INFECTION AND IMMUNITY, Jan. 2006, p. 769–772 Vol. 74, No. 1
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.1.769–772.2006

A σ^{28}-Regulated Nonflagella Gene Contributes to Virulence of *Campylobacter jejuni* 81-176

Scarlett Goon,[1] Cheryl P. Ewing,[1] Maria Lorenzo,[2] Dawn Pattarini,[1] Gary Majam,[1] and Patricia Guerry[1]*

Enteric Diseases Department, Naval Medial Research Center, Silver Spring, Maryland 20910,[1] *and Food and Drug Administration MOD1, Beltsville, Maryland*[2]

Received 9 August 2005/Returned for modification 19 September 2005/Accepted 25 October 2005

A *Campylobacter jejuni* 81–176 mutant in Cj0977 was fully motile but reduced >3 logs compared to the parent in invasion of intestinal epithelial cells in vitro. The mutant was also attenuated in a ferret diarrheal disease model. Expression of Cj0977 protein was dependent on a minimal flagella structure.

The motility imparted by the single polar flagella of *Campylobacter jejuni* is critical for intestinal colonization and for invasion into intestinal epithelial cells (IEC) in vitro (2, 4, 10, 22, 24, 26, 32, 34, 36). *C. jejuni* strains appear to use the flagella structure as a type III secretory organelle in the absence of specialized secretion systems for virulence factors. Thus, *C. jejuni* secretes a set of so-called Cia proteins upon coculture with IECs (19, 20, 29). Mutation of *ciaB* resulted in loss of secretion of all Cia proteins and reduced invasion of IECs (19, 20, 29). Although the Cia proteins are synthesized in nonmotile mutants, secretion required a minimum flagella filament structure (20). A second protein, FlaC, which shows sequence similarity to flagellin but which is not required for motility or flagella biogenesis, is also secreted through the flagella apparatus (31). FlaC, which is expressed and secreted independently of CiaB, binds to HEp2 cells in vitro and modulates invasion (31).

A recent microarray study by Carillo et al. (5) reported up-regulation of several σ^{28} and σ^{54} nonflagella genes in a more virulent variant of the genome strain NCTC 11168 (28). These same genes were also down-regulated in two flagella mutants of NCTC 11168. We have confirmed that many of these same genes are also down-regulated in flagella mutants in *C. jejuni* 81-176 (S. Goon, C. P. Ewing, and P. Guerry, unpublished data), and here we further characterize one of these σ^{28}-regulated genes, Cj0977.

The Cj0977 proteins (M_r, 21.2 kDa; pI 4.8) from 81-176 and NCTC 11168 were 98% identical. The Cj0977 protein lacks a leader sequence or transmembrane domains and is predicted to be cytoplasmic. The protein is conserved within the Proteobacteria and shares some similarity with initiation factor eIF-2B (GenBank accession no. NP_214448). A Cj0977::*cat* mutant in *C. jejuni* 81-176 was fully motile (Fig. 1A) and produced a normal flagella filament (Fig. 1B). Purified flagellin from the mutant displayed an isoelectric focusing (IEF) pattern identical to that of 81-176 (Fig. 1C), suggesting that Cj0977 is not involved in flagellin glycosylation (7, 9, 23, 33). There was no difference in growth kinetics of the mutant and the parent 81-176 in Mueller-Hinton (MH) broth, nor were there any changes in either capsular polysaccharide by immunoblot or lipooligosaccharide mobility on silver-stained gels (data not shown).

The Cj0977 mutant and a complement in which the wild-type allele linked to a Km^r marker was inserted into the *astA*

