, the monolayer was washed twice with Hanks' balanced salt solution and lysed with 0.01% Triton X-100 as above. Following serial dilution in phosphate-buffered saline, released intracellular bacteria were enumerated by the colony count method on MH agar cultured under microaerobic conditions. Invasion ability was expressed as the percentage of the inoculum surviving the gentamicin treatment, and adherent bacteria were expressed as the total number of bacteria counted without antibiotic treatment. Assays were repeated at least three times.

Invasion assays in the presence of biochemical inhibitors. Inhibitors of eukaryotic cell processes were added to the monolayer 1 h prior to the addition of bacteria and were maintained throughout the 2-h invasion period, as described previously (28, 38, 67). Mid-log-phase bacteria were grown in MH biphasic medium and added at multiplicity of infection of ca. 20. Following the invasion period, the infected monolayer was washed three times with Earle's balanced salt solution and incubated for 2 h in fresh tissue culture medium containing 100 μg of gentamicin per ml to kill extracellular bacteria. Subsequently, the infected monolayers were washed and lysed, and the internalized bacteria were enumerated by the plate count method. Control studies were conducted to verify that at the concentrations employed, each inhibitor did not affect epithelial cell viability or bacterial viability over the assay period, as measured by trypan blue staining and the viable plate count method, respectively. *Salmonella enterica* serovar Typhi was used as a microfilament (MF)-dependent invasion control. All invasion inhibition assays were repeated on three separate occasions. Invasion efficiency (i.e., the percentage of the inoculum internalized) is presented as the mean ± standard error of the mean for all assays. The relative percent invasiveness was determined as recovery in the presence of inhibitors divided by recovery in the absence of inhibitors, multiplied by 100.

Ferret experiments. The experiments reported herein were conducted according to the principles set forth in the 'Guide for the care and Use of Laboratory Animals', Institute of Laboratory Animals Resources, National Research Council, DHHS Publication No. (NIH) 86-23 (1985). Ferret feeding experiments were done as previously described (19, 67). Briefly, 6-week-old female ferrets (Marshall Farms) which were shown to be free of campylobacter were used. The animals were provided with Marshall Farms ferret chow and water ad libitum and were observed for 1 week prior to challenge for any signs of distress and diarrhea; on several days during this week, rectal swabs from each animal were cultured to confirm that the animals were campylobacter free.

Overnight biphasic cultures of bacteria were concentrated and used as the inoculum as described by Doig et al. (19). Viable counts were determined by serial dilution onto MH agar. Ferrets were anaesthetized with acepromazine-ketamine intramuscularly and fed 10.0 ml of bacterial culture via a pediatric intubation tube. At 1 h postchallenge, the animals were given 2.8 ml of tincture of opium per kg intraperitoneally to reduce peristalsis. After infection, the animals were monitored three times daily for signs of diarrhea.

Natural transformation of *C. jejuni*. The biphasic natural transformation procedure was used as previously described (62). *C. jejuni* strains were grown overnight on plates and resuspended in MH broth to an optical density at 600 nm of 1.0. Aliquots of 200 μl of each strain were grown for an additional 2 h at 37°C in biphasic cultures in tubes (26). DNA (1 μg) from a streptomycin-resistant mutant of 81-176 (26) was added to the cultures, and the incubation continued for 4 h at 37°C. The cultures were serially diluted and plated in duplicate to MH agar containing 20 μg of streptomycin per ml. The results were expressed as the number of transformants per microgram of Sm^r DNA. Negative controls were strains treated identically without the addition of DNA.

DNA hybridizations. Primers *tetO* F (5′-GGCGTTTTGTTTATGTGCG-3′) and *tetO* R (5′-ATGGACAACCCGACAGAAGC-3′) (35) were used to amplify a 559-bp product with 250 ng of total DNA from *C. jejuni* 81-176 as the template. The PCR conditions used were as follows: an initial melting temperature of 95°C for 2 min; 35 cycles of 95°C for 2 min, 52°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 2 min. Primers *virB11* F (5′-GAACAGGAAGTGG AAAAACTAGC-3′) and *virB11* R (5′-TTCCGCATTGGGCTATATG-3′) were used to amplify a 708-bp fragment within the *virB11* gene with 250 ng of total DNA from *C. jejuni* 81-176 as the template. The PCR conditions were as follows: an initial melting temperature of 95°C for 2 min; 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 2 min; and a final extension at 72°C for 2 min. The PCR products were gel purified using a Qiaex II kit (Qiagen) and randomly primed in the presence of [^{32}P]dCTP (NEN Life Science Products, Boston, Mass.). Hybridizations were performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.1% sodium dodecyl sulfate–100 μg of calf thymus DNA per ml (42) at 60°C for 18 h and were followed by four 30-min washes with 0.5× SSC at 60°C. Southern blots or colony lifts were exposed to X ray film for 18 to 24 h.

