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Campylobacter jejuni Motility and Invasion of Caco-2 Cells

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We investigated the influence of motility on *Campylobacter jejuni* binding and invasion of Caco-2 cells. *C. jejuni* was motile in soft agar at basic (pH 8.5) and neutral pH values representative of the intestinal environment. However, *C. jejuni* was immobilized at pH 5.0. The inability of *C. jejuni* to swarm on soft agar at pH 5.0 was not related to flagellar depolymerization or loss of viability. In tissue culture medium, *C. jejuni* displayed typical periods of straight swimming punctuated by tumbling behavior. This behavior was altered when the viscosity of the medium was adjusted to mimic the viscosity of intestinal mucus. *C. jejuni* showed longer periods of straight swimming with significantly increased velocity followed by pauses instead of tumbles. The binding and invasion of *C. jejuni* in Caco-2 cells also increased significantly in high-viscosity growth medium. We speculate that the swimming behavior of *C. jejuni* in a viscous environment may be an important factor in the interaction of these organisms with host epithelial cells. The pH, which affects *C. jejuni* motility, may also influence the tropism of these organisms.

The symptoms associated with *Campylobacter jejuni* enteritis range from cholera-like excretory diarrhea to shigella-like dysentery. The latter symptoms are reminiscent of an invasive infection, yet there is much skepticism over whether *C. jejuni* can invade eukaryotic cells. Early work by Bukholm and Kapperud demonstrated that *C. jejuni* invaded HEp-2 and A549 cells only when coinfected with other enteropathogenic bacteria, such as *Salmonella* or *Shigella* species or *Escherichia coli* (6). de Melo et al. showed, however, that *Campylobacter* species could exist alone intracellularly in HEp-2 cells (7). Konkel and Joens also found that *Campylobacter* species were invasive in HEp-2 cells (13). Nonetheless, they demonstrated higher levels of HEp-2 cell invasion when *C. jejuni* was coinfected with enteroviruses (14).

The cell lines used in many of the early *Campylobacter* invasion experiments were derived from epidermoid carcinoma of the human larynx (HEp-2) and carcinoma of the human lung (A549). *Campylobacter* spp. may not have invaded these cells efficiently because they do not accurately mimic intestinal epithelial cells. The most convincing data were obtained by Konkel et al. (15). They infected polarized Caco-2 cells, originally derived from a human colon adenocarcinoma. They showed that *C. jejuni* can invade Caco-2 cell monolayers to the levels found for *Salmonella* species and was able to translocate across Caco-2 polarized monolayers.

The results of animal studies of *Campylobacter* enteritis suggest that invasion is a key mechanism in pathogenesis. These model studies include work with infant chickens (21, 24, 30), infant mice (20, 31), newborn piglets (3), and infant monkeys (23). Finally, Van Spreeuwel et al. demonstrated intracellular *C. jejuni* in colonic epithelial cells from patients with colitis and positive stool samples for *C. jejuni* (28). It is now generally accepted that *Campylobacter* species can invade intestinal epithelial cells in vivo.

The *Campylobacter* literature clearly shows that flagella are significant virulence determinants. Yet, the role of flagella in pathogenesis is poorly understood. Some investigators believe flagella may act as adhesins, while others believe flagella play a role in *Campylobacter* penetration of host epithelial cells.

To address some of the remaining deficiencies in our understanding of pathogenic mechanisms of *Campylobacter* organisms, we have taken a holistic approach to examining *C. jejuni* binding and invasion of eukaryotic cells. Our studies involved examination of the effects of pH and viscosity on *C. jejuni* swimming behavior and binding and invasion of Caco-2 cells. The motivation for the studies was to establish a more reliable, in vitro model system for investigating the interactions of *Campylobacter* organisms and perhaps other enteropathogens with epithelial cells of the gastrointestinal tract.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* E863 and ER1109 were obtained from the Provincial Laboratory of Northern Alberta. *C. jejuni* UA580 and UA581 were kindly provided by D. E. Taylor, University of Alberta. *Salmonella enteritidis* 710063 was kindly provided by M. Finlayson, University of Alberta.