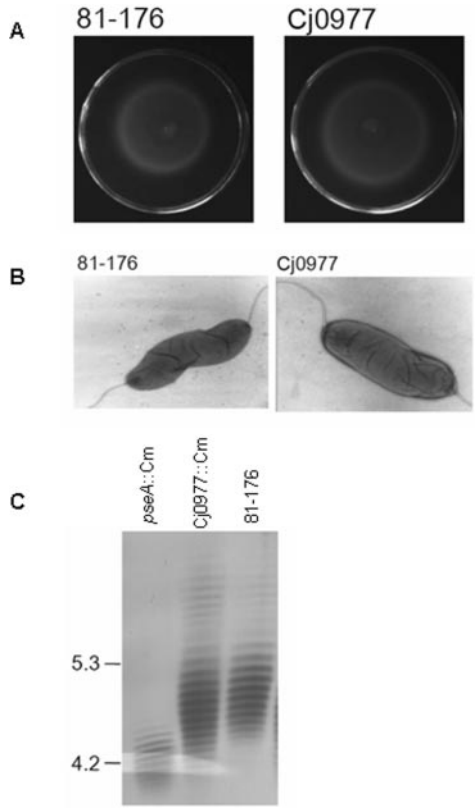

FIG. 1. Characterization of the Cj0977 mutant. A. Motility agar plates. Wild-type 81–176 and the Cj0977 mutant were inoculated into 0.4% MH agar plates and grown for 48 h at 37°C. B. Electron micrographs of the wild type and the Cj0977 mutant. C. Isoelectric focusing gel of purified flagellins (7). Flagellin from a *pseA* mutant that lacks an acetamidino form of pseudaminic acid (33) is shown for comparison. The position of pI standards is indicated on the left.

* Corresponding author. Mailing address: Enteric Diseases Dept., Naval Medical Research Center, 503 Robert Grant Ave., Silver Spring, MD 20910. Phone: (301) 319-7662. Fax: (301) 319-7679. E-mail: guerryp@nmrc.navy.mil.

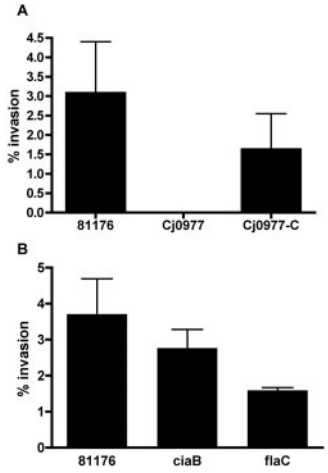

FIG. 2. Invasion of *C. jejuni* strains into INT407 cells. The data are presented as the percentage of the input inoculum that survived gentamicin killing (1, 27, 36). A. Invasion of 81-176, the Cj0977 mutant, and the complement (Cj0977-C). The complement was constructed as follows. A wild-type copy of Cj0977 with its promoter was PCR amplified with primers that introduced HindIII and EcoRI sites at either end, and the amplicon was cloned into pBluescript. After sequence analysis to confirm that no mutations had been introduced by PCR, a kanamycin resistance cassette from pILL600 (21) was cloned into the BamHI site in the multiple cloning site to generate pSG977-Km7. Plasmid pSG977-Km7 was digested with XhoI and XbaI to release a fragment of approximately 2.1 kb. This fragment was gel purified, blunted with Klenow, and cloned into a unique EcoRV site within the arylsulfatase (*astA*) gene of 81-176 as cloned into pYG660 (40), and this plasmid was used to electroporate the Cj0977 mutant to Km^r. A double crossover was confirmed by PCR. B. Invasion of 81-176 and isogenic mutants of *ciaB* and *flaC*. These mutants were constructed by transposition using an in vitro Tn*5*-based transposition system (Epicentre, Madison, WI) with a *Campylobacter* chloramphenicol resistance (*cat*) cassette into *Escherichia coli* clones of the 81-176 genes, as described previously (11, 12). The position of the inserts was determined by DNA sequence analysis prior to electroporation into 81-176. The position of the *cat* insert into *flaC* was at base pair 167 of the 759-bp open reading frame; the position of the insertion into *ciaB* was at base pair 1227 of the 1,833-bp open reading frame.

gene of the mutant (37) were compared to 81-176 for their ability to invade INT407 cells. The mutant invaded at <0.0015% of the input inoculum (>1,000-fold reduced compared to the wild type), as shown in Fig. 2A, and invasion was restored in the complement. For comparison, mutations were constructed in 81-176 in *ciaB* and *flaC*. As seen in Fig. 2B, both