Nucleotide sequence accession number. The sequences of the genes described in this paper have been submitted to GenBank under accession number AF226280.

RESULTS

Plasmid content of 81-176. Agarose gel electrophoretic analyses of plasmid DNA isolated from 81-176 revealed the presence of two plasmids (data not shown). An antibiogram determined that 81-176 is resistant to tetracycline (Tet^r). When individual colonies of 81-176 were picked from MH agar to MH supplemented with tetracycline, 20 of 172 colonies (12%) had spontaneously lost tetracycline resistance. Comparison of the *Bgl*II digestion patterns of plasmids from 81-176 and a Tet^S derivative, DB179 (Fig. 1) indicates that DB179 has lost one of the two plasmids. Restriction analyses with several different enzymes, similar to that in Fig. 1, indicated that the plasmids were each approximately 35 kb in size. PCR primers specific for the *Campylobacter tetO* gene (35) were used to amplify a 559-bp product with 81-176 DNA as template. Partial DNA sequence analysis of this PCR product confirmed that it contained part of the *tetO* gene (data not shown). Southern hybridization using this PCR product as probe indicated that a *Bgl*II fragment of approximately 10 kb from one of the plasmids, corresponding to the one indicated by the circle in Fig. 1, lane 1, contained *tetO*. The R factor has been termed pTet and the cryptic plasmid has been called pVir, for reasons described below. Attempts to transfer pTet or pVir which had been tagged with a kanamycin resistance gene (see below) conjugally from 81-176 to *C. jejuni* VC83 Nal^r Str^r were uniformly unsuccessful (data not shown). Similarly, attempts to mobilize a nonconjugative chloramphenicol resistance shuttle plasmid, pRY111 (66), from 81-176 to VC83 Nal^r Str^r were unsuccessful.

DNA sequence analysis of part of plasmid pVir. A 6-kb *Bgl*II fragment of pVir (arrow in Fig. 1), was cloned into pBluescript to generate pDB100. DNA sequencing of part of that fragment revealed the presence of four ORFs (Fig. 2). ORF1 through
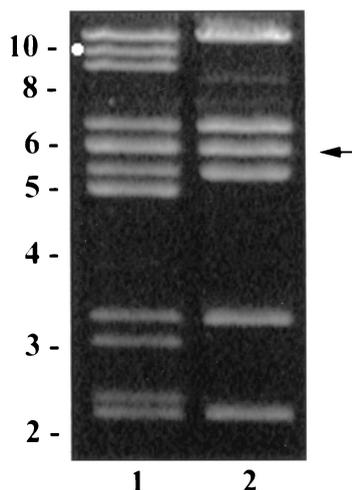
FIG. 1. *Bgl*II digestion pattern of plasmids isolated from *C. jejuni*. Lanes: 1, 81-176; 2, DB179, a Tet^S derivative of 81-176. The circle marks the fragment which hybridized to a *tetO* probe, and the arrow indicates the 6-kb *Bgl*II fragment cloned in pDB100. The positions of size markers in kilobase pairs are indicated to the left.
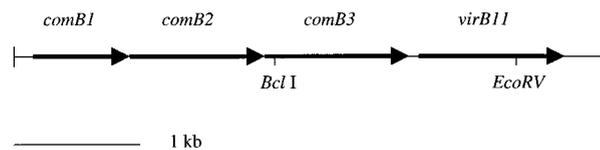

FIG. 2. Schematic of the ORFs identified by sequence analysis of the pDB100 plasmid. The restriction sites mark the position of insertion of the Km^r cassette in the mutants.