Campylobacter organisms were stored in brain heart infusion (BHI [Difco Laboratories]) broth with 10% glycerol (BDH) at −70°C. Cultures were plated on supplemented blood agar plates (Triage Microbiological Systems, Ardrossan, Alberta, Canada) overnight at 37°C under microaerophilic conditions (10% CO_2, 5% O_2, 85% N_2). The organisms were then inoculated into BHI broth and incubated overnight, with constant shaking, under the same conditions. After centrifugation at 6,000 × *g* the bacteria were resuspended in BHI broth and used in further experiments.

S. enteritidis was maintained on BHI agar. Experiments were conducted with overnight cultures prepared in BHI broth.

Culture of Caco-2 cells. The human epithelial cell line Caco-2 (American Type Culture Collection, HTB 37) was originally derived from a human colon adenocarcinoma and is widely employed in studies of pathogen-host cell interactions because of its ability to form well-differentiated cell monolayers. The cell line is very similar to small intestinal enterocytes with respect to their structure, brush border enzymes, and time courses of differentiation.

Caco-2 cells were grown at 37°C in minimal essential medium (MEM [Gibco Laboratories]) supplemented with 10% (vol/vol) fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 and 95% air. Confluent cells were harvested by trypsinization in 0.01% EDTA (BDH) and plated in a 24-well Multiwell (Falcon) tissue culture plate. Polarized Caco-2 cell monolayers were used after 10 to 14 days of growth, similar to those used by Konkel et al. (15).

Effect of pH on *Campylobacter* motility. The effect of pH on the motility of *C. jejuni* UA580, E863, ER1109, and UA581 was investigated with BHI agar (0.4% [wt/vol]) plates adjusted to pH 5.0, 7.3, and 8.5. Five microliters of culture was inoculated into the center of the plates, and the diameter of the resulting swarms was measured the next day.

C. jejuni UA580 was also inoculated into BHI broth, adjusted to pH 5.0, 7.3, and 8.5, and incubated overnight. The next day, the organisms were negatively

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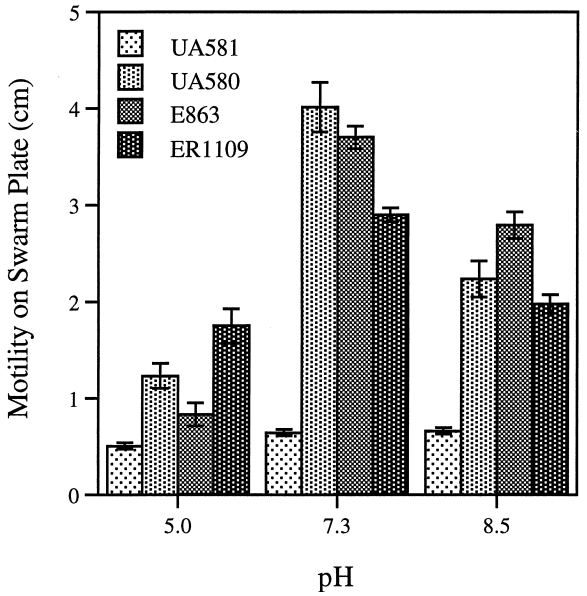

FIG. 1. *Campylobacter* motility on swarm plates at various pH values. Inocula: UA581, $1.0 \times 10^7 \pm 0.06 \times 10^7$; UA580, $1.1 \times 10^7 \pm 0.25 \times 10^7$; E863, $0.75 \times 10^7 \pm 0.16 \times 10^7$; ER1109, $0.75 \times 10^7 \pm 0.10 \times 10^7$.

stained and observed by transmission electron microscopy. The pH of the broth was also checked to ensure that there was no change. The viability of the organisms was determined by plating of serial dilutions at 5-h intervals throughout the incubation period.

Invasion and binding assays. Caco-2 cell monolayers in MEM-FBS or MEM-FBS and carboxymethylcellulose (CMC [Sigma]) were infected with approximately 10^9 *C. jejuni* organisms for 5 h at 37°C in a humidified atmosphere. Caco-2 cell monolayers were infected with *S. enteritidis* for 2 h. The infected cells were then washed and incubated with 100 μg of gentamicin (Gibco BRL) per ml in MEM-FBS at 37°C for 1 h to kill any extracellular organisms. The cells were washed again and treated with 1% Triton X-100 (Sigma) in PBS at 37°C for 5 min to release intracellular organisms. Dilutions from each well were plated on BHI agar. We have previously determined that the Triton X-100 treatment does not affect the viability of *C. jejuni.*

The total number of organisms bound was determined simultaneously by performing the invasion assay but excluding gentamicin treatment. Since the modified version of the assay yielded the number of organisms bound plus internalized, the difference between the total number bound and the total number internalized was taken to give the number of bound organisms.

