Isolation of Nonchemotactic Mutants of *Campylobacter jejuni* and Their Colonization of the Mouse Intestinal Tract

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Three nonchemotactic mutants (D54, Y14, and N74) of *Campylobacter jejuni* were isolated from wild-type strain FUM158432 by either the negative swarming or liquid gradient method with brucella broth as the attractive substance. Strains D54 and Y14 were isolated after mutagenesis with methyl methanesulfonate, and N74 was isolated from a nonmutagenized culture. These mutants all failed to swarm on a semisolid medium and did not show any chemotactic behavior in the hard-agar plug assay method for any of the chemicals which act as attractants for the wild-type strain. They had intact flagella and were actively motile. Swimming behavior examined by a video tracking technique showed that the mutants swim only straight, without any tumbling. When suckling mice were challenged orally with approximately 10^5 CFU of these mutant strains, all of the mutants were cleared from the intestinal tract by 48 h. In contrast, the wild-type strain colonized the intestinal tracts of all mice challenged with 10^2 CFU. We concluded that chemotactic movement is important for colonization of the intestinal tract of suckling mice by *C. jejuni*.

Campylobacter jejuni is one of the most common pathogens causing acute bacterial gastroenteritis in humans (6, 7, 21). The pathophysiology of infection with this bacterium is not thoroughly understood. In vivo experiments with experimental animals and human volunteer studies suggested that for colonization of the intestinal tract, the motility mediated by the flagellum is important (5, 8, 19, 20).

Chemotaxis is said to be an important mechanism controlling the colonization of the intestinal tract by motile bacteria (2). In *Vibrio cholerae*, the importance of chemotactic movement in association with the intestinal mucosa has been shown in both in vitro and in vivo model systems (9, 12). However, there have been opposing reports on infection with *Escherichia coli* F-18 (16) and *Salmonella typhimurium* (17). In this study, we isolated nonchemotactic mutants of *C. jejuni* and investigated their ability to colonize the mouse intestine. Our results show that chemotactic movement is important for intestinal colonization by this bacterium.

MATERIALS AND METHODS

Bacteria and culture media. *C. jejuni* FUM158432 and its aflagellated mutant M1 were used. The origins and characteristics of these strains were described previously (19). The strains were stored in brucella broth (Difco Laboratories, Detroit, Mich.) in a freezer at −80°C. For use, they were cultured on brucella agar plates or in brucella broth at 42°C in a GasPak anaerobic system without a catalyst (BBL Microbiology Systems, Cockeysville, Md.). Brucella broth (Difco) consists of Bacto Tryptone, Bacto Peptamin, Bacto Dextrose, Bacto Yeast Extract, sodium chloride, and sodium bisulfite. For motility testing, a semisolid brucella agar containing 0.35% agar was used.

Isolation of chemotactic mutants. Mutants were isolated by two methods, the negative swarming method and the liquid gradient method.

(i) Negative swarming method. The method was used essentially as described by Armstrong et al. (3). A bacterial

suspension of strain FUM158432 (5×10^8 CFU per ml) was mutagenized with 50 mM methyl methanesulfonate as described elsewhere (19). One drop of the suspension was then plated on the center of a semisolid brucella agar plate. After incubation at 42°C overnight, the bacteria formed a widely spreading colony on the plate because of their swarming movement. Nonmotile or nonchemotactic mutants could be expected to remain in the center of the colony. Bacteria were picked from the center of the colony, transferred to fresh medium, and incubated. This process was repeated several times until the bacteria did not show extensive swarming on the semisolid agar plate. After the selection was repeated more than 10 times, single colonies were streaked on brucella agar plates, and the bacteria that formed small compact colonies were isolated. Among these isolates, nonmotile and/or aflagellated strains were eliminated by observation by the dark-field microscopy and electron microscopy.

