

Role of Chemotaxis in the Association of Motile Bacteria with Intestinal Mucosa: In Vivo Studies

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In vivo loops were prepared in the small intestine of rabbits and injected with mixtures of *Vibrio cholerae* and polystyrene spheres (1.1- μ m diameter). The loops were removed and frozen after 15 min and then sectioned in a cryostat. The locations of particles and vibrios were determined microscopically. The vibrio/particle ratio was unity in the lumen of the loops, but increased 10-fold in the deep intervillous spaces, indicating active invasion of the mucus gel by the chemotactic parent strain. Motile nonchemotactic mutants and nonmotile mutants of this strain invaded the mucus at the same rate as inert particles. Similar results were obtained with intestinal loops prepared in germfree mice. When germfree mice were diassociated with mixtures of chemotactic (parent or revertant) and nonchemotactic mutant vibrios in equal proportions, the chemotactic strain rapidly outgrew its nonchemotactic counterpart in the intestine. Nonchemotactic mutants introduced as monoassociates into germfree mice were rapidly overgrown by nonmotile mutants which apparently arose spontaneously in the gut. Motility was therefore beneficial to survival only when it was directed by chemotactic stimuli, whereas it was a liability in the absence of such stimuli. Growth of chemotactic vibrios in small intestinal loops of rabbits paralleled that of nonchemotactic mutants for the first 4 to 6 h. Thereafter, the growth rate of the chemotactic vibrios was significantly faster. This was correlated with a significantly higher degree of association with the mucosa on the part of the chemotactic vibrios.

The preceding paper in this series (2) has presented data showing that motile bacteria guided by chemotactic stimuli can actively penetrate the mucus gel of slices of intestinal tissues. The present article deals with the obvious corollary to that study, namely, whether similarly enhanced penetration of mucus gel also occurs under in vivo conditions and, if so, whether this phenomenon confers an ecological advantage on motile, chemotactically active bacteria. As will be seen, both questions can be answered in the affirmative.

(These data have been presented at the 13th and 14th Symposia on Cholera of the U.S.-Japan Cooperative Medical Science Program. [Freter et al., Proceedings of the 13th Joint Conference on Cholera, The U.S.-Japan Cooperative Medical Science Program, p. 152-181, 1978, U.S. Government Printing Office, Washington, D.C.; Freter et al., in K. Takeya and Y. Zinnaka, ed., Proceedings of the 14th Joint Conference, U.S.-Japan Cooperative Medical Science Program, Japanese Cholera Panel, Tokyo, Japan].)

MATERIALS AND METHODS

Vibrio strains. The parent and nonchemotactic mutants of *Vibrio cholerae* are described in an accom-

panying paper (4). Inocula for animal experiments were overnight broth cultures in Trypticase soy broth without glucose (BBL Microbiology Systems). These cultures were inoculated from individual single colony isolates of the stock cultures.

Yeast cells. *Saccharomyces cerevisiae* was grown overnight at 37°C on Sabourand-dextrose agar, washed off in saline, and heated to 60°C for 30 min.

Krebs-Ringer-Tris buffer. Krebs-Ringer-Tris buffer was identical to that used for in vitro studies of chemotaxis (4), except that the Triton X-100 was omitted.

Mice. The germfree mice were Charles River strain CD-1, maintained in Trexler-type plastic isolators. The animals were infected by introducing the inoculum either directly into the stomach or by contaminating the drinking water. At intervals after infection the animals were sacrificed, the entire ceca were homogenized in a Virtis blender, and colony counts of parent and nonchemotactic mutants were determined in semisolid agar as described in an accompanying paper (4).

Acute intestinal loops in rabbits. Acute intestinal loops were prepared as described earlier (1). Loops were inoculated with a mixture containing 2×10^6 cells each of the parent vibrio and nonchemotactic mutant 31. At various times after inoculation loops were removed, rinsed gently with 10 ml of sterile broth, and homogenized in a Waring blender. Colony counts of the parent and the mutant in the homogenate of

washed intestine and in the separately homogenized rinse fluid were then determined in semisolid tryptone agar, as described in an accompanying publication (4).

Invasion of intestinal mucus gel. Six-month-old female germfree mice or adult Dutch belted male rabbits were anaesthetized with approximately 3 mg and 75 mg of pentobarbital, respectively. The drug was given in divided doses until complete narcosis was obtained.

