

Role of Chemotaxis in the Association of Motile Bacteria with Intestinal Mucosa: Chemotactic Responses of *Vibrio cholerae* and Description of Motile Nonchemotactic Mutants

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A motile, chemotactic, Ogawa strain of *Vibrio cholerae* was attracted by all 20 L-amino acids tested, in contrast to *Escherichia coli* AW 405, which did not react to several of these. The maximum number of vibrios entering a capillary was much lower when the capillary contained carbohydrates rather than amino acids, but the minimum effective concentrations of the carbohydrates and amino acids tested were of the same order of magnitude. L-Fucose, a sugar known to inhibit the adhesion of this vibrio strain to brush border membranes, had no attraction (taxin activity) for it. A pepsin digest of rabbit mucosal scrapings or tryptone attracted vibrios as strongly as the most active amino acids. Several nonchemotactic and one nonmotile mutant were selected from the parent vibrio. The nonchemotactic mutants were indistinguishable from the parent in their ability to attach in vitro to isolated intestinal brush border membranes, whereas the nonmotile mutant had lost this ability. Parent and nonchemotactic mutants had equal growth rates in stirred and still continuous flow cultures that were maintained in an anaerobic environment.

As reviewed by Weibull (19), little is known about the chemotactic reactions of *Vibrio cholerae*. Pfeffer, in his original work at the end of the last century, found only a low degree of chemotactic responsiveness among cholera and typhoid bacteria and, for this reason, considered it unlikely that chemotaxis played an important role in the in vivo distribution of these bacteria (quoted in reference 17). This was quickly refuted, however, by the demonstration that potato juice was a potent attractant (taxin) for cholera vibrios, and the suggestion was made to use this reaction as an enrichment procedure for stool cultures by dipping capillaries filled with potato juice into patients' stools (17).

Preliminary reports from this laboratory (3, 13, 14) had indicated that chemotaxis appears to have a profound effect on the interaction of cholera vibrios with intestinal mucosa, both in vivo and in vitro. The present paper is the first in a series reporting the results of the completed study. It is concerned with the description, in vitro reactions, growth, and adhesive properties of the parent and nonchemotactic mutants used. The accompanying papers (9, 11, 12) report the in vitro interactions of parent and mutants with intestinal mucosa and, finally, their behavior in various in vivo situations.

(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

***V. cholerae* strains.** The streptomycin-resistant parent strain (Ogawa type) has been described in earlier publications, where it had been designated as strain P (15). Nonchemotactic mutants were selected from this strain by the method of Aswad and Koshland, using tryptone (Difco Laboratories) as the counterselecting taxin (6). One mutant (no. 8) was obtained after treatment of the parent culture with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Three other mutants were spontaneous, i.e., were selected without mutagenesis from a population of approximately 10^{10} cells of the parent strain (no. 31, 40, and 50). All four mutants were obtained in separate experiments employing cultures grown from single bacterial colonies and therefore must be regarded as descendants of separate mutational events. The spontaneous mutants were quite stable. Only once was it possible to isolate a revertant strain from mutant 40 growing in monoassociated mice. This revertant was designated 40R. The

mutants were smooth swimming and did not differ from the parent strain in their rate of motility as determined by direct microscopic observation.

The nonmotile mutant of *V. cholerae* used in these studies (designated 31NM) was isolated from a gnotobiotic mouse inoculated with motile, nonchemotactic mutant 31. Details of this procedure are described in an accompanying paper (11). The nonmotile mutant lacked a flagellum when grown in broth cultures under conditions where the parent strain regularly synthesized single polar flagella as determined by Gray's flagella strain.

For purposes of comparison, *Escherichia coli* strain AW405, kindly supplied by Dr. Julius Adler, was used in some experiments. This strain has been described by Adler and his co-workers in some of their basic studies of chemotactic reactivity of *E. coli* (5).

Culture media. Parent and nonchemotactic mutants were quantitated on pour plates of semisolid tryptone agar containing 1% tryptone, 0.5% NaCl, 0.4% agar (Difco), 0.01% 2,3,5-triphenyl tetrazolium chloride, and 1 mg of streptomycin per ml. Colonies of the parent strain in this medium are large, diffuse, and pink, whereas the nonchemotactic mutants form pinpoint, dark red colonies which can be detected with ease even on a crowded plate.