ORF3 are overlapping and appear to be organized in an operon. ORF4 begins 207 bp downstream of the 3′ end of ORF3, and there are no other ORFs present for >400 bp on either strand downstream of ORF4 (data not shown). ORF1 through ORF3 encode proteins that display significant identity to the products of the *comB1*, *comB2*, and *comB3* genes, respectively, of *H. pylori* P1, which are involved in competence for natural transformation and DNA uptake in *H. pylori* (27). Orthologs of the *comB1* through *comB3* genes have also been found in both of the completed *H. pylori* genome sequences (Table 1). In *H. pylori* J99, the genes are complete and encode products with predicted molecular masses of 25.9, 40.9, and 42.8 kDa for ComB1, ComB2, and ComB3, respectively (3, 18). However, in *H. pylori* 26695, both the *comB2* and *comB3* genes appear not to be expressed, since they carry a frameshift mutation, and are represented by HP0039/HP0040 (ComB2) and HP0041/HP0042 (ComB3) (61). *H. pylori* ComB2 and ComB3, but not ComB1, possess typical N-terminal signal sequences. In addition, ComB1 and ComB3 are predicted to contain an α-helical transmembrane region (27). The *C. jejuni* ComB1 protein also lacks a leader sequence and is predicted to

be associated with the inner membrane. *C. jejuni* ComB2 and ComB3 proteins have signal sequences and are predicted to be localized to the periplasmic space or the outer membrane. These three predicted *C. jejuni* proteins also have weaker homology to components of type IV secretion systems which are involved in transfer of various macromolecules. Type IV secretion systems are involved in transfer of the Ti plasmid DNA and proteins from *Agrobacterium* to plant cells (6, 14, 49), transfer of conjugative plasmids in *E. coli* (23, 69), transport of proteins required for intracellular survival of *Legionella pneumophila* (7, 12, 47), *Brucella suis* (36), and *Rickettsia prowazekii* (4), and transport of pertussis toxin across the outer membrane of *Bordetella pertussis* (63, 65). Thus, ComB1 shows between 16 to 23% identity and 32 to 34% similarity to VirB8 proteins from *B. suis* (36) and *R. prowazekii* (4); ComB2 shows 18% identity and 30% similarity to LvhB9 from a newly described type IV secretion system of *L. pneumophila* (47); and ComB3 shows 22% identity and 33% similarity to TrbI of plasmid RK2 (33) and 12% identity and 27% similarity to LvhB10 of *L. pneumophila* (47).

ORF4 of pDB100 (Fig. 2) encodes a protein whose highest identity match by BLASTP analysis is to the *H. pylori* protein JHP1316/HP1421 (Table 1). This *H. pylori* protein is a member of a paralogous family that also contains a protein encoded by a gene that maps in the *cag* pathogenicity island (ORF11, HP0525, and JHP474) (1, 3, 39, 61). This paralogous family has significant identity to VirB11 of the *Agrobacterium* type IV secretion system (6, 49). The *H. pylori* VirB11-like paralogous family (HP0525 and HP1421) has been proposed to function in a type IV secretion system required for virulence (1, 16, 37, 44). The predicted protein encoded by ORF4 of pDB100 is soluble and contains a consensus ATP-GTP binding domain at amino acids 150 to 157 (GGTGSGKT), like other VirB11 homologs (49, 65).

TABLE 1. Homology of pVir-encoded proteins to *H. pylori* proteins

ORF	Protein	Length (aa)[a]	Mass (kDa)	pI	Genomic *H. pylori* ortholog[b]	Length (aa)	% Identity	% Similarity[c]
ORF1	ComB1	225	25.9	8.10	HP0038	245	31	60
					JHP34[d]	247	31	60
ORF2	ComB2	356	40.9	8.77	HP0039/HP0040	40/247	30[e]	49[e]
					JHP35	328	30	50
ORF3	ComB3	378	42.8	7.95	HP0041/HP0042	123/233	33[e]	58[e]
					JHP36	376	31	56
ORF4	VirB11	317	36.4	5.79	HP1421	304	35	59
					JHP1316	304	35	59

[a] aa, amino acids.

[b] The HP and JHP proteins represent the orthologs from *H. pylori* strains 26695 (59) and J99 (3), respectively.

[c] The percent relatedness between orthologs is calculated as the number of identical or identical and conserved residues from an optimal alignment as a percentage of the length of the *C. jejuni* protein.