TABLE 1. Flagella mutants of *C. jejuni* 81–176 used in this study

Gene	Cj no.	Function	Insertion point/length of gene in bp	Reference
fliR	Cj1179c	Export	557/768	This work
flgE	Cj1729c	Hook	1,321/2,598	This work
flhA	Cj0882c	Export	1,351/2,175	This work
flhB	Cj0335	Export	524/1,089	This work
rpoN	Cj0670	σ^{54}	Deletion	13
flgR	Cj1024c	Regulator	548/1,302	This work
flgS	Cj0793	Kinase	280/1,020	This work
fliA	Cj0061	σ^{28}	Deletion	13
fliD	Cj0548	Filament cap	127/1,929	This work
flaA flaB	Cj1339c/1338c	Flagellins	Substitution	This work

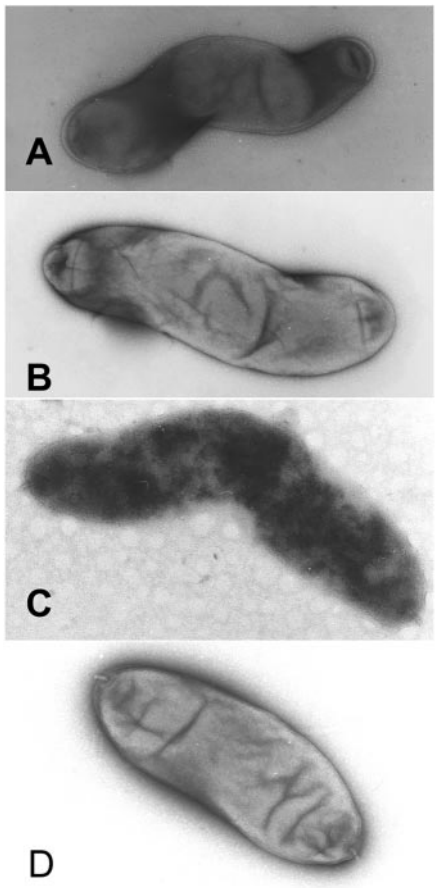

FIG. 3. Electron micrographs of selected flagella mutants. A. *fliR*; B. *flgE*; C. *flaA flaB*; D. *fliD*. The *fliR* and *flgE* mutants were bald, but the *flaA flaB* double mutant and the *fliD* mutant produced hook structures, visible at both poles. An image of wild-type 81–176 is shown in Fig. 1. The *flaA flaB* mutant was constructed as follows. A 446-bp PCR product was generated using primers within Cj1341, the gene adjacent to *flaA*, and the 5′ end of *flaA*; these primers introduced SacII and BamHI sites at either end. This amplicon was cloned into pBluescript to generate plasmid pCE101. A 310-bp PCR product was generated using primers within the 3′ end of *flaB* and the adjacent Cj1337 gene that introduced XhoI and BamHI sites, respectively, and the amplicon was cloned into pCE101. After DNA sequence analysis of the insert in the resulting plasmid (pCE102) insert, a chloramphenicol cassette from pRY109 (35) was cloned into the unique BamHI site to generate pCE103, and this plasmid was used to electroporate 81–176 to Cm resistance. All other mutants were generated using an in vitro Tn*5*-based transposition system, as described previously and in the legend to Fig. 2 (11, 12).

mutants were reduced in invasion compared to 81-176 but much less so than the Cj0977 mutant and less than the corresponding mutations in other strains (19, 20, 29, 31).

We examined expression of the Cj0977 protein in 10 mutants in the flagella regulon of 81-176 as shown in Table 1. All of the mutants in Table 1 were nonmotile, except for the Δ*fliA* mutant, which has a truncated flagella filament that confers reduced motility (13). Electron microscopic examination indicated that the remaining mutants were bald, except for the *flaA flaB* mutant and the *fliD* mutant, both of which produced hook structures. Representative electron micrographs are shown in Fig. 3. The Cj0977 protein could be observed in immunoblots