Unless stated otherwise below, bacterial suspensions were prepared from overnight still cultures in Trypticase soy broth without glucose (BBL Microbiology Systems).

In some instances, bacterial suspensions were prepared in a minimal medium of the following composition: 11.2 g of K_2HPO_4 , 4.8 g of KH_2PO_4 , 2.0 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.5 mg of $Fe_3(SO_4)_2$, 1 liter of water, plus 1 g of the carbon source specified below.

Continuous flow cultures. Continuous flow cultures were housed entirely within an anaerobic glove box (4). The growth medium had the following composition: 5% veal infusion broth supplemented with yeast extract, 100 mg of hemin per liter, and 0.5 mg of menadione per liter as described earlier (8). The medium was pumped into the growth tube by means of a peristaltic pump. The growth tube had a volume of 5 ml, and the flow rate of medium was 0.84 ml/h.

Pepsinized mucosal scrapings. Pepsinized mucosal scrapings were prepared from rabbit intestine as described earlier (10), except that the material was passed through an Amicon PM30 ultrafiltration membrane before use in the present studies.

Capillary test for bacterial chemotaxis. This capillary test was performed by following the principles described by Adler (1). The vibrios were harvested by centrifugation from overnight cultures in Trypticase soy broth without glucose or in minimal medium and suspended in Krebs-Ringer-Tris buffer (15), pH 7.4, to which 0.1% Triton X-100 had been added to prevent excessive adhesion of the vibrios to glass. Capillaries of 0.27 mm diameter (5- μ l size Micropipets; Clay Adams) were filled with 5 μ l of the attractive substance dissolved in this buffer or with pure buffer as controls.

Conventional capillary tests are usually carried out by dipping the capillaries into a suspension of a single

bacterial strain and determining the number of bacteria that enter the capillary. In the experiments reported here, reproducibility was greatly increased by using mixtures of chemotactic parent vibrio and nonchemotactic mutant strain 31. The capillaries were dipped into vibrio suspensions which contained 6×10^3 cells of the parent strain per ml and 2.4×10^9 cells of nonchemotactic mutant 31 per ml. Quadruplicate capillaries were tested for each concentration of attractant or for the buffer controls. Tryptone (1%) was used as a positive control attractant in each experiment. At the end of the experiment (1-h incubation at 37°C), the contents of the capillaries were expelled into petri dishes, and pour plates were prepared with liquid semisolid tryptone agar at 45°C. A given concentration of a taxin was considered chemotactically active only when both the following criteria were fulfilled: (i) an average of at least 10 chemotactic vibrios had entered each capillary, and (ii) the ratio of parent/nonchemotactic vibrios in the capillary was at least three times higher than in control capillaries containing only buffer. Fulfillment of these criteria results in a difference of high statistical significance ($P < 0.01$) between capillaries containing taxin and control capillaries. The density of the vibrio suspensions had been chosen such that the number of parent vibrios in control capillaries ranged from 0 to 2, whereas 20 to 40 mutant vibrios entered a given capillary by random motility (regardless of its contents). The vibrio suspensions were placed into small (6- by 50-mm) test tubes. The capillaries were filled with the appropriate solutions and closed at the upper end with grease. By means of adhesive tape applied to the outside of the test tubes and extending approximately 1 cm above the lip, the capillaries were then attached vertically to the test tubes containing bacterial suspension (one capillary per tube) such that the open tip of the capillaries was submerged a few millimeters below the surface of the suspension. All of the above-described manipulations were carried out in a cold room at 4°C. At this point the tubes were transferred to a 37°C water bath and incubated for 1 h; then the contents of the capillaries were cultured. The same method was used for testing *E. coli*, except that this strain was grown in minimal medium as described by Adler (1). The bacteria were then washed and suspended in ethylenediaminetetraacetic acid-phosphate buffer (1).

Adhesion of vibrios to brush border membranes. The method of testing adhesion to brush border membranes has been described in an earlier publication (15). It should be noted that suspensions of isolated membranes were stored for prolonged periods of time (several weeks) and that the membranes were washed extensively before storage and again before use. Therefore, such membranes cannot be expected to still elaborate soluble taxins when they are finally used in the adhesion assay. This was verified experimentally by the fact that brush borders failed to attract "bands" of chemotactic vibrios or salmonellae, an observation which is strikingly apparent when pieces of fresh mucosa are exposed to bacterial suspensions (illustrated in reference 3).