(ii) Liquid gradient method. The method was essentially the same as that described by Aswad and Koshland (4). Brucella broth was used as the attractant. Three liquid layers, the gradient of glycerol, the bacterial suspension, and the layer of the attractant, were made in a small test tube (12 mm in diameter and 120 mm in length) in this order from bottom to top. In the bottom of the tube, the linear gradient of glycerol (4 to 3% in 0.1 M phosphate-buffered saline, pH 7.0) was made. Over the gradient, 0.2 ml of the bacterial suspension (5×10^7 CFU per ml) in diluted brucella broth (1:3) containing 1.5% glycerol was gently applied. The top layer containing the attractant consisted of two layers of diluted brucella broth. Directly over the bacterial suspension, 2.5 ml of one-half of the concentration of brucella broth containing 1% glycerol was layered, and 2 ml of the same broth without glycerol was placed over this layer. After incubation at 42°C for 1 h, the contents were gently removed from the top of the tube with a 5-ml syringe with an intradermal needle, leaving the final 1 ml in the bottom of the tube. The bacteria in this final 1 ml were cultured on a brucella agar plate, and the new culture was used for the second selection. Selection was repeated four times, and
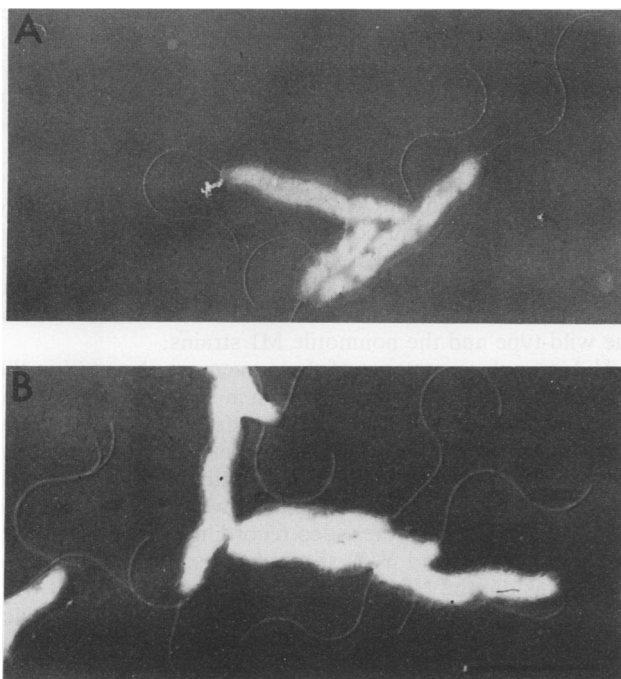
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FIG. 1. Electron micrographs of the wild-type strain (A) and the nonchemotactic strain (N74) (B). Bar represents 1 μm.

small compact colonies were isolated from the final culture as in the negative swarming method.

Electron microscopy. Bacteria on a Formvar-coated grid were shadowed with chromium at an angle of 20° and examined with a JEM-2000 EX electron microscope.

Video tracking of bacterial motility. A drop of bacterial suspension in brucella broth (5×10^8 CFU per ml) on a glass slide was examined with dark-field illumination (Nikon type MC X; Nikon Corp., Tokyo, Japan), and bacterial movement was recorded on videotapes through a video camera (Hamamatsu Photonics K. K., Hamamatsu, Japan). Bacterial movement was tracked on the recorded video images by an image analyzer (DVS-1000; Hamamatsu Photonics).

Chemotaxis assay. Chemotaxis was evaluated by the hard-agar plug (HAP) assay of Hugdahl et al. (14). A bacterial suspension (5×10^8 CFU per ml) was made in phosphate-buffered saline containing 0.35% agar from a plate culture of each mutant strain and transferred to a plastic petri dish. The chemoattractant to be tested was soaked in an agar plug and placed on the soft-agar plate. The plate was incubated at 42°C in a GasPak jar for 3 h. The chemicals used as chemoattractants for *C. jejuni* were L-aspartate, L-glutamate, L-serine, L-fucose, pyruvate, succinate, fumarate, citrate, L-malate, α-ketoglutarate, and hog gastric mucin, all of which have been reported to act as chemoattractants for *C. jejuni* (14). All of the chemicals were of analytical grade and were used at the highest concentration that had been reported to cause a positive chemotactic response (0.1 M except for L-glutamate (0.125 M), pyruvate (1 M), and hog gastric mucin (0.1%). The wild-type strain formed a zone of the accumulated bacteria around the agar plug.

Inoculation of suckling mice. For the test for colonization of the intestinal tract of suckling mice, bacterial suspensions of the wild-type and mutant strains were inoculated into suckling ddY mice (1 to 4 days old) as described previously (19). More than four mice were used in each experiment.

Several days after inoculation, the mice were sacrificed, the intestinal tracts were removed, and the number of *C. jejuni* in them was determined by the colony-counting method.