[d] The *C. jejuni* ComB1 protein is also related to the *H. pylori* J99 protein JHP921, which is located in that strain's hypervariable plasticity zone (3). The percent identity and similarity to this ortholog are 36 and 62%, respectively.

[e] Due to the splitting of the orthologs in *H. pylori* 26695 by a frameshift mutation, the percent identity and similarity were calculated by aligning each of the predicted portions and combining the totals.

TABLE 2. Adherence and invasion of 81-176 and derivatives

	Adherence		Invasion	
Strain	% Adherence	% of wild-type value	% Invasion	% of wild-type value
81-176	7.6 ± 0.87	100	4.67 ± 0.86	100
DB179	9.4 ± 0.81	123	5.6 ± 0.85	120
81-176 _comB3_::Km	2.7 ± 1.0[a]	29	1.3 ± 0.49[b]	28
81-176 _virB11_::Km	1.34 ± 0.41[b]	13	0.39 ± 0.27[b]	9

[a] $P < 0.01$ compared to strain 81-176.
[b] $P < 0.01$ compared to strain 81-176.

Effect of mutation of _comB3_ and _virB11_ on adherence and invasion in vitro. Site-specific insertional mutants were constructed in the _C. jejuni_ homologs of _comB3_ and _virB11_ using a nonpolar (43) kanamycin resistance cassette (32) as described in Materials and Methods. Growth of the mutants on motility agar demonstrated that both mutants were unperturbed in flagellar function and were fully motile (data not shown). The mutants were then compared to wild-type _C. jejuni_ 81-176 in in vitro adherence and invasion assays as previously described (28, 38, 67), and the results are summarized in Table 2. The _comB3_ mutant adhered to and invaded INT407 cells at levels that were approximately one-third those of the wild-type strain. However, the effects were more pronounced with the _virB11_ mutant, whose adherence and invasion levels were only 17.1% ($P < 0.001$) and 8.5% ($P < 0.001$) those of the wild-type strain, respectively. Strain DB179, which lacks the pTet plasmid, adhered to and invaded the INT407 cells at levels equivalent to wild-type levels. In contrast, _C. jejuni_ strain NCTC 11168, whose complete genome has been sequenced by the Sanger Centre, and _E. coli_ DH5α invaded INT407 cells at levels of 0.035% ± 0.02% and 0.08% ± 0.02%, respectively.

Involvement of MT versus MF in the residual invasion of 81-176 _virB11_::Km. Strain 81-176 is internalized into eukaryotic cells by a microtubule (MT)-dependent mechanism (28, 38). We assessed different inhibitors of MF polymerization (cytochalasin D), MT polymerization (colchicine, demecolcine, and nocodazole), or MT depolymerization (taxol) to examine the cytoskeletal requirements for the residual invasion of INT407 cells seen in the _virB11_::Km mutant of 81-176. Compounds that affect MF or MT polymerization were individually used to pretreat INT407 cell monolayers before and during the invasion period, as described in Materials and Methods. Cytochalasin D pretreatment resulted in reduction of the MF-dependent invasion by the control _S. enterica_ serovar Typhi strain from 19.6% ± 0.7% to 0.86% ± 0.79% (95.7% reduction). In contrast, the invasion ability of _C. jejuni_ 81-176 was not inhibited by either 1 or 2 μM cytochalasin D, as previously demonstrated (28). Mutant _C. jejuni_ 81-176 _virB11_::Km had a 91% decrease in invasion efficiency compared to the wild-type parent strain, 81-176 in the absence of inhibitors, and the remaining invasion ability was unaffected by cytochalasin D (Fig. 3). When host cells in concomitant studies were pretreated to depolymerize MTs with any of three inhibitors, the control _S. enterica_ serovar Typhi strain was not reduced in its entry ability (data not shown). However, _C. jejuni_ 81-176 and mutant _virB11_::Km were typically reduced more than 75% in their ability to invade INT407 cells by this latter treatment (Fig. 3). Taxol pretreatment of the monolayer showed no marked effect on the invasion ability of these _Campylobacter_ strains. Thus, mutant 81-176 _virB11_::Km exhibited the same
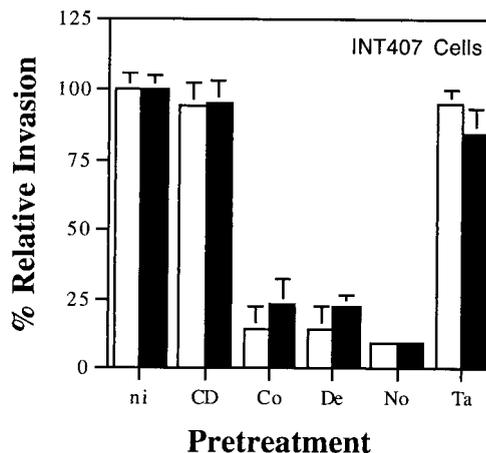