Viscosity measurements. Viscosity measurements were done with a Cannon-Fenske routine type viscometer no. 475. All measurements were done at 37°C. The kinematic viscosity of the sample, in centistokes, was obtained by multiplying the efflux time, in seconds, by the viscometer constant at 37°C. To determine the viscosity in centipoise (cP), the kinematic viscosity was multiplied by the density of the solutions in grams per milliliter.

Bacterial vibrational frequency and velocity measurements. *C. jejuni* UA580, E863, and ER1109 were grown in BHI broth in a microaerobic environment overnight at 37°C with constant shaking. *E. coli* and *S. enteritidis* standing cultures were grown in BHI broth overnight at 37°C.

The next day, the organisms were added to a 24-well tissue culture plate containing MEM-FBS alone or MEM-FBS with 0.6% (wt/vol) CMC and then incubated for 5 h at 37°C in a humidified atmosphere. A small drop of each culture was placed under a sealed coverslip on a microscope slide. Video recordings of the bacteria were obtained with a Carl Zeiss microscope, a JVC CCD camera, and a Sony SL-2500 video cassette recorder. When the videotape was played back on the monitor, the tape speed was manually slowed to allow measurement of bacterial vibrational frequencies and traveling velocities.

Tracing bacterial movements. Photographs of the videotape were taken with an Appligene high-performance CCD imaging system at intervals of 0.2 s. The images were saved in tagged image file format (TIFF) and analyzed.

RESULTS

Effect of pH on *Campylobacter* motility. The control strain (UA581) used in the motility experiments was a nonmotile,