RESULTS

Isolation of the mutants. In the negative swarming method, 214 small compact colonies were picked. Twelve of them were found to be composed of motile organisms by dark-field microscopy, and finally two strains (D54 and Y14) were determined to be nonchemotactic by the HAP assay. In the liquid gradient method, 56 colonies were picked as compact colonies, and five of them were composed of motile bacteria. One strain (N74) was selected as a nonchemotactic strain. Strains D54 and Y14 were selected from methyl methane-sulfonate-mutagenized cultures, and N74 was selected from a nonmutagenized culture. All of the mutants were obtained in separate experiments, so they should be derived from different ancestors.

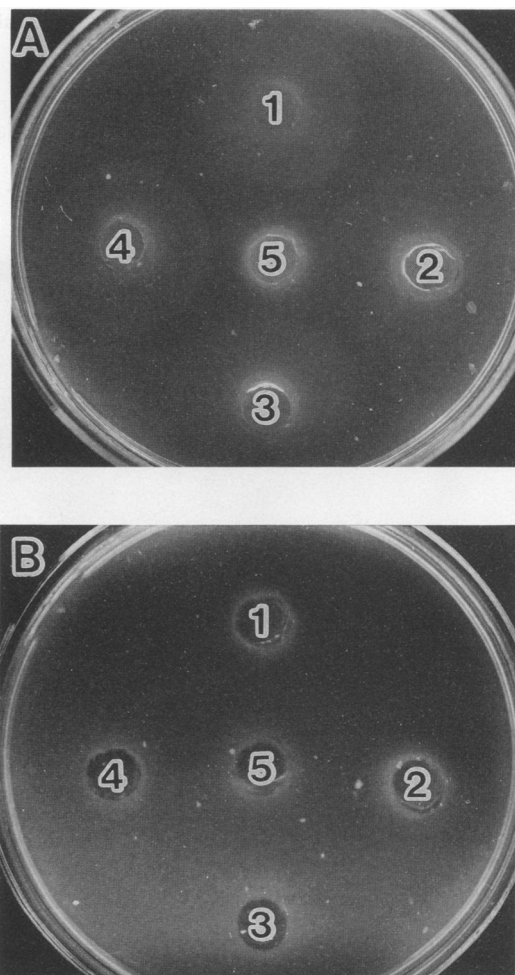Morphologically, these three strains did not show any

FIG. 2. Chemotactic response by HAP assay of the wild-type strain (FUM158432) (A) and the nonchemotactic strain (N74) (B) after 3 h of incubation at 42°C. Plugs 1 to 5 contained, respectively, 0.1 M L-serine (chemoattractant, bacterial accumulation around the plug), 0.125 M L-glutamate (chemoattractant), 0.1 M α-ketoglutarate (chemoattractant); 0.1 M L-aspartate (chemoattractant); and 0.1 M galactose (control, no chemotactic response).
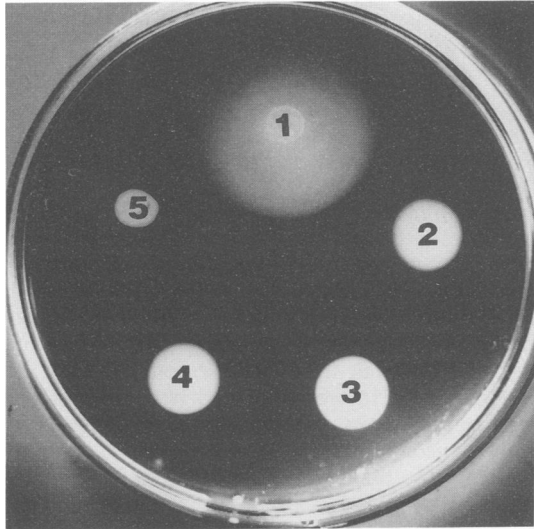
FIG. 3. Brucella broth semisolid agar plate showing swarm phenotypes of the wild-type and mutant strains. Colonies: 1, wild type; 2, D54; 3, N74; 4, Y14; 5, nonmotile mutant M1. Incubation was for 18 h at 42°C. Inocula were 2 µl of late-logarithmic-phase cultures.

differences from the wild-type strain in cell shape, length and width of the flagella, and shape of the wave of the flagellar filament (Fig. 1).

Responses of the mutant strains to chemoattractants. In the

HAP assay, the wild type showed active chemotactic movement toward all of the attractants used and formed a halo of accumulated bacteria around the agar plugs (Fig. 2A). However, no such accumulation of the bacteria was found in mutant strains (Fig. 2B). They did not respond to any of the 11 chemoattractants used in this study.

