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In Vivo Antigenic Variation of *Campylobacter* Flagellin

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***Campylobacter coli* VC167 cells producing either antigenic phase 1 (P1) or phase 2 (P2) flagellins (as determined by characteristic protein and DNA patterns) were used to infect rabbits by the removable intestinal tie-adult rabbit diarrhea (RITARD) procedure. Rabbits infected with P2 cells shed predominantly P2 cells throughout the infection; in rabbits infected with P1 cells, a transition of fecal isolates from P1 to P2 was observed.**

The thermophilic campylobacters, *Campylobacter jejuni* and *Campylobacter coli*, are recognized as the leading causes of acute gastroenteritis in humans (3, 7, 21). These microaerophilic, spiral organisms are motile by means of polar flagella, and this motility is thought to facilitate colonization of the mucous lining of the intestinal tract (12, 18, 19). Some strains of campylobacters have been shown to undergo a bidirectional transition, termed phase variation, between flagellated and nonflagellated phenotypes (4). When nonflagellated variants of such strains are used to infect either rabbits (4) or humans (2), only flagellated cells can be recovered from fecal samples, suggesting that nonflagellated forms are less able to colonize the host. Other campylobacter strains have been shown to reversibly express two distinct flagellum types that are distinguishable antigenically and by differences in the apparent M_r of the flagellin subunit (9, 14). The best-studied example is *C. coli* VC167, the type strain of the Lior 8 serogroup. When VC167 cells producing P1 flagellin (M_r 61,500) are grown in the presence of Lior 8 antiserum, motile cells producing P2 flagellin (M_r 59,500) are selected. When P2 cells are grown in the presence of an antiserum specific to P2 flagellin (LAH2;9), motile cells producing P1 flagellin are selected. This antigenic variation is accompanied by a reversible DNA rearrangement (8) in a piece of DNA that maps next to one of the genes coding for rRNA (S. M. Logan, P. Guerry, and T. J. Trust, manuscript in preparation). Since flagella have been implicated as virulence determinants of *Campylobacter* sp., we investigated the ability of *C. coli* VC167 to undergo antigenic variation in experimentally infected rabbits.

Cultures of *C. coli* VC167 cells producing antigenic phase 1 (P1) or phase 2 (P2) flagella were used to infect rabbits by the removable intestinal tie-adult rabbit diarrhea (RITARD) procedure as previously described (4, 22, 23). Briefly, after sedation, the abdomen of the rabbit was opened surgically and the small intestine and cecum were externalized. The cecum was ligated at the ileocecal junction with sterile umbilical tape, and a slip knot was used to temporarily ligate the terminal ileum anterior to the mesoappendix. Ten milliliters of an overnight culture (5) of campylobacter (approximately 10^8/ml) was injected directly into the mid-small bowel. The incision was closed with suture in two layers, and the ends of the slip knot were externalized and secured with sterile metal clips. Four hours later, the slip knot was removed and the incision was closed. Three rabbits were infected with P1 cells, and three rabbits were infected with P2 cells. Stools of infected animals were diluted in sterile saline and cultured on sheep blood agar with campylobacter-selective supplement (Remel, Lenexa, Kans.) at various times for up to 1 week after infection. Cultures were incubated at 42°C in an atmosphere of 85% nitrogen, 10% CO_2, and 5% O_2.

Samples of each inoculum were processed as follows. The cells in one sample were treated in 0.2 M glycine buffer (pH 2.2) (15), and the extracted proteins were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis on 7.5% acrylamide gels as previously described (16). DNA was purified from samples of each inocula by the method of Hull et al. (10) and was analyzed by Southern blot hybridization analysis, using plasmid pGK109 as a probe (8). This plasmid carries a 5.2-kilobase fragment of the VC167 chromosome, which maps adjacent to the site of the DNA rearrangement associated with the antigenic variation of flagella in *C. coli* VC167. As predicted from previous work (9), the protein analysis indicated that the P1 inoculum cells produced a flagellin of M_r 61,500 and the P2 cells produced a flagellin of M_r 59,500 (Fig. 1A, lanes 2 and 3). The hybridization data (Fig. 1B, lanes 1 and 2) indicate that the cells displayed the characteristic hybridization pattern reported for P1 and P2 cells, respectively (8).

Campylobacters isolated from the stools of infected rabbits were analyzed for both the M_r of the flagellin produced and for the DNA pattern obtained in Southern blot hybridization analysis, using plasmid pGK109 as the probe as follows. Representative samplings from each time point were obtained by subculture of as many campylobacter colonies as possible from the primary plates onto four fresh campylobacter blood agar plates. These were subcultured for 18 h at 42°C, and cells were harvested in sterile water. One sample of the cells was used for DNA extraction (10) for use in Southern hybridization analysis, and the other sample underwent glycine extraction (15) for SDS-PAGE analysis of flagellin subunit M_r. In addition, individual colonies (ranging in number from 5 to a maximum of 20, depending on availability) were used to inoculate an area of approximately 2 cm^2 of a campylobacter blood agar plate. After 18 h of incubation, each patch of cells was subjected to glycine extraction and analysis of flagellin by SDS-PAGE.