The germfree mice were removed from their isolator immediately before the start of an experiment. Two to four loops approximately 2.5 cm long were prepared in a region of the small intestine that was approximately equidistant from stomach and cecum. The loops were injected with a mixture of three components: (i) 5×10^8 polystyrene particles (1.1- μ m diameter), (ii) 5×10^8 cells of *S. cerevisiae*, and (iii) 5×10^8 cells of one of the vibrio strains. The inoculum was contained in 0.05 ml of Krebs-Ringer-Tris buffer. In rabbits the inoculum consisted of 0.25 ml of Krebs-Ringer-Tris buffer containing 7.5×10^8 each of the above three components; the inoculum was injected into small intestinal loops approximately 6 cm long prepared at a site equidistant between stomach and cecum.

The first two components of the inocula were the same in all experiments. The third component (i.e., the vibrio) was either (i) the parent strain, (ii) a motile, nonchemotactic mutant, or (iii) a nonmotile mutant. At 15 min after inoculation the loops were removed from the animals, frozen in liquid nitrogen, sectioned in a cryostat, stained without fixation for 5 s with 1% Giemsa stain, and washed for approximately 1 s in tap water (2). This rapid staining procedure with concentrated dye was necessary to prevent loss of mucus gel from the preparations. The sections were observed at $\times 500$ magnification. Particles and microorganisms were counted in the mucus gel overlying the villi and in the spaces between villi. The latter spaces were divided by inspection into two areas of equal length (luminal half and basal half), and counts were recorded separately for each. In view of the fact that exact quantitation of bacteria in histological sections is very difficult, the ratio between the number of vibrios and polystyrene particles was chosen as the significant parameter for comparing the parent with the two mutant strains.

RESULTS

Table 1 presents the data of an experiment testing the penetration of mucus gel in rabbit intestinal loops. As described above, the loops had been injected with a triple mixture of polystyrene indicator particles, yeast cells, plus one vibrio strain (either the parent or a nonchemotactic or nonmotile mutant). When the parent strain was used, the ratio of vibrios to polystyrene particles increased from approximately unity in the inoculum to 9.99 when the mucus gel at the base of the villi was scored (Table 1). This indicates that the parent strain was considerably more efficient than the inert particles in penetrating mucus gel. Similar results were noted when the vibrio/yeast cell ratios were calculated (not shown in Table 1). In contrast, no consistent

TABLE 1. Penetration of vibrios into the mucus gel of rabbit intestinal loops inoculated with a suspension of the bacteria, yeast, and indicator particles

Vibrio strain	Vibrio/particle ratio ^a in:			
	Inoculum	Mucus gel		
		Above villi	Between villi (luminal half)	Between villi (basal half)
Parent	1.03 (31)	1.22 (442)	4.62 ^b (833)	9.99 ^b (349)
Nonchemotactic	1.07 (30)	1.00 (427)	3.08 (474)	1.72 (83)
Nonmotile	0.97 (31)	1.08 (338)	1.25 (171)	1.09 (42)
Control ^c		0.11 (39)	0.43 (55)	0.59 (17)

^a Corrected for ratio of control slices. Total number of vibrios is shown within parentheses.

^b Difference from ratio of nonchemotactic strains is highly significant ($P \ll 0.001$).

^c The inoculum of the control loops contained polystyrene particles and yeasts, but no vibrios.

changes in vibrio/particle ratios occurred in experiments where the motile but nonchemotactic or the nonmotile mutants were used instead of the parent strain (Table 1). As with similar *in vitro* experiments with intestinal slices reported elsewhere (2), one must conclude that the superior ability of the parent strain to penetrate intestinal mucus gel *in vivo* was related to its active motility guided by chemotaxis along a taxin gradient which extends deeply into the mucus gel.

In view of the fact that the rabbits used in these experiments were conventional and therefore harbored indigenous bacteria, it was necessary to determine to what extent the above results might have been affected by mistakenly including such bacteria or other artifacts among the counts of vibrios. For this purpose intestinal loops were inoculated as in the preceding experiments, except that the vibrios were omitted from the inoculum. The ratios of "vibrios" (i.e., other bacteria or artifacts) to particles were then determined in the usual manner, counting the same number of microscopic fields as in the preceding experiments. As may be seen (Table 1) other bacteria or artifacts that could be mistaken for vibrios were too few in number to significantly affect the ratios of vibrios to particles calculated in the preceding experiments. Nevertheless, the fraction of vibrio-like structures determined in these controls has been deducted from the ratios of vibrios in the other experimental groups shown in Table 1.