Analysis of motility. Three strains showed less active swarming than did the wild-type strain but more active swarming than did the nonmotile and nonflagellated strain M1. These differences in motility were reflected in the size of the colonies on a semisolid agar plate (Fig. 3). The mutant strain formed colonies intermediate in size between those of the wild-type and the nonmotile M1 strains.

Video tracking patterns of the movement observed in the wild-type and N74 strains are shown in Fig. 4. The pattern of movement of strain N74 was quite different from that of the wild type. The wild-type strain frequently changed direction (tumbling), and consequently the continuous moving path was shorter than that of the mutant, which did not change direction during 3 s of the video recording period. The other two strains (D54 and Y14) showed the same characteristic motility as did the N74 strain. This result is consistent with the observation that nonchemotactic mutants of other bacterial species lost the capacity to tumble during movement (1, 4, 10, 13).

The rate of reversion of these mutant strains to the wild phenotype seemed to be low in in vitro culture. After three successive cultures on brucella agar plates, we did not have any revertants among the three mutants.
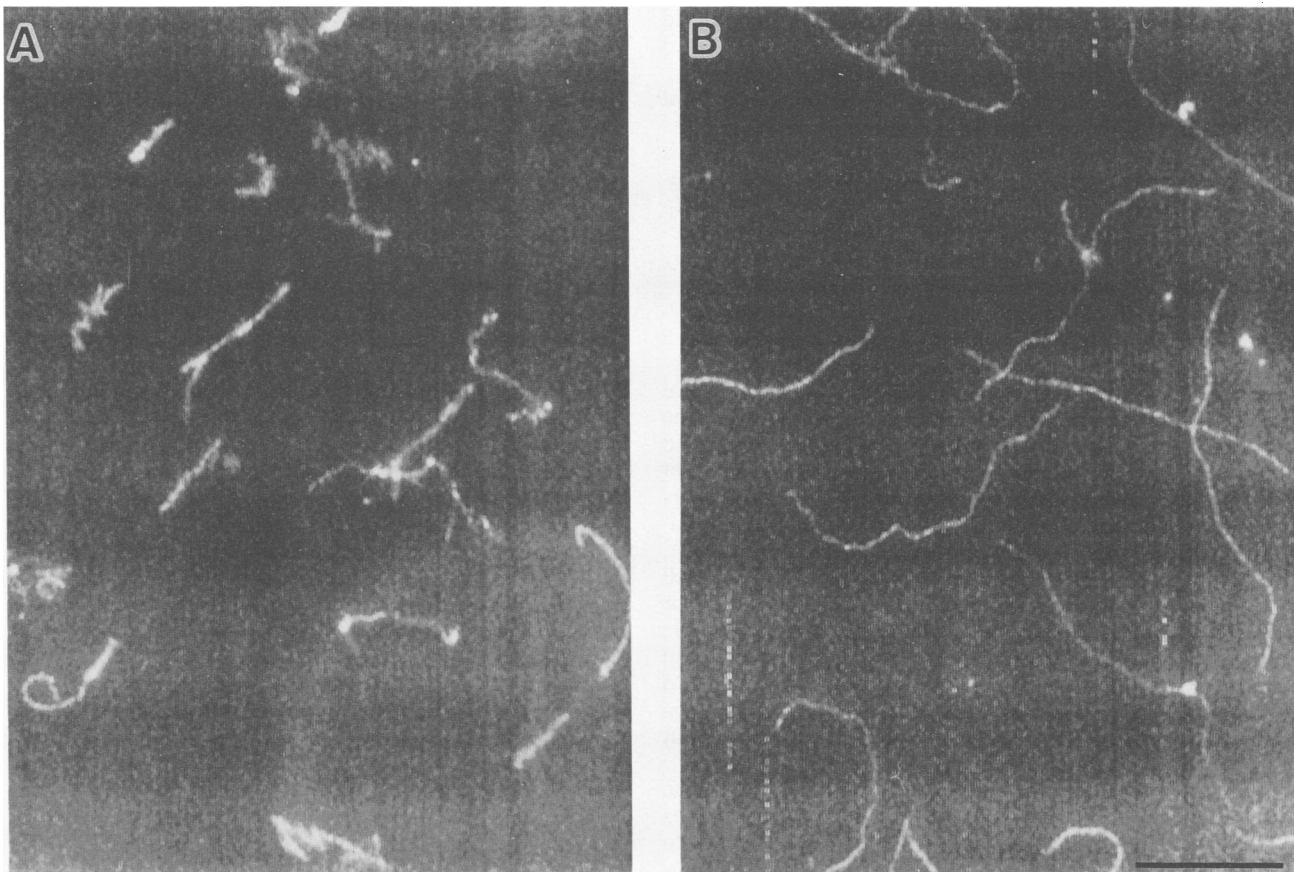