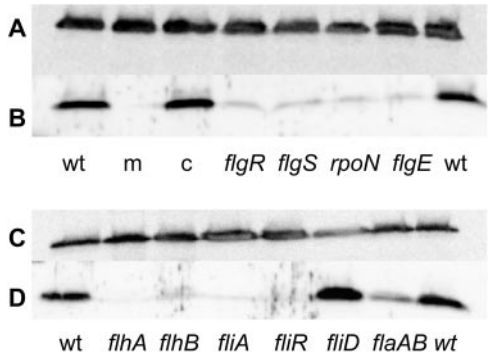

FIG. 4. Coregulation of Cj0977 with the flagella regulon. Whole-cell preparations were electrophoresed on sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis gels, transferred to nitrocellulose, and immunoblotted with anti-Omp50 antiserum (A and C) or anti-Cj0977 antiserum (B and D). Both antisera were generated in rabbits (Harlan, Madison, WI) against recombinant forms of the proteins. In panels A and B, "m" refers to the Cj0977 mutant and "c" refers to the complement. The bands in panels A and C were run with a 50-kDa protein marker, and the bands in panels B and D were between a 20- and 25-kDa protein marker. wt, wild type.

of whole-cell preparations of wild-type 81-176 and the complement but was missing in the mutant, as seen in Fig. 4B. Figure 4 also shows that no Cj0977 protein could be detected in the *fliA* mutant, and Cj0977 protein levels were significantly reduced in mutants in *flgR*, *flgS*, *rpoN*, *flgE*, *flhA*, *flhB*, and *fliR*. The level of expression appeared to be slightly reduced in a *flaA flaB* double mutant, and expression appeared to be equivalent to or perhaps increased in the *fliD* mutant. The same preparations were also immunoblotted with anti-Omp50 (3) antibody to confirm equal loading (Fig. 4A and C).

The Cj0977 mutant and the complemented strain were compared to wild-type 81-176 in the ferret diarrhea model (1). The Cj0977 mutant was attenuated compared to both the parent and the complement, as shown in Fig. 5. Thus, fewer animals developed diarrhea over the course of the experiment when fed the mutant (3 of 16 ferrets) compared to the wild type (12 out of 16; $P = 0.0038$) or complement (11 out of 16; $P = 0.0113$), and those animals that did develop diarrhea following feeding of the mutant displayed symptoms later than those fed the wild type or complement.

Although we have not been able to demonstrate secretion of Cj0977 into the supernatant (data not shown), the invasion defect in the Cj0977 mutant of 81-176 was greater than mutation of either *ciaB* or *flaC* in the same strain. In TGH9011, the *flaC* mutant invaded at about 14% of the level of the wild type compared to 43% of the level of the wild type in 81-176. Similarly, while the *ciaB* mutant in F38011 invaded at levels that were about 50-fold lower than those of the parent, the attenuation in 81-176 was 72% of the wild type. The discrepancy may be due, in part, to technical differences in invasion assays and cell lines used among different laboratories. However, the observed variations may reflect inherent differences in microtubule and microfilament uptake mechanisms among strains (6, 16–18, 25, 27, 30).

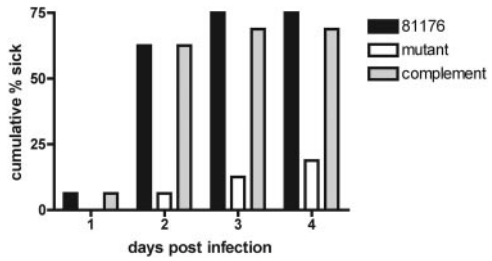

FIG. 5. Cj0977 mutant is attenuated in the ferret diarrheal disease model. The cumulative percentages of the total animals that developed diarrhea over 4 days postinfection are shown. The total number of animals per group was 16.

While much additional work remains to be done to understand the mechanism by which Cj0977 contributes to virulence of 81-176 at the molecular and cellular levels, these preliminary data establish a role for this protein in pathogenesis. This is the first report of coregulation of a virulence determinant with the *C. jejuni* flagella regulon, and it is similar to reports of σ^{28}-regulated virulence genes in other pathogens (8, 14, 15). The data also provide an additional explanation of the role of flagella in pathogenesis of *C. jejuni*, namely that this virulence gene, and perhaps others that are also σ^{28} or σ^{54} regulated (5 and Goon et al., unpublished), form an integral part of the flagella regulon.