The data in Table 2 represent yeast/polystyrene particle ratios in the same preparations and microscopic fields as those shown in Table 1. As may be seen, the yeast/particle ratios decreased slightly with increasing penetration of the mucus gel. This indicates that the observed differences

in vibrio/particle ratios in the various areas of the mucosa (Table 1) were real rather than artifacts that might have been caused by shifts in the mucus gel during preparation or staining of the sections or by other factors unrelated to the nature of the vibrio strains.

Tables 3 and 4 show data obtained with germ-free mice analogous to those presented for rabbits in Tables 1 and 2. As may be seen, similar trends were observed indicating that a taxin gradient attracted motile, chemotactically reactive vibrios into the deep layers of small intestinal mucus gel of this animal species in the same manner as had been observed in the rabbit. In some experiments of this type the ratio of nonchemotactic vibrios to particles was somewhat elevated in the luminal half of the mucus gel, a difference which in some instances reached statistical significance (e.g., $P < 0.01$ in Table 1). The phenomenon was not observed consistently, however (e.g., Table 3), in contrast to the consistent increase in vibrio/particle ratio that was observed with high statistical significance in all

in vivo and in vitro (2) experiments of this type that involved chemotactic vibrios. The significance of this observation, if any, is not clear. One possible explanation may be that nonchemotactic vibrios accumulate in the more superficial layers because of weak adherence to mucus gel, in contrast to nonmotile vibrios which also lack adhesive capacity (4, 8). This phenomenon certainly deserves further study.

To compare the fitness of chemotactic and nonchemotactic vibrios in vivo, germfree mice were associated with mixtures of the parent (or revertant) strain plus a nonchemotactic mutant in approximately equal proportions. The bacteria were either added to the drinking water or were introduced directly into the stomach in 0.5 ml of 3.5% NaHCO_3 . All three spontaneous mutants were used in different experiments. All nonchemotactic mutants reacted similarly, and the chemotactic revertant strain behaved like the chemotactic parent strain. The data obtained in several experiments were therefore pooled. These are illustrated in Fig. 1. It is

TABLE 2. Penetration of yeast cells and polystyrene indicator particles into the mucus gel of rabbit intestinal loops inoculated with a mixed suspension^a

Vibrio strain	Yeast/particle ratio ^b in:			
	Inoculum	Mucus gel		
		Above villi	Between villi (luminal half)	Between villi (basal half)
Parent	1.07 (32)	1.64 (545)	0.67 (111)	0.85 (28)
Nonchemotactic	1.11 (31)	1.49 (571)	0.72 (97)	0.56 (20)
Nonmotile	0.91 (29)	1.65 (466)	0.78 (80)	0.48 (12)
Control ^c	0.97 (32)	0.91 (370)	0.50 (64)	0.48 (14)

^a Results are based on the same histological sections scored in Table 1.

^b Not corrected for ratio of control slices. Total number of yeast cells is shown within parentheses.

^c The inoculum of the control loops contained polystyrene particles and yeasts, but no vibrios.

TABLE 4. Penetration of yeast cells and polystyrene indicator particles into the mucus gel of germfree mouse intestinal loops inoculated with a mixed suspension^a

Vibrio strain	Yeast/particle ratio ^b in:			
	Inoculum	Mucus gel		
		Above villi	Between villi (luminal half)	Between villi (basal half)
Parent	0.45 (43)	0.33 (288)	0.24 (119)	0.047 (11)
Nonchemotactic	0.51 (48)	0.36 (339)	0.33 (135)	0.063 (11)
Nonmotile	0.47 (49)	0.37 (320)	0.34 (145)	0.051 (13)
Control ^c	0.48 (47)	0.44 (374)	0.35 (160)	0.040 (9)

^a Results are based on the same histological sections scored in Table 3.

^b Not corrected for ratio of control slices. Total number of yeast cells is shown within parentheses.

^c The inoculum of the control loops contained polystyrene particles and yeasts, but no vibrios.

TABLE 3. Penetration of vibrios into mucus gel of germfree mouse intestinal loops inoculated with a suspension of the bacteria, yeasts, and indicator particles

Vibrio strain	Vibrio/particle ratio ^a in:			
	Inoculum	Mucus gel		
		Above villi	Between villi (luminal half)	Between villi (basal half)
Parent	1.14 (109)	0.79 (999)	1.55 ^b (926)	4.12 ^b (1,067)
Nonchemotactic	1.18 (112)	0.79 (1,060)	1.00 (531)	0.60 (183)
Nonmotile	0.97 (101)	0.76 (953)	0.42 (302)	0.16 (153)
Control ^c		0.34 (294)	0.29 (134)	0.44 (99)

^a Corrected for ratio of control slices. Total number of vibrios is shown within parentheses.