Statistical methods. The 95% confidence intervals

for proportions were calculated as described by Bowker and Lieberman (7). Standard methods were used for chi-square and *t*-test evaluations (7).

RESULTS

Table 1 shows the results of testing, by means of the capillary test, the taxin activity of 20 amino acids. The parent vibrio strain was used as well as *E. coli* strain AW405. The capillary test does not differentiate negative taxis from indifference; for this reason, amino acids for which *E. coli* is reported to exhibit negative taxis are listed in Table 1 as showing no attraction. The reactions of the *E. coli* strain tested show the same pattern of positive and neutral (or negative) reactions reported by Adler (2) for *E. coli* (Table 1). This indicates that the test method as well as the purity of the amino acids used were comparable to those of the earlier workers. The vibrio strain reacted differently from the *E. coli* in being very responsive to all of the amino acids tested at concentrations in the order of 10^{-4} to 10^{-6} M (Table 1).

The kinetics of chemotactic attraction of the parent strain by carbohydrates were qualitatively and quantitatively different from those observed with amino acids. The data presented in Fig. 1 show that the maximum chemotactic

response to the carbohydrates tested was approximately 1 order of magnitude lower than that to amino acids (note the different scales for amino acids and sugars in Fig. 1). In spite of this low maximal response, the sensitivity of the chemotactic reaction to carbohydrates was comparable or even higher than that determined with amino acids, because the minimal attractive concentrations were in the order of 10^{-5} to 10^{-6} M. Thus, one might conclude from the data in Fig. 1 that the vibrio parent strain had a 100 times stronger response to galactose than to proline (lowest effective concentrations, 10^{-6} and 10^{-4} M, respectively). However, on the basis of the maximal attraction to these two agents, one may also conclude the opposite, namely, that the response to proline was approximately 20 times stronger than that to galactose (220 versus 12 vibrios per capillary at 0.1 M concentration). The significance of these qualitative differences is not clear, but it is obvious that the nature of chemotactic responsiveness of this vibrio strain cannot be described adequately by a single parameter such as the lowest effective concentration of a given attractant. All of the carbohydrates tested that attracted vibrios at all (cf. Table 2) exhibited patterns of taxin activity similar to those shown in Fig. 1, i.e., in comparison to amino acids they exhibited considerably lower maximal responses, but nevertheless showed minimal attractive concentrations in the order of 10^{-5} to 10^{-6} M, i.e., of the same order of magnitude as those of amino acids. The chemotactic activity of the parent strain did not change when the bacterial culture to be used in the capillary test was grown in minimal medium with the carbohydrate under test as the only carbon source, rather than in Trypticase soy broth.

Several carbohydrates frequently found in mucopolysaccharides were tested for taxin activity and for their ability to support the growth of the parent vibrio when they were the only carbon sources in a minimal medium. In view of the low maximal chemotactic response of the parent strain to carbohydrates, the sensitivity of the capillary test was increased by using equal concentrations (2.4×10^5 cells per ml) of parent and mutant 31 in the suspensions (rather than the usual, 40-times-lower concentration of the parent). The results are shown in Table 2. As has been reported for other species (2), the ability to utilize a given carbohydrate did not necessarily correlate with chemotactic reactivity toward it. It should also be noted that the nonchemotactic mutants and revertant strain 40R described in this and the accompanying publications reacted exactly like the parent strain with respect to utilization of the various carbohydrates.

TABLE 1. Chemotaxis of *V. cholerae* and *E. coli* AW405 toward L-amino acids in the capillary assay

Amino acid	Lowest effective molar concn (log for:	
	<i>V. cholerae</i>	<i>E. coli</i>
Alanine	-6	-3
Arginine	-6	N ^a or -1
Asparagine	-4	-4
Aspartic acid	-4	≤-6
Cysteine	-4	-4
Glutamic acid	-5	-4
Glutamine	-5	N
Glycine	-5	-2
Histidine	-5	N
Isoleucine	-4	N
Leucine	-4	N
Lysine	-5	-1
Methionine	-5	-2
Phenylalanine	-5	N
Proline	-4	N
Serine	-5	-5
Threonine	-4	-1
Tryptophan	-5	N
Tyrosine	-5	<-2.61 ^b
Valine	-5	N

^a N, No attraction at the highest concentration tested (10^{-1} M).

^b No attraction in saturated solution (2.48×10^{-3} M).