We thank Dave Hendrixson and Vic DiRita for the *rpoN* and *fliA* deletion mutants of 81–176, Lindsay Holder and Anahita Kiavand for technical assistance, and Robert Williams for electron microscopy.

This work was supported by RO1 AI043559 and NMRC work unit 6000.RAD1.DA3.A0308 from the Military Infectious Diseases Research Program.

REFERENCES

1. **Bacon, D. J., C. M. Szymanski, D. H. Burr, R. P. Silver, R. A. Alm, and P. Guerry.** 2001. A phase variable capsule is involved in virulence of *Campylobacter jejuni* 81–176. Mol. Microbiol. **40:**769–777.
2. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infections in humans. J. Infect. Dis. **157:**472–479.
3. **Bolla, J. M., E. De, A. Dorez, and J. M. Pages.** 2000. Purification, characterization and sequence analysis of Omp50, a new porin isolated from *Campylobacter jejuni*. Biochem. J. **352:**637–643.
4. **Caldwell, M. B., P. Guerry, E. C. Lee, J. P. Burans, and R. I. Walker.** 1985. Reversible expression of flagella in *Campylobacter jejuni*. Infect. Immun. **50:**941–943.
5. **Carrillo, C. D., E. Taboada, J. H. E. Nash, P. H. Lanthier, J. Kelly, P. C. Lau, R. Verhulp, O. Mykytczuk, J. Sy, W. A. Findlay, K. Amoako, G. Gomis, P. Willson, J. W. Austin, A. Potters, L. Babiuk, B. Allan, and C. M. Szymanski.** 2004. Genome-wide expression analyses of *Campylobacter jejun*i NCTC 11168 reveals coordinate regulation of motility and virulence by *flhA*. J. Biol. Chem. **279:**20327–23008.
6. **deMelo, M. A., G. Gabbiani, and J. C. Pechere.** 1989. Cellular events and intracellular survival of *Campylobacter jejuni* during infection of HEp-2 cells. Infect. Immun. **57:**2214–2222.
7. **Doig, P., N. Kinsella, P. Guerry, and T. J. Trust.** 1996. Characterization of a post-translational modification of *Campylobacter* flagellin: identification of a sero-specific glycosyl moiety. Mol. Microbiol. **19:**379–387.
8. **Eichelberg, K., and J. E. Galan.** 2000. The flagellar sigma factor FliA (σ^{28}) regulates the expression of *Salmonella* genes associated with the centisome 63 type III secretion system. Infect. Immun. **68:**2735–2743.
9. **Goon, S., J. F. Kelly, S. M. Logan, C. P. Ewing, and P. Guerry.** 2003. Pseudaminic acid, the major modification on *Campylobacter flagellin*, is synthesized via the Cj1293 gene. Mol. Microbiol. **50:**659–671.
10. **Grant, C. C. R., M. E. Konkel, W. Cieplak, Jr., and L. S. Tompkins.** 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. Infect. Immun. **61:**1764–1771.
11. **Guerry, P., R. Yao, R. A. Alm, D. H. Burr, and T. J. Trust.** 1994. Systems of experimental genetics for *Campylobacter* species. Methods Enzymol. **235:** 474–481.
12. **Guerry, P., C. P. Ewing, T. E. Hickey, M. M. Prendergast, and A. P. Moran.** 2000. Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*. Infect. Immun. **68:**6656–6662.