^b Difference from ratio of nonchemotactic strains is highly significant ($P < 0.001$).

^c The inoculum of the control loops contained polystyrene particles and yeasts, but no vibrios.

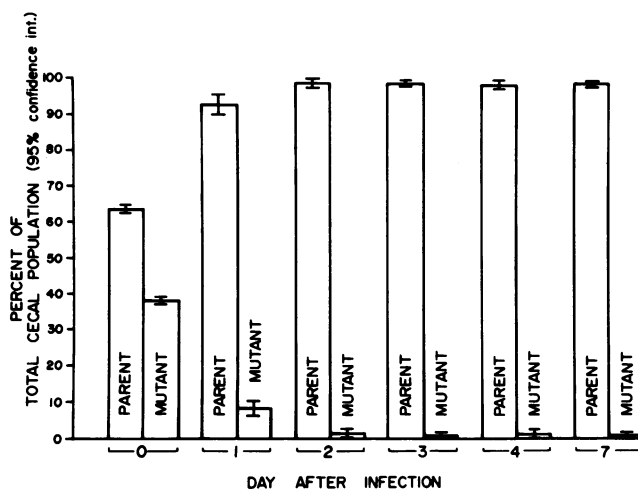


FIG. 1. Recovery of the parent and nonchemotactic, motile mutants of *V. cholerae* from the cecum of diassociated gnotobiotic adult mice inoculated with approximately equal proportions of these strains on day 0.

obvious that the nonchemotactic mutants were rapidly displaced by the parent (or chemotactic revertant) strain in the cecum of germfree mice. When the original inoculum had been introduced into the drinking water, the nonchemotactic mutants and the parent strain retained their original proportions in the water, in spite of the fact that the mutants became undetectable in the mouse cecum. When germfree mice were monoassociated with the nonchemotactic mutant only, nonmotile mutants rapidly displaced the original inoculum (Table 5). One must conclude, therefore, that motility was a liability to the vibrios in the gut of these animals, unless this motility was also directional, i.e., was also guided by chemotactic stimuli. It is interesting to note that Lauffenburger et al. (D. Lauffenburger, R. Aris, and K. H. Keller, *Microb. Ecol.*, in press) have predicted these results on the basis of an entirely theoretical mathematical model of the effects of motility and chemotaxis in natural environments, stating that "we can make the prediction that, in a non-mixed environment, an immotile species will often grow to a larger population than a randomly-motile species that has a greater growth-rate constant."

The in vivo fitness of the chemotactic parent and nonchemotactic mutants was studied also in intestinal loops of rabbits (Fig. 2). When such loops were injected with a mixture containing 2×10^6 cells each of the *V. cholerae* chemotactic parent and nonchemotactic mutant strains, multiplication of these two types of bacteria was equal for the first 4 h after inoculation. Thereafter, the parent strain continued to multiply, whereas the mutant population increased only at a very slow rate. A number of control animals

TABLE 5. Selection of nonmotile mutants in the cecum of germfree mice monoassociated with motile, nonchemotactic mutants of *V. cholerae*^a

Day after monoassociation	% of nonmotile vibrios in cecum	No. of mice cultured
0 (inoculum)	0.0	
1	44 (27) ^b	6
2	50 (35)	11
3	39 (36)	3
5	96	1
10 or more	93 (7.9)	11

^a Data obtained with three mutants are pooled.

^b Number within parentheses indicates the standard deviation.

were inoculated with the nonchemotactic mutant only (i.e., the parent strain was omitted from the control inoculum). Under these conditions the mutant vibrios still grew at a significantly slower rate than the parent strain (Fig. 2), indicating that the reduced growth rate of the mutant in mixed populations cannot be attributed solely to competition with the parent strain.