FIG. 4. Typical motility tracks of the wild-type strain (A) and the nonchemotactic strain (N74) (B). Bar represents 100 µm.
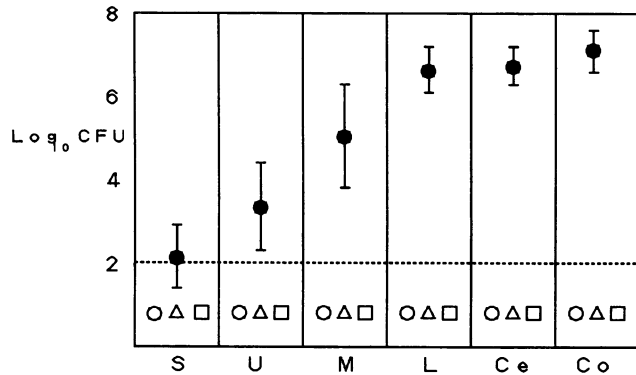
FIG. 5. Distribution of nonchemotactic mutant strains of *C. jejuni* in the gastrointestinal tract of suckling mice 1 week after inoculation. The segments of the gastrointestinal tract that were tested here and in Fig. 6 were stomach (S), upper (U), middle (M), and lower (L) thirds of the small intestine; cecum (Ce), and colon (Co). The inoculum was 5×10^5 CFU per mouse. Each point represents the mean number of colonies recovered from the organs ± standard deviation. Symbols and numbers of mice used (in parentheses): ●, wild type (10); ○, D54 (8); △, N74 (8); □, Y14 (8). The area under the broken line indicates an amount of bacteria undetectable by our colony assay.
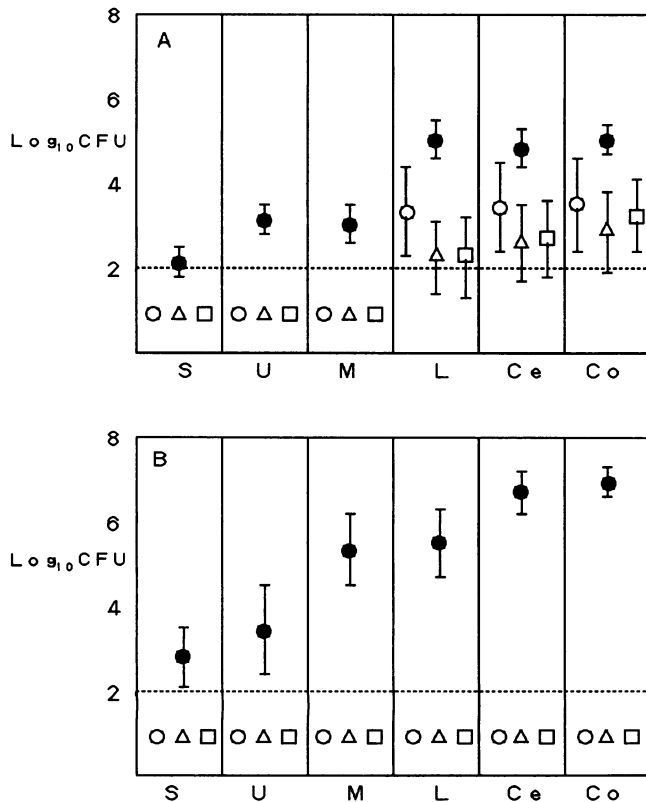

FIG. 6. Distribution of the nonchemotactic mutant strains in the suckling mouse gastrointestinal tract in early stages (10 h [A] and 48 h [B]) after inoculation. The inoculum was 5×10^5 CFU per mouse. Symbols are as in Fig. 5. The number of mice used (in parentheses) were as follows: (A) wild type (5), D54 (6), N74 (6), and Y14 (5); (B) wild type (5), D54 (8), N74 (8), and Y14 (6).

TABLE 1. Effect of inoculum size on intestinal colonization by wild-type and nonchemotactic mutant strains of *C. jejuni* 7 days after inoculation

Strain	Inoculum (CFU/mouse)	No. of colonized mice/total no. of mice
Wild type	1.1×10^2	10/10
D54	5.0×10^5	0/10
	5.0×10^6	3/8
	4.8×10^7	4/9
N74	5.0×10^7	0/11
Y14	4.9×10^7	0/8

Colonization of the mouse intestinal tract. In mice inoculated with the wild-type strain (5×10^5 CFU per mouse), more bacteria than the inoculum were recovered from the gastrointestinal tract 1 week after challenge (Fig. 5). However, in mice inoculated with the nonchemotactic strains, no campylobacters were detected in any portions of the intestinal tract 1 week after injection.