FIG. 3. Effect of various inhibitors on internalization into INT407 cells of _C. jejuni_ 81-176 (□) and the mutant _virB11_::Km strain (■). At 1 h prior to the addition of bacteria to the monolayer, the epithelial cells were incubated either with no inhibitor (ni) or with 1 μM cytochalasin D (CD), 10 μM colchicine (Co), 1 μM demecolcine (De), 20 μM nocodazole (No), or 50 μM taxol (Ta). Each inhibitor was maintained throughout the 2-h invasion period. The relative percent invasiveness was determined as 100 multiplied by recovery in the presence of inhibitors divided by recovery in the absence of inhibitors (i.e., 100% relative invasiveness). _S. enterica_ serovar Typhi Ty2W served as an MF-dependent invasive control. No other inhibitor showed a significant effect on _S. enterica_ serovar Typhi internalization. Results are presented as the mean of at least three separate experiments and standard error, shown as bars above or below the mean.

cytoskeletal requirements for invasion ability as the parent 81-176 strain (i.e., MT dependence).

Effect of the _virB11_ mutation on virulence in the ferret. To determine the effect of the _virB11_ mutation on virulence in vivo, the mutant was compared to wild-type 81-176 in the ferret diarrhea model (19, 67). Groups of ferrets were fed a high dose of bacteria (ranging from 9×10^9 to 8×10^{10} CFU/ml) and a lower dose (ranging from 9×10^8 to 8×10^9 CFU/ml), and their symptoms were monitored. The results, shown in Table 3, indicate that eight of eight animals fed the high dose of 81-176 developed mucous diarrhea but that only four of eight animals fed the same dose of the _virB11_ mutant became ill ($P < 0.05$). In contrast, only one out of nine animals fed similar doses of NCTC 11168 developed any diarrheal symptoms, which is consistent with the inability of this strain to adhere to or invade INT407 cells in vitro. At the lower dose, 50% of the animals fed _C. jejuni_ 81-176 developed diarrhea, compared to none of the animals fed the _virB11_ mutant ($P < 0.05$). Furthermore, at this low dose, none of the eight animals fed NCTC 11168 became ill. We have previously demonstrated that insertion of the Km[r] cassette into a gene unrelated to virulence (arylsulfatase) does not affect disease in the ferret model (67, 68).

TABLE 3. Virulence of _C. jejuni_ strains in ferrets

Strain	No. of animals with diarrhea/total no. given:	
	Low dose[a]	High dose[a]
81-176	4/8	8/8
81-176 _virB11_::Km	0/8[b]	4/8[b]
NCTC 11168	0/8[b]	1/9[c]

[a] The low dose of bacteria ranged from 9×10^8 to 8×10^9 CFU and the high dose ranged from 9×10^9 to 8×10^{10} CFU in two separate experiments.
[b] $P < 0.05$ compared to strain 81-176 by Fisher's exact test.
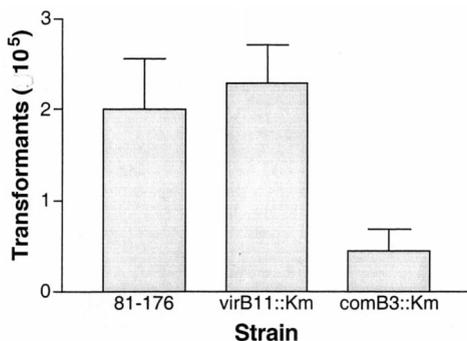[c] $P < 0.005$ compared to strain 81-176 by Fisher's exact test.

FIG. 4. Relative transformation frequency of strain 81-176 and mutants. DNA (1 μg) from a streptomycin-resistant mutant of 81-176 (26) was used to transform *C. jejuni* strains. Results are expressed as the total number of transformants per microgram of DNA and represent the mean and standard deviation of three independent experiments.