The results of both the protein and DNA analyses are summarized in Table 1, and representative data is shown in Fig. 1. Rabbits 309, 310, and 311 were infected with VC167

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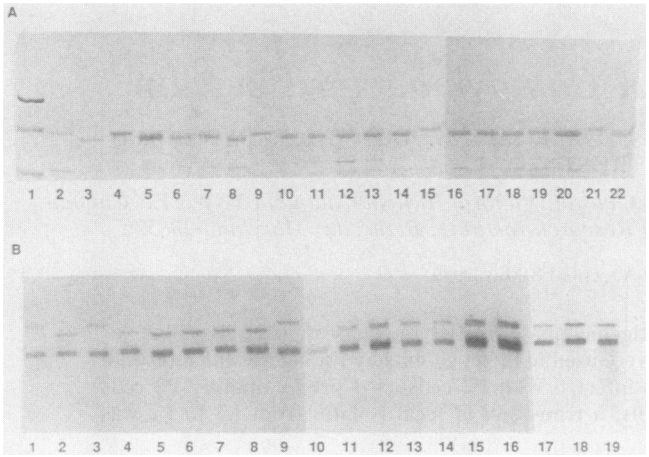

FIG. 1. Antigenic variation of VC167 flagellin. (A) SDS-PAGE analysis of glycine extracts of VC167 cells. Lanes 9 through 15 show individual colonies from one rabbit at one time point; all other samples shown are from the total population of cells at that time point. Lane 1, M_r size markers (from top to bottom, the three bands shown have M_rs of 97,400, 66,200, and 42,600); lane 2, P1 inoculum; lane 3, P2 inoculum; lane 4, rabbit 305, day 3; lane 5, rabbit 306, day 3; lane 6, rabbit 309, day 3; lane 7, rabbit 310, day 3; lane 8, rabbit 311, day 3; lanes 9 through 15, seven individual colonies of rabbit 305, day 6; lane 16, rabbit 305, day 6; lane 17, rabbit 306, day 6; lane 18, rabbit 310, day 6; lane 19, rabbit 305, day 7; lane 20, rabbit 306, day 7; lane 21, rabbit 307, day 7; lane 22, rabbit 311, day 7. (B) Southern blot hybridization patterns of DNAs of VC167 isolates from rabbits. DNAs were digested with restriction enzyme *Eco*RV, electrophoresed on a 0.7% agarose gel, transferred to a nitrocellulose membrane, and probed with pGK109 as previously described (8). Lane 1, P1 inoculum (bands shown are approximately 9.4 kilobases and a 6.6-kilobase doublet); lane 2, P2 inoculum (bands are approximately 8.4 kilobases and a 6.6-kilobase doublet); lane 3, rabbit 305, day 3; lane 4, rabbit 305, day 6; lane 5, rabbit 305, day 7; lane 6, rabbit 306, day 3; lane 7, rabbit 306, day 6; lane 8, rabbit 306, day 7; lane 9, rabbit 307, day 7; lane 10, rabbit 309, day 1; lane 11, rabbit 309, day 2; lane 12, rabbit 309, day 3; lane 13, rabbit 310, day 1; lane 14, rabbit 310, day 2; lane 15, rabbit 310, day 3; lane 16, rabbit 310, day 6; lane 17, rabbit 311, day 1; lane 18, rabbit 311, day 3; lane 19, rabbit 311, day 7.

P2 cells. Campylobacters were isolated from the stools of these rabbits as early as day 1 (rabbits 309 and 310) or day 2 (rabbit 311). All campylobacter isolates from their stools for up to 7 days postinfection were P2, as determined by both protein and DNA analyses, with one exception. One colony (of five examined) from rabbit 311 on day 3 was a mixture of P1 and P2 by SDS-PAGE. The total protein (Fig. 1A, lane 8) and DNA (Fig. 1B, lane 18) patterns from that sampling indicated only a P2 configuration, suggesting that P1 cells represented a minor component at that time point. Of the three rabbits infected with VC167 P1 cells, one, rabbit 307, did not shed detectable campylobacters in its stool until day 7, and these cells remained in P1 (Fig. 1A, lane 21, and Fig. 1B, lane 9). However, rabbit 305, also infected with P1 cells, shed only P1 cells on day 3 after infection (the first positive culture; Fig. 1A, lane 4, and Fig. 1B, lane 3) but by day 6 (the next time point taken) had shifted to production of P2 flagellin by SDS-PAGE determination on the total population (Fig. 1A, lane 16). The DNA analysis confirmed that the predicted DNA rearrangement had occurred in most of the population, although a faint background of the P1 pattern is barely visible (Fig. 1B, lane 4). Protein analysis of individual colonies from this sample confirmed a minor component (4 of 17 colonies) in the P1 configuration (Fig. 1A, lanes 9 through 15). By day 7, the shift to P2 was completed by all criteria (Fig. 1A, lane 19, and Fig. 1B, lane 5). Rabbit 306 also yielded VC167 cells that had undergone antigenic variation. In this rabbit, however, the transition seemed complete by the analyses of the total population of cells at day 3, the first positive culture (Fig. 1A, lane 5, and Fig. 1B, lane 6); the population of cells at day 6 (Fig. 1A, lane 17, and Fig. 1B, lane 7) and day 7 (Fig. 1A, lane 20, and Fig. 1B, lane 8) also showed a P2 configuration. However, individual colonies examined at day 6 indicated that 2 of 16 total colonies remained in the P1 configuration (Table 1; also data not shown). By day 7, all individual colonies tested were in the P2 configuration (Table 1; also data not shown).