To monitor the fate of the inoculated nonchemotactic strains in the gastrointestinal tract at an earlier stage after injection than 1 week, recovery of the mutant strains within 48 h after inoculation was analyzed. As shown in Fig. 6A, at 10 h after inoculation of 5×10^5 CFU per mouse, most of the bacteria were recovered from the lower intestinal tract. The lack of increase in the total numbers of bacteria recovered from the intestinal tract at this stage suggested that no significant growth of the bacteria of these mutant strains had occurred in the intestinal tract. At 48 h after inoculation, bacteria were no longer recovered from the intestinal tract (Fig. 6B).

Colonization by the mutant strain could be related to the size of the inoculum. We previously observed that the larger the inoculum, the more colonization that was attained by a mutant which has a partial defect in motility (19). The effect of the size of the inoculum on the colonization by the three mutant strains was determined (Table 1). With an inoculum of less than 5×10^5 CFU per mouse, none of the strains colonized in any part of the gastrointestinal tract. With an inoculum of 5×10^6 CFU per mouse, colonization was observed only with strain D54 and in only a few mice. With strains N74 and Y14, no colonization was observed even after inoculation of 5×10^7 CFU per mouse, which was the largest number of bacteria used in this study.

The bacteria recovered from the intestinal tract of mice inoculated with strain D54 showed the same chemotactic response as did the parental D54 strain, indicating that the colonized bacteria were not revertants.

DISCUSSION

The HAP assay developed by Hugdahl et al. (14) for demonstration of the chemotactic behavior of *C. jejuni* was found to be useful in this study. The bacteria moved toward and accumulated around the plug if the chemical in it was an attractant. Since we incubated the plates at 42°C for 3 h, which was not sufficient for the rapid growth of this bacterium, the turbid zone formed around the plug was a zone of accumulation but not growth of the bacteria.

In this study, we isolated three mutant strains which did not respond to several chemicals known to be chemoattractants for *C. jejuni*. On the basis of the following observations, we concluded that these mutants are nonchemotactic: (i) the bacteria were morphologically normal in cell shape

and flagellar structure, (ii) they did not swarm on a semisolid agar surface, (iii) they did not respond to any of several known chemoattractants, and (iv) they could swim but with less frequent tumbling than did the wild-type strain.

These mutants did not colonize the mouse intestinal tract even when 5×10^7 CFU of the bacteria per mouse was orally inoculated, although the wild-type strain colonized with only 10^2 CFU per mouse. This efficiency of colonization is almost the same as that of nonflagellated mutant strains (19). For colonization of the mouse intestinal tract, the active movement mediated by the flagella was found to be an important factor, and the antiflagellar antibody has the capacity to reduce colonization of the mouse intestinal tract by this bacterium (19, 23). These observations, however, did not completely eliminate the possibility that the flagellum filament itself acts as an adhesin for intestinal cells (18). The observations that we made in this study definitely support the idea that chemotactic movement is the major colonization factor for C. jejuni.

Bacterial movement is generally controlled by chemotactic responses, and for infection by several species of motile bacteria, chemotactic movement is reported to have an important role (2, 22). Of the mechanisms of colonization, the role of chemotactic movement is not thoroughly understood.

Since the surface of the intestinal tract is covered with a thick layer of mucus, the following steps are thought to be involved in the establishment of colonization of the intestinal tract (11): adhesion to the surface of the mucus gel, penetration into the mucus gel, and attachment to the intestinal epithelial cells. Lee et al. showed in a gnotobiotic or germ-free adult mouse model that C. jejuni colonizes the intestinal tract via association with the intestinal mucus layer on the surface mucosa or in the intestinal crypts and does not adhere to the epithelial cells (15). Analysis of colonization of the rabbit intestinal surface by V. cholerae suggested that chemotactic motility has an important role in adhesion to and penetration of the mucus (9, 12). Like V. cholerae, C. jejuni could require chemotactic movement for effective colonization of the intestinal mucus layer. It is conceivable that more chemoattractant might be presented in the mucus layer than in the gut space, and this gradient of the concentration of the attractant could be the driving force for colonization by this bacterium.

ACKNOWLEDGMENTS

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