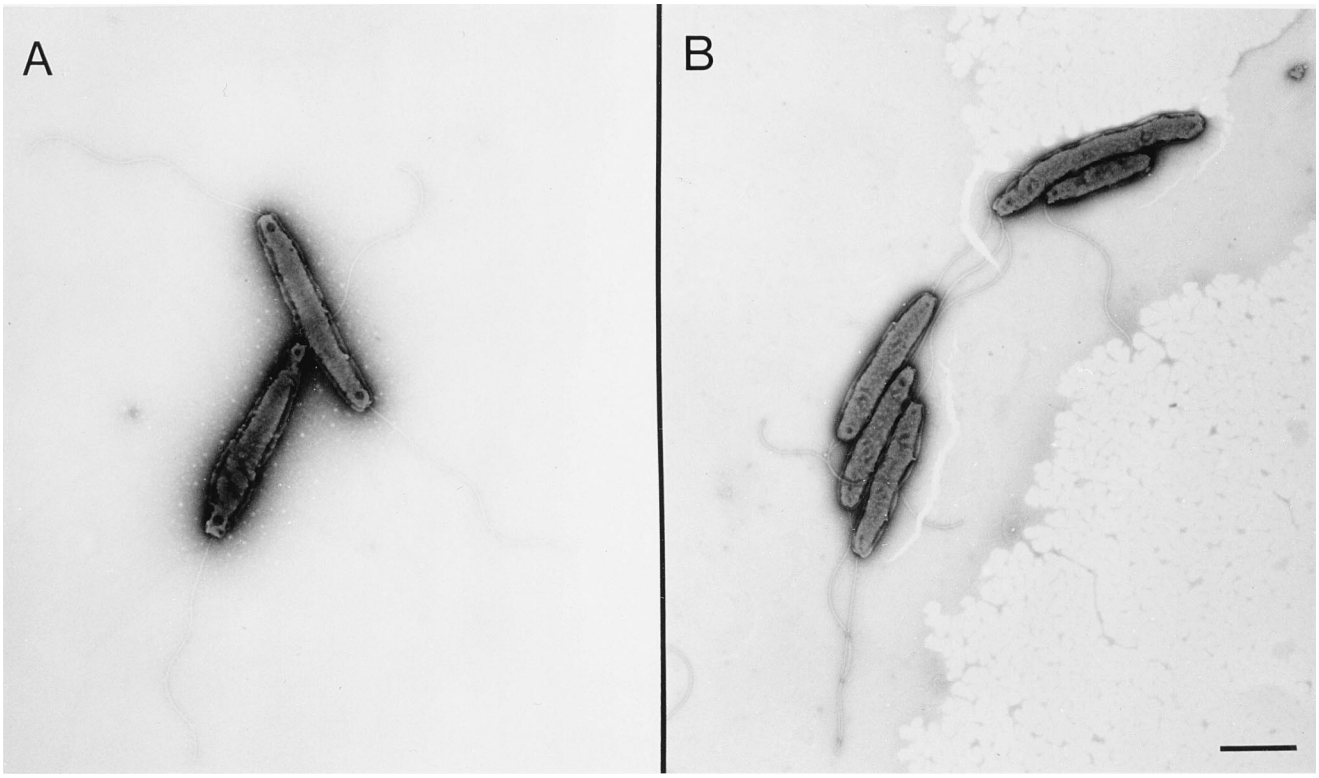

FIG. 2. Electron micrographs illustrating the morphology of overnight cultures of *C. jejuni* UA580 grown at pH 5.0 (A) and pH 7.3 (B). Note that the flagella remain intact without any observable deformations. The solid bar represents 1 μm.

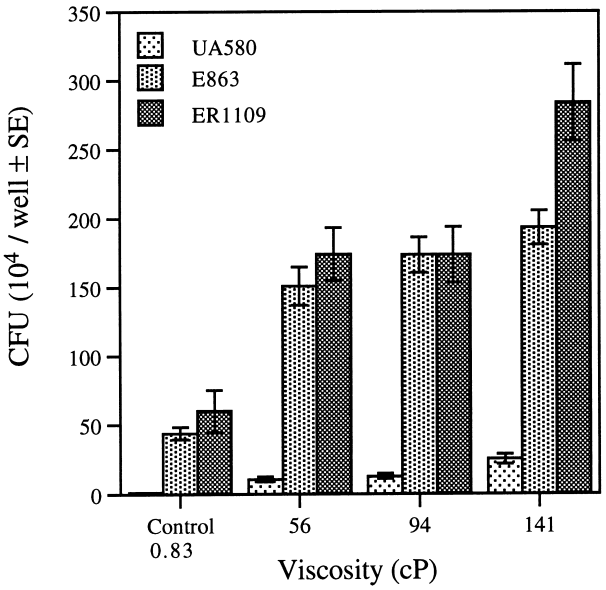

FIG. 3. Concentration-dependent increase in *C. jejuni* invasion of Caco-2 cells in CMC-supplemented MEM solutions. Control represents *C. jejuni* invasion of Caco-2 cells in MEM alone (conventional invasion assay). Mean numbers of CFU per well were determined from duplicate sets of wells per experiment. Each experiment was done in duplicate. All increases in invasion were determined to be statistically significant ($P < 0.05$).

nonflagellated *C. jejuni* strain. We included this strain to monitor nonpropulsive bacterial motion on motility agar. The other strains were motile and flagellated. Strain UA580 was a laboratory strain passaged multiple times since its isolation. The other strains were recent clinical isolates. We found that lowering the pH from 7.3 to 5.0 decreased the ability of all three fla^+ mot^+ *C. jejuni* strains to swarm on motility agar (Fig. 1). Electron microscopy observations revealed that the morphology of the UA580 flagella was unaltered at pH 5.0 (Fig. 2). Moreover, the viability of the *C. jejuni* strains did not decrease at the lower pH levels. Increasing the pH to 8.5 had little effect on *C. jejuni* motility.

Binding and invasion in viscous solutions. We speculated that although rapidly swimming *C. jejuni* organisms may make frequent contact with the Caco-2 cells, the rapid movement of the organisms may prevent them from becoming firmly attached to the surface of these cells. If the organisms were moving more slowly, *C. jejuni* may make less frequent contacts with the Caco-2 cells, but more of these collisions may result in irreversible binding and internalization.

To test this hypothesis, we modified the standard tissue culture invasion assay so it more closely resembled the conditions in the human intestines. We specifically wanted to reproduce the physical characteristics of the mucoid secretions covering intestinal epithelial cells. We chose to use CMC because it is more chemically defined than purified mucin, is nontoxic to tissue culture cells, and approximates the viscosity of intestinal mucus.