The chemotactic parent strain showed a strong association with the mucosa, more than one-third of its population being retained there after rinsing of the tissue (Fig. 2). In contrast, the nonchemotactic mutant associated with the mucosa to a markedly lower degree. We speculate that the bacterial population in the lumen has exhausted the original supply of oxygen and of fermentable substrates in that location between 4 and 6 h after inoculation. It is likely, however, that a continued supply of oxygen or substrates (or both) was available via mucosal

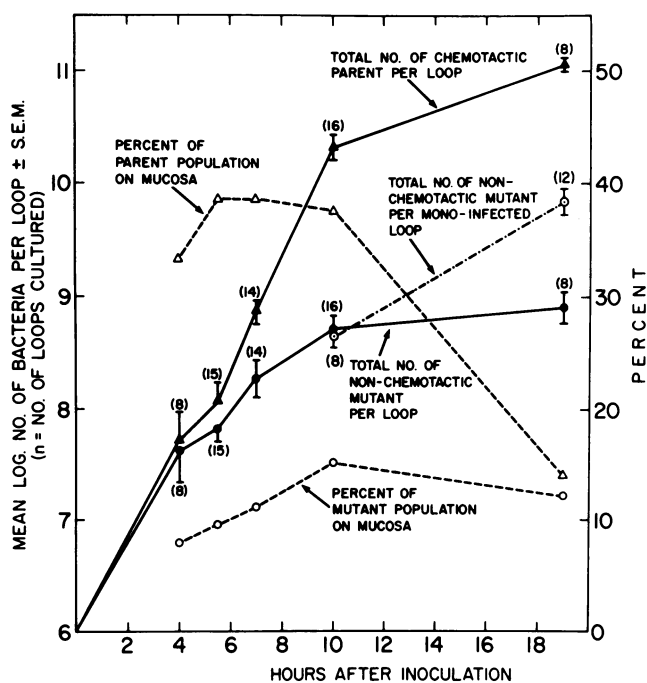


FIG. 2. Growth and association with the mucosa of chemotactic parent and motile, nonchemotactic mutant 31 in intestinal loops of adult rabbits.

secretions to vibrios residing deep in the intervillous spaces, thus giving an advantage to the mucosa-associated parent strain.

DISCUSSION

The data presented in this paper show that the superior ability of chemotactically reactive vibrios to associate with intestinal mucosa *in vitro* (2) does indeed have an *in vivo* counterpart. These data, together with those reported earlier from this and other laboratories (3, 7, 11), have stressed the importance of the following three distinct steps in the association of bacteria with intestinal mucosa.

(i) The ability to approach and make contact with the surface of the mucus gel. This step is apparently facilitated by motility of the bacterium and by its ability to respond to chemotactic stimuli.

(ii) Penetration of or trapping in the mucus gel (or both). Our experiments with intestinal slices and intestinal loops demonstrate that the taxin gradient which attracted the chemotactic parent strain to the surface of the mucus gel actually extended deeply toward the base of the villi. Somewhat surprising was the finding that inert particles can also penetrate the mucus gel, though with considerably less efficiency than the parent strain vibrios. Equally surprising are the data which show that penetration of inert particles and vibrios into the mucus gel and deep

into the intervillous spaces occurred within a matter of minutes. The fact that such rapid penetration was observed with intestinal loops in rabbits as well as in mice makes it unlikely that our earlier similar finding with intestinal slices (2) was an artifact of the *in vitro* experimental technique employed.

(iii) Adhesion to the surface of the epithelial cells. To the extent that this reaction is represented by the adhesion of bacteria to isolated brush border membranes (9), this adhesion involves the interaction of specific receptors on the bacterial and mammalian cells.

The present finding that intestinal mucus gel can be penetrated by motile, chemotactic bacteria with relative ease seems at first glance to contradict an earlier finding (8) that extensive *in vitro* penetration by vibrios of mucus gel stripped from rabbit intestine occurred only to a minor degree. This apparent discrepancy might perhaps be resolved by the finding of Gibbons and Sellwood (6) that mucus gel develops capillary channels along planes of stress. It is possible that mucus gel flowing *in vivo* from the goblet cells toward the lumen forms such stress planes running roughly parallel to the villi and that penetration of colloidal particles and bacteria occurs via the channels thus formed. In contrast, when the mucus gel is removed by stripping, the channels may be destroyed in the process, and bacterial penetration of the col-

lected gel may become difficult to observe.