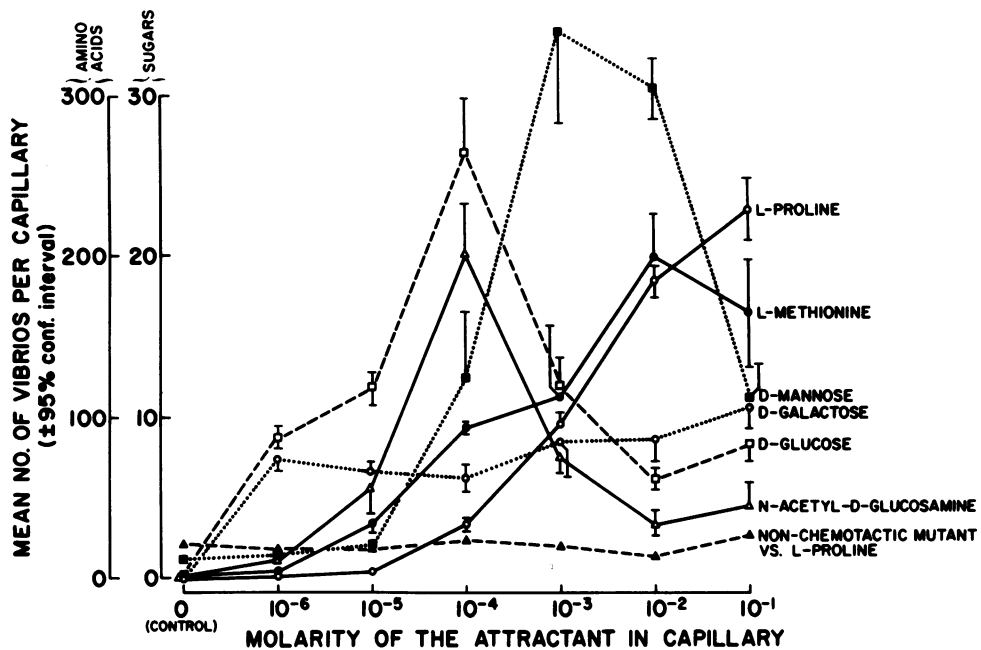


FIG. 1. Chemotactic attraction of parent strain vibrios into capillaries containing various concentrations of taxins.

TABLE 2. Growth-promoting and chemotactic attractant activity of carbohydrates for *V. cholerae*

Carbohydrate	Growth of <i>V. cholerae</i> ^a	Attractant for <i>V. cholerae</i> ^b
D-Fucose	No	-
L-Fucose	No	-
<i>p</i> -Nitrophenyl α -L fucoside	Not done	(-) ^c
D-Glucose	Yes	++
D-Galactose	No	+
D-Mannose	Yes	++
<i>N</i> -Acetyl neuraminic acid	No	++
<i>N</i> -Acetyl D-glucosamine	Yes	+
<i>N</i> -Acetyl D-galactosamine	No	+

^a Support of growth of *V. cholerae* at a concentration of 0.1% in minimal medium.

^b Capillary test results were scored as follows: ++, attractive over a wide range of concentrations (at least to 10^{-5} M); +, attractive to at least 10^{-5} M, but the total number of bacteria entering the capillary at any concentration was significantly lower than with glucose as the attractant; -, not attractive at 10^{-1} M.

^c Poorly soluble in water, tested for chemotactic activity in saturated solution.

In an earlier publication (10), a pepsin digest of intestinal mucosa had been shown to inhibit the association of cholera vibrios or *Salmonella enteritidis* with rabbit intestine. Figure 2 presents the kinetics of the taxin activity of pepsinized mucosal scrapings toward the parent strain, showing that this material is indeed a strong

attractant, stronger than the carbohydrates tested and comparable to amino acids in its ability to attract vibrios into the capillaries. A similar response was obtained with tryptone as the attractant.

Figure 3 shows the ability of parent strain, mutant 31, and the nonmotile mutant 31NM to adhere to isolated brush border membranes of rabbit ileum. As is apparent, the nonchemotactic mutant was not impaired in this respect. This was true also with the other nonchemotactic mutants studied (i.e., 40 and 50). In contrast, the nonmotile mutant had lost the ability to adhere to brush borders, a phenomenon that is consistent with the behavior of other nonmotile mutants of the same parent strain described earlier (16).