Effect of plasmid mutations on competence for natural transformation. The *comB3* gene product plays a role in natural transformation in *H. pylori* (27), where isogenic mutants demonstrate a marked reduction in the efficiency of transformation. We examined the *C. jejuni* 81-176 *comB3* mutant to determine whether this gene product played a similar role in the natural transformation of *C. jejuni*. The transformation frequency for a chromosomally located Str^r marker (26) was determined for the wild type and the two mutants. As seen in Fig. 4, there was no difference in transformation frequency between the wild-type strain and the *virB11* mutant. However, similar to the phenotype observed with the *H. pylori* mutant, the *C. jejuni* 81-176 *comB3* mutant carried a transformation-defective phenotype, which resulted in an 80% reduction in the natural transformation efficiency compared to the level for wild-type *C. jejuni* 81-176.

Presence of the *virB11* gene in other strains of *C. jejuni*. None of the four genes from the pVir plasmid described here was found to be present in the genome of *C. jejuni* NCTC 11168, which has been sequenced by the Sanger Centre (http://microbios1.mds.qmw.ac.uk/campylobacter/). Furthermore, DNA hybridization studies failed to detect the presence of a *virB11* homolog in nine laboratory strains of *C. jejuni* unrelated to 81-176 (data not shown). These probe-negative strains included A3249, which, like 81-176, caused diarrheal disease when fed to human volunteers, although the symptoms were less severe than those caused by 81-176 (8). Given that these *C. jejuni* strains had been passaged extensively under laboratory conditions, we used colony blot hybridization to examine the frequency of the *virB11* gene in freshly isolated *Campylobacter* strains obtained from U.S. military personnel with diarrheal disease in Thailand. The strains screened included 58 fresh clinical isolates of *C. jejuni* and 4 of *C. coli*. These results indicated that 10.3% (6 of 58) of the *C. jejuni* strains and none of the *C. coli* strains were positive for the *virB11* gene probe. Furthermore, the *virB11* gene probe hybridized to a large plasmid in all six of these isolates (data not shown).

DISCUSSION

The perception that plasmids are not involved in pathogenesis of *Campylobacter* spp. is based on a limited number of early studies indicating that a relatively low proportion of different strains contained plasmids and one study in which plasmid content was not associated with disease in a guinea pig model (55). However, the relevance of this model, which mea-

sures abortion of pregnant guinea pigs following intraperitoneal injection (15, 50, 55), to diarrheal disease is arguable. The experimental observation that strain 81-176 contains plasmids, coupled with its relatively high levels of both internalization into intestinal epithelial cells in vitro (28, 38) and virulence in ferrets (19, 67) and human volunteers (8), led us to reevaluate the role of plasmids in the pathogenesis of this well-characterized strain of *C. jejuni*. The version of 81-176 with which we routinely work is an isolate from the human feeding studies conducted by Black et al. (8). We have also examined an isolate obtained prior to the human feeding study and one from a primate feeding study (40), and the plasmid complement appears identical in all three strains. However, repeated attempts to transfer the tetracycline resistance plasmid (pTet) or a kanamycin-tagged version of pVir conjugatively from 81-176 to another *C. jejuni* strain or to mobilize a shuttle plasmid using either resident plasmid have been uniformly unsuccessful. Nonetheless, the high frequency of spontaneous loss of tetracycline resistance in the absence of selective pressure suggests that there must be some genetic transfer system, perhaps natural transformation, operating to maintain this plasmid in a population of 81-176 cells.

The partial sequence of the pVir plasmid identified four genes, whose products all had identifiable orthologs in the closely related pathogen *H. pylori*, and one of these is found in the *cag* pathogenicity island. The *cag* pathogenicity island is a region of approximately 37 kb which distinguishes virulent type I strains of *H. pylori* from the less virulent type II strains (13). Type I strains are characterized by their ability to induce numerous cellular changes following attachment to eukaryotic cells in vitro, including effacement of microvilli, cup and pedestal formation, cytoskeletal rearrangements, synthesis of interleukin-8, and morphological changes typical of those induced by exposure to growth factors (44–46). Among the proteins encoded by the *cag* pathogenicity island are the 145-kDa CagA protein and several homologs of type IV secretion systems (1). It has been recently shown that insertion of the 145-kDa CagA protein into the host cell is a key step in induction of the observed host cellular changes, and it has been shown that the type IV secretion genes encode proteins responsible for this transfer process (37, 44, 48).