13. **Hendrixson, D. R., and V. J. DiRita.** 2003. Transcription of σ^{54}-dependent but not σ^{28}-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus. Mol. Microbiol. **50:**687–702.
14. **Heuner, K., C. Dietrich, C. Skriwan, M. Steinert, and J. Hacker.** 2002. Influence of the alternate σ^{28} factor on virulence and flagellum expression of *Legionella pneumophila*. Infect. Immun. **70:**1604–1608.
15. **Heuner, K., and M. Steinert.** 2003. The flagellum of *Legionella pneumophila* and its link to the expression of the virulence phenotype. Int. J. Med. Microbiol. **293:**133–143.
16. **Hu, L., and D. J. Kopecko.** 1999. *Campylobacter jejuni* associates with microtubules and dynein during invasion into human intestinal cells. Infect. Immun. **67:**88–93.
17. **Konkel, M. E., S. F. Hayes, L. A. Joens, and W. Cieplak, Jr.** 1992. Characteristics of the internalization and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. Microb. Pathogen. **13:**357–370.
18. **Konkel, M. E., and L. A. Joens.** 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. **57:**2984–2990.
19. **Konkel, M. E., B. J. Kim, V. Rivera-Amill, and S. G. Garvis.** 1999. Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured epithelial cells. Mol. Microbiol. **32:**691–701.
20. **Konkel, M. E., J. D. Klena, V. Rivera-Amill, M. R. Monteville, D. Biswas, B. Raphael, and J. Mickelson.** 2004. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagella export apparatus. J. Bacteriol. **186:**3296–3303.
21. **Labigne-Roussel, A., P. Couroux, and L. S. Tompkins.** 1988. Gene disruption and replacement as a feasible approach for mutagenesis of *Campylobacter jejuni*. J. Bacteriol. **170:**1704–1708.
22. **Lee, A., J. L. O'Rourke, P. J., Barrington, and T. J. Trust.** 1986. Mucus colonization as a determinant of pathogenicity in intestinal infection by *Campylobacter jejuni*: a mouse cecal model. Infect. Immun. **51:**536–546.
23. **Logan, S. M., J. F. Kelly, P. Thibault, C. P. Ewing, and P. Guerry.** 2002. Structural heterogeneity of carbohydrate modifications affects serospecificity of campylobacter flagellins. Mol. Microbiol. **46:**587–597.
24. **Morooka, T., A. Umeda, and K. Amako.** 1985. Motility as an intestinal colonization factor for *Campylobacter jejuni*. J. Gen. Microbiol. **131:**1973–1980.
25. **Monteville, M. R., J. E. Yoon, and M. E. Konkel.** 2003. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. Microbiology **149:**153–165.
26. **Newell, D. G., H. McBride, and J. M. Dolby.** 1985. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. J. Hyg. **95:**217–227.
27. **Oelschlaeger, T. A., P. Guerry, and D. J. Kopecko.** 1993. Novel microtubule dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc. Natl. Acad. Sci. USA **90:**6884–6888.
28. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665–668.
29. **Rivera-Amill, V., B. J. Kim, J. Seshu, and M. E. Konkel.** 2001. Secretion of the virulence-associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. J. Infect. Dis. **183:**1607–1616.
30. **Russell, R. G., and D. C. Blake, Jr.** 1994. Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. Infect. Immun. **62:**3773–3779.
31. **Song, Y. C., S. Jin, H. Louie, D. Ng, R. Lau, Y. Zhang, R. Weerasekera, S. A. Raschid, L. A. Ward, S. D. Der, and V. L. Chan.** 2004. FlaC, a protein of *Campylobacter jejuni* TGH9011 (ATCC43431) secreted through the flagella apparatus, binds epithelial cells and influences cell invasion. Mol. Microbiol. **53:**541–553.
32. **Szymanksi, C. M., M. King, M. Haardt, and G. D. Armstrong.** 1995. *Campylobacter jejuni* motility and invasion of Caco2 cells. Infect. Immun. **63:**4295–4300.
33. **Thibault, P., S. M. Logan, J. F. Kelly, J.-R. Brisson, C. P. Ewing, T. J. Trust, and P. Guerry.** 2001. Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. J. Biol. Chem. **276:**34862–34870.
34. **Wassenaar, T. M., N. M. C. Bleumink-Pluym, and B. A. M. van der Zeijst.** 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. EMBO J. **10:**2055–2061.
35. **Yao, R. R., A. Alm, T. J. Trust, and P. Guerry.** 1993. Construction of new *Campylobacter* cloning vectors and a new chloramphenicol mutational cassette. Gene **130:**127–130.
36. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry.** 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. Mol. Microbiol. **14:**883–893.
37. **Yao, R., and P. Guerry.** 1996. Molecular cloning and site-specific mutagenesis of a gene involved in arylsulfatase synthesis in *Campylobacter jejuni*. J. Bacteriol. **178:**3335–3338.

Editor: J. T. Barbieri