As predicted by our hypothesis, *C. jejuni* invasion increased when we used CMC to mimic the viscosity of intestinal mucus on the Caco-2 cells (Fig. 3). However, the binding of *C. jejuni* also increased (Fig. 4). We also examined *S. enteritidis* in the conventional and modified Caco-2 invasion assay. In contrast to *C. jejuni*, binding and invasion of *S. enteritidis* did not change in CMC-supplemented medium (Fig. 4).

Effect of viscosity on motility. To examine the relationship between viscosity and the swimming behavior of the microorganisms, we used a videotaping system to record the movements of *C. jejuni* and *S. enteritidis* in regular and high-viscosity culture medium. In regular tissue culture medium, all of the bacteria displayed typical smooth swimming behavior (runs) punctuated by periods of tumbling (Fig. 5). However, *S. enteritidis* traveled more slowly for shorter distances during periods of smooth swimming than any of the *C. jejuni* isolates.

In viscous solutions, *C. jejuni* and *S. enteritidis* showed smooth swimming patterns punctuated by pauses in motion rather than tumbling behavior. At higher viscosities, *C. jejuni* exhibited a darting motility. During periods of smooth swimming, *C. jejuni* traveled much farther with greater velocity in viscous medium (Fig. 6). In contrast, the distance traveled and velocity of *S. enteritidis* were significantly lower during periods of smooth swimming in viscous solutions.

DISCUSSION

Motility should play an important role in *Campylobacter* colonization, since the organism has to penetrate mucus in order to adhere to and invade surface epithelial cells. *C. jejuni* is motile by means of one or two polar flagella. The organism contains two copies of the flagellin gene. The expression of these genes is controlled by two unique promoters, at least one of which responds to environmental signals (1). The evolution of this complex flagellar expression system in *C. jejuni* emphasizes the importance of motility to its survival.

Increases in viscosity resulting in increased bacterial motility have previously been described as a general behavioral phenomenon in bacteria (10, 25, 27). However, the low viscosity range at which these observations were made is unrepresenta-

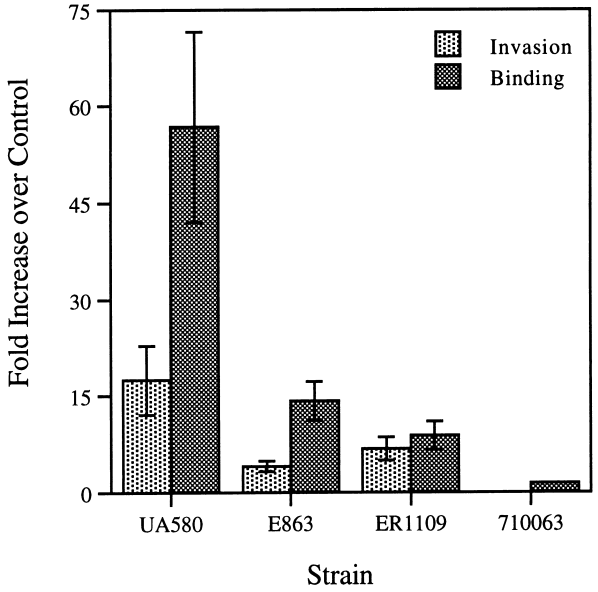

FIG. 4. Comparison between binding and invasion in 141-cP (0.6% [wt/vol]) CMC solutions. *S. enteritidis* 710063 is also shown. Mean numbers of CFU per well were determined from duplicate (invasion) or triplicate (binding) sets of wells per experiment. Each experiment was done in duplicate or triplicate. The fold increase over the control was determined (± standard error). Control values for binding and invasion, respectively, are as follows: UA580, $56.0 \times 10^5 \pm 7.2 \times 10^5$ and $14.7 \times 10^4 \pm 2.3 \times 10^4$; E863, $14.8 \times 10^7 \pm 2.7 \times 10^7$ and $27.1 \times 10^5 \pm 3.5 \times 10^5$; ER1109, $55.2 \times 10^6 \pm 12.9 \times 10^6$ and $38.9 \times 10^5 \pm 4.7 \times 10^5$; 710063, $13.2 \times 10^6 \pm 1.4 \times 10^6$ and $11.1 \times 10^4 \pm 1.8 \times 10^4$. All changes in *Campylobacter* binding and invasion were determined to be statistically significant ($P < 0.05$).