It hardly needs emphasizing that bacteria can associate with the epithelial surface of the intestine in the absence of one, or even all of the above mechanisms. For example, *Shigella* is an efficient intestinal pathogen lacking motility. Moreover, a number of laboratories have shown that even inert particles such as polystyrene or starch, which presumably lack specific adhesive mechanisms, can penetrate the intestinal barrier (12). Conceivably, then, a bacterium lacking chemotaxis, motility, and special adhesive properties may still be a successful pathogen if it can, for example, multiply in the lumen of the intestine to build up high population levels. In such an instance some of the bacteria must be expected to reach and even penetrate the epithelial surface with the same efficiency that has been demonstrated for inert particles. For these reasons, motility, chemotaxis, and specific adhesion must not be regarded as *sine qua non* but rather as mechanisms which can facilitate bacterial association with the mucosa. In other words, all of these factors (and, of course, many others) singly and collectively affect the probability that a given bacterium will succeed in establishing itself in the intestinal environment. Consequently, the importance of any one of these mechanisms should not be underestimated because in the face of the body's defense mechanisms such as peristalsis, mucus flow, or local immunity, they may well supply that extra increment in virulence which determines whether a given bacterium will succeed in establishing itself in or on the mucosa, or whether the body will succeed in eliminating it.

The findings that the parent vibrio was more successful than the nonchemotactic mutants in germfree mice and in the late stages of the adult rabbit loop are consistent with our original hypothesis that chemotaxis promotes association with the mucosa. Alternative explanations are equally plausible: when gnotobiotic mice monoassociated with the parent strain of *V. cholerae* are autopsied in an anaerobic chamber and the cecal contents are observed under a phase-contrast microscope placed inside the chamber, the vibrios are largely immobile. However, the addition of a (prereduced) glucose solution or removal of the specimen into air instantly restores full motility. This suggests that the physiological activities (and presumably multiplication) of the vibrios in the cecum of gnotobiotic mice were limited by the availability of a carbon/energy source which could be utilized under anaerobic conditions in a manner described earlier for *Escherichia coli* (10). Chemotaxis may thus aid the bacteria to seek out and closely approach areas of high substrate concentration, such as

food particles undergoing digestion. Alternatively, chemotactic bacteria may be able to seek out areas of higher oxygen concentration, e.g. near the mucosa, or negative chemotaxis may enable them to avoid areas containing toxic substances such as volatile fatty acids.

Anaerobic conditions similar to those in the large intestine of monoassociated mice may also prevail in the later stages of infection of isolated intestinal loops of adult rabbits. The observed superiority of the chemotactic parent strain in these two *in vivo* situations may therefore be mediated by similar mechanisms that control access to substrates or oxygen (or to both). In contrast, in the early stages of an infected loop, vibrio multiplication is rapid and the availability of substrates in the lumen does not appear to be a growth-limiting factor. If these assumptions were correct one would predict that chemotactic bacteria should have no advantage over nonchemotactic mutants in early intestinal loops. This expectation was indeed borne out by the observations of population dynamics in early and late loops reported in this paper (Fig. 2), another piece of evidence showing that there was no intrinsic difference in the growth rates of the parent and the mutant.

In summary, then, the data presented clearly show that chemotactic responsiveness, or the lack of it, was of great importance in the interactions between cholera vibrios and host in the two *in vivo* models studied. The extent to which these observations represent interactions occurring in cholera or other human or animal diseases cannot be decided with the information currently on hand. It is entirely possible, for example, that some or even all of the interactions of cholera vibrios with the large intestine of gnotobiotic adult mice (or with the other *in vivo* models employed in this series of studies) may have no counterpart in human cholera. Nevertheless, they give us a clue as to what one should look for in studying similar interactions of *E. coli* and other facultative or anaerobic species with mucosae (of the intestine, bladder, respiratory tract, etc.) of humans or animals. For this reason, the present studies should be regarded as a delineation of the various types of interactions that can occur between bacteria and mucosal surfaces in general, and the relevance of the findings reported is therefore not limited to human cholera. Chemotaxis was of considerable importance to bacterial growth in every one of the *in vitro* and *in vivo* models studied so far; therefore, it would be very surprising if chemotaxis were not important in a number of other *in vivo* situations as well. It should be remembered in this regard that all natural isolates of motile bacteria which have been examined also show

chemotaxis (J. Adler, personal communication, 1977). In other words, natural environments generally select against nonchemotactic mutants, consequently, chemotaxis must confer a considerable advantage to motile bacteria in their natural habitat. An accompanying paper (5) reports an apparent contradiction to this rule, namely, that chemotactic vibrios may be at a relative disadvantage in the infant mouse model of cholera.

ACKNOWLEDGMENT

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