The pVir plasmid contains an apparent operon of three *comB* genes in a tandem physical arrangement identical to that seen in *H. pylori* isolates. Mutation of the *comB3* gene significantly impaired the natural transformation ability of *C. jejuni* 81-176. Since strain 81-176 shows a higher transformation frequency than many other *C. jejuni* strains examined (P. Guerry, unpublished observations), it may be that the presence of the membrane-associated ComB3 protein enhances the level of natural transformation, perhaps by affecting surface changes that promote DNA binding. This apparent association of competence and virulence, while probably indirect, needs to be studied in more detail.

Although the level of homology for ORF1 (*comB1*) and ORF2 (*comB2*) is low, all four genes encode proteins with homology to proteins of type IV secretion systems, specifically VirB8, VirB9, VirB10, and VirB11, all of which are involved in formation of a channel or gate though which DNA and/or proteins are transferred. Mutation of either the *comB3* (*virB10*) or *virB11* genes in *C. jejuni* 81-176 resulted in a statistically significant reduction of adherence and internalization in vitro. However, the levels of invasion of both mutants still remained higher than those of both the negative control, *E. coli* DH5α, and another strain of *C. jejuni*, NCTC 11168. The internalization of the *virB11* mutant displays the same pattern of inhibition by host cytoskeletal inhibitors as that of 81-176,

suggesting that the residual invasion in the mutant is not likely to be due to the presence of a second invasion system. It is important to note that the invasion efficiency for wild-type strain 81-176 observed in this study was approximately double that reported previously (28, 38, 67). Invasion efficiency is most directly affected by the number of host cells in the monolayer, the number of bacteria added (i.e., multiplicity of infection), and the volume of medium in which the assay is conducted. In the present studies, all manipulations were the same as previously reported (28, 38, 67), except that the host cell number was inadvertently doubled. This resulted in, as expected, a doubling in the number of bacteria entering the monolayer. However, the percent reduction in invasion of the mutant 81-176 strains remained the same, even when the host cell concentration was lowered by twofold to the level used previously (28). Differences in invasion efficiency (i.e., the percentage of the inoculum internalized after a set time) of severalfold can occur easily unless the host cell and bacterial concentrations are carefully controlled; this phenomenon may largely explain laboratory-to-laboratory variation in observed invasion efficiencies for the same strain. Unlike determination of the average number of bacteria internalized per host cell (obtained by dividing the total number of internalized bacteria by the number of host cells per assay well), invasion efficiency is a relative number, which can vary when the experimental conditions are altered.

Reduction of adherence and invasion in vitro has previously been shown to correlate with reduced virulence in vivo in the ferret diarrhea model (67). This correlation is supported in this study, since the isogenic *virB11* mutant, which displayed significantly reduced levels of adherence and invasion in vitro, also was shown to cause significantly less severe symptoms in vivo. However, the present data do not specify if the primary defect is in adherence, invasion, or other undefined factors. Although we cannot exclude the possibility that virulence plasmids in most strains of *C. jejuni* are lost rapidly upon subculture, the remarkable stability of pVir in strain 81-176 in the absence of selective pressure would suggest otherwise. Rather, the data suggest that there are different mechanisms by which different strains of *C. jejuni* can cause disease. This hypothesis is consistent with reports of distinct clinical presentations of campylobacter enteritis, with differences observed in virulence among isolates, including NCTC 11168, in the ferret model (D. H. Burr, unpublished data) and in in vitro invasion assays (38), and with the observation that only approximately 10% of the fresh clinical isolates of *C. jejuni* tested were positive for the *virB11* gene probe. Genetic differences among strains may also explain some of the apparent discrepancies observed in different laboratories in terms of the MT versus MF dependency of campylobacter invasion (17, 28, 29, 38). Taken together, all of these data suggest that there may be distinct mechanisms of virulence among different strains of *C. jejuni*, analogous to the situation seen with diarrheagenic *E. coli* strains (34). The identification of this putative type IV secretion system in strain 81-176 should help elucidate the mechanism of virulence of this class of pathogenic *C. jejuni*.

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