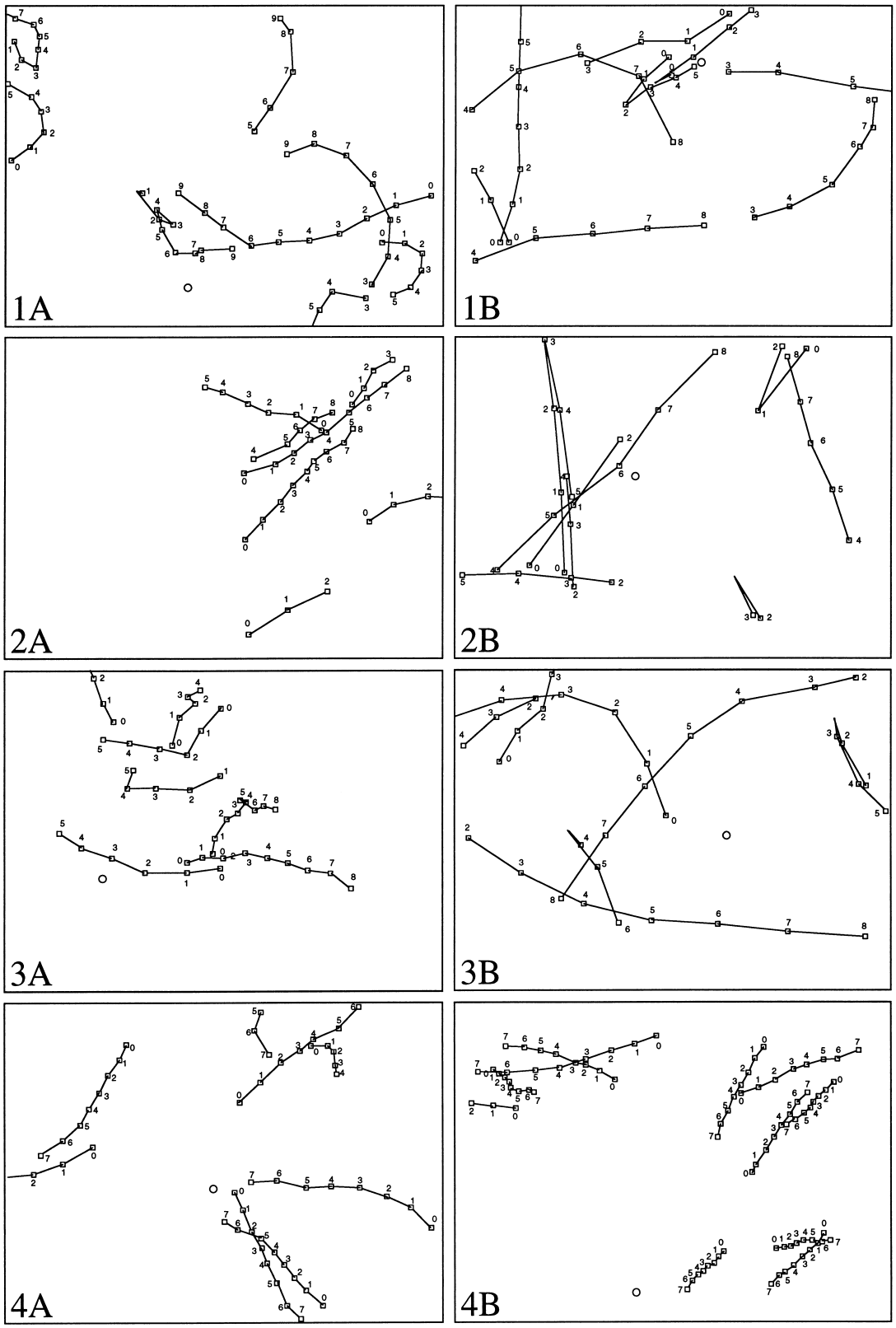

FIG. 5. Movements of *C. jejuni* (panels 1 to 3) and *S. enteritidis* (panels 4) in regular medium (A) and viscous medium (0.6% [wt/vol] CMC) (B). Photographs of the videotape were taken at intervals of 0.2 s, and the positions of the bacteria at each interval are designated by squares. The circle represents the reference point on the television screen. (1A and B) *C. jejuni* UA580. (2A and B) *C. jejuni* E863. (3A and B) *C. jejuni* ER1109. (4A and B) *S. enteritidis* 710063.