TABLE 1. Antigenic variation of VC167 in rabbits infected by RITARD procedure

Rabbit no.	Phase of inoculum	Day of isolation[a]	Phase[b] (no. of colonies) of fecal isolates		
			Protein		Total DNA
			Individual	Total	
305	P1	3	ND	P1	P1
		6	P1 (4); P2 (13)	P2	P1/P2
		7	ND	P2	P2
306	P1	3	ND	P2	P2
		6	P1 (2); P2 (14)	P2	P2
		7	P1 (0); P2 (10)	P2	P2
307	P1	7	ND	P1	P1
309	P2	1	P1 (0); P2 (10)	P2	P2
		2	P1 (0); P2 (12)	P2	P2
		3	P1 (0); P2 (8)	P2	P2
310	P2	1	P1 (0); P2 (10)	P2	P2
		2	P1 (0); P2 (9)	P2	P2
		3	ND	P2	P2
		6	P1 (0); P2 (5)	P2	P2
311	P2	2	ND	P2	P2
		3	P1 (0); P2 (4); P1 + P2 (1)	P2	P2
		7	ND	P2	P2

[a] Samples were taken at days 1, 2, 3, 6, and 7 postinfection. Numbers indicate all days at which campylobacters were isolated in stool cultures.

[b] Phase was determined by examining the total population of cells for flagellin M_r and DNA configuration as described in the text. In some cases, when sufficient numbers of well-isolated colonies were present on the primary plates, individual colonies were expanded as decribed in the text, and the flagellin M_r was determined. ND, Not done.

The data clearly demonstrate that antigenic variation of flagella occurs in vivo and that it correlates with the same DNA rearrangement previously described to be associated with in vitro flagellar variation. However, this observed DNA rearrangement has not been shown to be directly related to flagellar antigenic variation. Mutational analysis of the region of DNA that rearranges will allow us to determine if a causal relationship exists between the rearrangement and variation in flagellar antigenic expression. Moreover, it is possible that other surface properties are changing during animal passage, and these properties may or may not be coregulated with flagellin. Nonetheless, the observed flagellar changes suggest a role for P2 flagella in pathogenesis, even though no major differences in symptomatology were observed between animals infected with P1 or P2 cells. All of the infected animals displayed some diarrhea, and some had occult blood in the stools. The mild symptoms seen with these strains in rabbits, compared with published data for other strains, may be due to the fact that VC167 is a *C. coli*, which is thought to be less virulent than *C. jejuni* (2). All rabbits infected with P2 cells shed campylobacters earlier after infection than the animals infected with P1 cells, suggesting that P2 cells may have an advantage in establish-

ing an infection or in their ability to survive in the host. Antigenic variation of surface antigens of several pathogens, including the flagella of *Salmonella typhimurium*, is thought to be involved in avoidance of the immune response of the host (20). However, in the data presented here for campylobacter, antigenic variation occurred in nonimmune animals, suggesting a more direct, functional role in pathogenesis. Flagella have been suggested as virulence factors for other enteric pathogens (1, 6, 13, 24). In *Vibrio cholerae*, flagella are thought to be required for attachment in in vitro assays (1). Recent genetic data indicate that mutations affecting flagella synthesis in *Salmonella typhi* prevent invasion of HeLa cells (13). The data presented here and the data from another experiment with VC167 P1 and P2 cells, in which fewer samples were examined (data not shown), strongly suggest a biological preference for P2 flagella during some undetermined early stage of campylobacter infection. The exact nature of the functional differences between P1 and P2 flagella remains to be elucidated. Two flagellin genes from VC167 have been cloned and sequenced (17; P. Guerry, S. M. Logan, and T. J. Trust, manuscript in preparation). Mutational analysis of these genes (11) is under way and should allow us to better assess the molecular mechanism of antigenic variation and the role of each phase of flagellin in pathogenesis in the RITARD model.

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