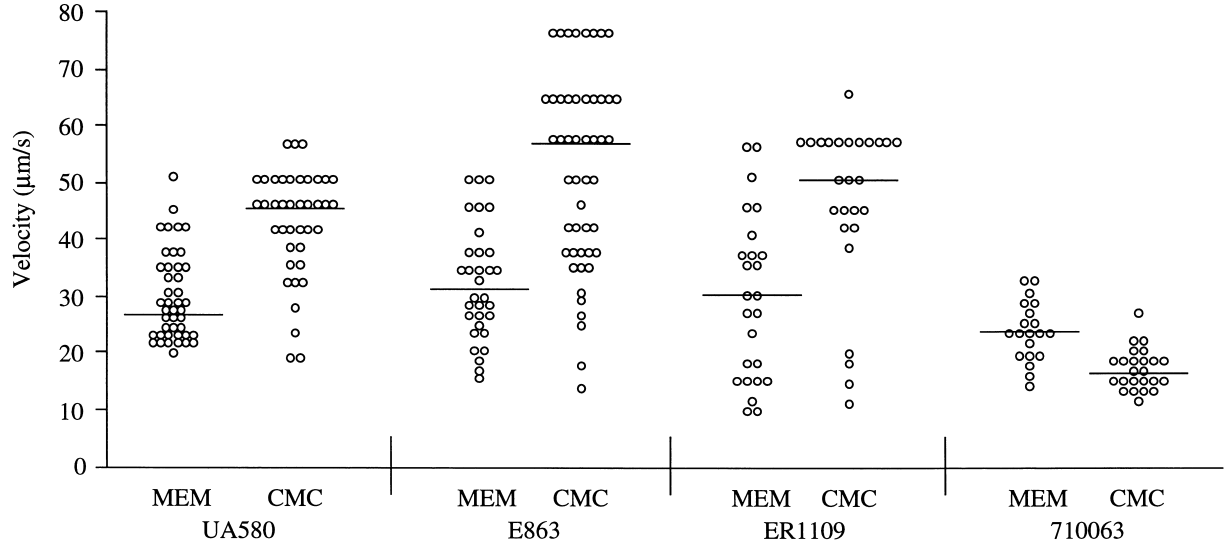

FIG. 6. Velocity of *C. jejuni* UA580, E863, and ER1109 and *S. enteritidis* 710063 in regular tissue culture medium (MEM) and viscous medium (0.6% [wt/vol] CMC-MEM). The median of each group of datum points is shown.

tive of the viscosity of mucoid secretions found in the gastrointestinal tract. Ferrero and Lee compared the motilities of *C. jejuni* and conventional rod-shaped bacteria in a viscous environment (9). Although they observed that *Campylobacter* organisms were motile in viscous, mucus-like solutions that immobilized other bacteria, they did not extend their investigations to examining the effect of viscosity on infectivity (9). Other groups described the effect of mucus or mucin on the pathogenesis of *C. jejuni*, but these investigations were directed at determining whether mucus components mediated bacterial attachment to epithelial cells (8, 18, 19).

In our experiments, we examined the physical effect of viscosity on attachment and invasion of *C. jejuni* and *S. enteritidis* in Caco-2 cells. However, in determining the impact of viscosity on the behavior of microorganisms, it is necessary to account for the effect of motility on the viscosity of the solution through which the bacteria are traveling. As microorganisms swim, they vibrate with a characteristic frequency that applies a strain to the viscous solution. With any gel-like material, the apparent viscosity varies with the strain (12). In practical terms, this means that different microorganisms vibrating at different frequencies may actually behave as if they were in solutions of quite different viscosity. The corollary to this is that microorganisms modify the viscosity of a solution as they swim through it.

The vibrational frequency of the organisms used in our experiments was determined from the videotape records to be approximately 20 to 30 Hz. In experiments by Mantle et al., Sellers et al., and Bell and Allen, the viscosity of reconstituted intestinal mucus ranged between 178 and 316 cP at 16 Hz and could be estimated to be approximately 140 cP at 30 Hz, on the basis of the typical frequency dependence in this range (4, 17, 26). Therefore, although we cannot be certain of the actual viscosity experienced by *C. jejuni* and *S. enteritidis*, this should be similar for each of them. As a control, we also measured the vibrational frequency of *E. coli* (5 to 10 Hz), which we found to be similar to the frequency of *E. coli* (8 to 13 Hz) determined by Berg and Turner (5).

In the presence of viscous solutions, the invasion of *C. jejuni* increases as a result of increased attachment. We believe that increases in attachment may be due to more contacts or, perhaps, the impact between *Campylobacter* organisms and the host cell surface. Berg and Turner showed that viscous solutions form a highly structured network which can be easily penetrated by microscopic organisms (5). From our videotaping observations, *C. jejuni* can easily maneuver through the gel matrix of a 0.6% (wt/vol) CMC-MEM solution. The velocity of *C. jejuni* in CMC-MEM was significantly greater than its velocity in MEM alone. In contrast the velocity of *S. enteritidis* significantly decreased in CMC-MEM.

Lee et al. examined scrapings of mucosa from cecal tissue of infected mice (16). They also found that *C. jejuni* was highly motile in the tissue. Interestingly, the organisms moved extremely rapidly across the field of view in parallel streams. The bacteria seemed to track along the mucus strands. Ferrero and Lee measured the path lengths of *C. jejuni* in two solutions of different viscosities (9). They found an abrupt increase in the proportion of cells that displayed longer path lengths during smooth swimming in a high-viscosity medium. It is obvious from our tracings that all three strains of *C. jejuni* also showed longer swimming path lengths in viscous solutions. In contrast, movement of *S. enteritidis* was reduced in solutions of similar viscosity, as has been previously described (9). We suggest that the increased directionality of *Campylobacter* motility in viscous solutions may favor an increase in the frequency of collisions with host epithelial cells. This phenomenon would increase the amount of contacts between the organisms and the host cells and therefore increase binding.

It was previously believed that *Campylobacter* flagella acted as adhesins. However, Wassenaar et al. then found that antibodies directed against the flagella did not inhibit attachment to INT-407 cells (29). McSweegan and Walker found that sheared flagella were not effective in blocking the attachment of whole *C. jejuni* cells to INT-407 cells (19). Later, several laboratories demonstrated that flagella and/or motility is required for the internalization of *C. jejuni* in vitro and in vivo (2, 22, 29, 31, 32).

The motility of an organism can be broken down into two components: translational motion, represented by velocity, and vibrational motion, represented by frequency. Flagella rotate about their long axes with a measurable frequency (11). We speculate that *Campylobacter* species may have adopted a

unique form of motility that allows the organisms to function efficiently in a viscous medium such as mucus. Greenberg and Canale-Parola suggested that certain motile bacteria were able to swim through viscous environments because they may possess a specialized motility apparatus (10). Ferrero and Lee also described two mechanisms of motility for *C. jejuni* (9). At low viscosities, they observed that *Campylobacter* organisms behaved much like other flagellated bacteria. The organisms relied primarily on their flagella for propulsion. At higher viscosities, Ferrero and Lee speculated that *C. jejuni* was able to overcome the problem of "flagellar dampening" caused by viscous media by developing a different mechanism of motility. While other organisms have a low minimum inhibitory viscosity, *Campylobacter* organisms remain motile in highly viscous solutions (i.e., >100 cP) (9). Also, *Campylobacter* organisms move much faster than other intestinal bacteria.

The importance of motility to *C. jejuni* has already been emphasized. The organism contains two copies of the flagellin gene, at least one of which is independently and environmentally regulated. The organism has repeatedly been shown to be well adapted to movement in viscous environments. Our results also showed that although the flagella remain intact without any observable morphological changes, the organisms lose motility but remain viable at pH 5. Thus, even if the organisms remain viable in an acidic environment, such as that in the stomach, *C. jejuni* may not be able to initiate an infection in this environment. It has been shown that expression of *flaB* is reduced when the organisms are grown at pH 5 at 42°C (1). However, when *C. jejuni* was grown at 37°C, *flaB* expression slightly increased (1). Since our experiments were done at 37°C, it is unlikely that changes in *flaB* expression contributed to the reduced motility of *C. jejuni* at pH 5.

Our results suggest that the motility of *C. jejuni* may play several key roles in pathogenesis: (i) involvement in tissue tropism, (ii) transport through mucus toward surface epithelial cells, and (iii) increases in the efficiency of attachment and invasion of host cells. Experiments are currently under way to further elucidate the role of *C. jejuni* motility in pathogenesis.

ACKNOWLEDGMENTS

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