As is detailed in the accompanying publications (11, 12), parent and nonchemotactic mutant vibrios showed different rates of growth in vivo. It was important, therefore, to rule out basic differences in the rate of growth of these strains under circumstances in which chemotaxis was not likely to confer an ecological advantage. Anaerobic continuous flow cultures were chosen for this purpose because, in the absence of oxygen, there could be no growth stimulation of bacteria attracted to the surface by aerotaxis and because even minor differences in growth rates lead to rapid population shifts in continuous flow cultures. In one set of experiments the growth tube was rapidly stirred to

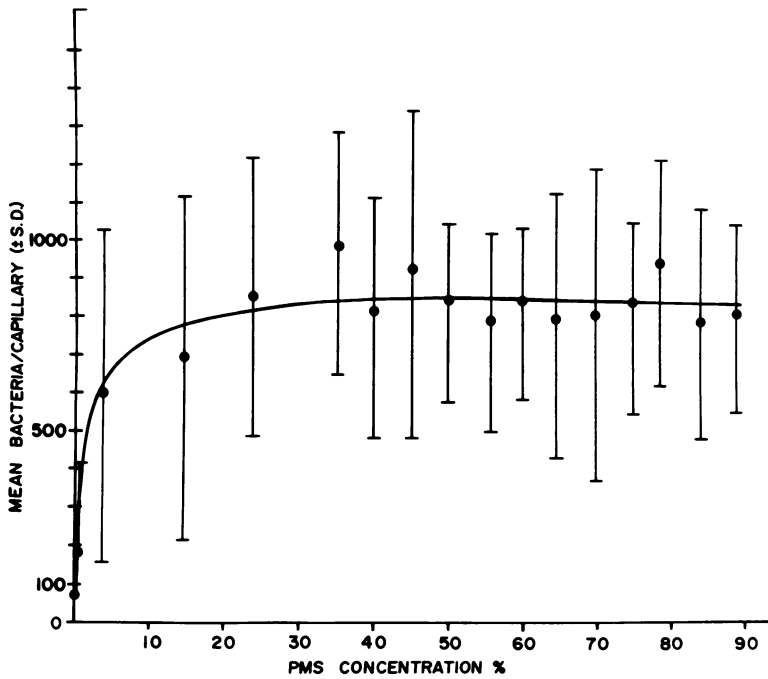


FIG. 2. Attraction of parent strain vibrios into capillaries containing pepsinized mucosal scrapings (PMS).

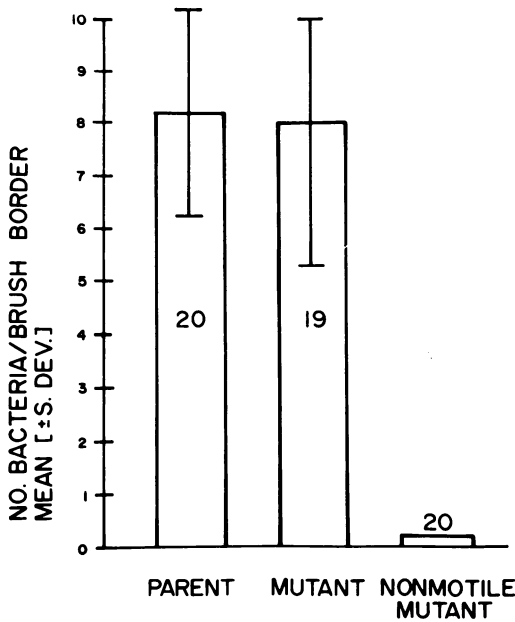


FIG. 3. Adherence of parent strain, nonchemotactic mutant 31, and nonmotile mutant 31NM to isolated intestinal brush borders *in vitro*. The figures inside the bars indicate the number of tests on which the data are based.

prevent the possible establishment of taxin gradients; in others the growth tube was left unstirred. The data in Table 3 were obtained in

TABLE 3. Growth of nonchemotactic mutant 31 and the parent strain of *V. cholerae* in an anaerobic continuous flow culture

Continuous flow culture	% of nonchemotactic mutants on day:				
	0 (inoculum)	1	2	3	4
A (stirred)	64	55	50	53	62
B (not stirred)	12	28	29	31	F ^b

^a The total bacterial population was in the order of 2×10^8 cells per ml throughout the experiments.

^b Filamentous, motile mutants predominated.

two representative experiments. As may be seen, parent and mutant populations retained their original proportions under these conditions throughout the experiment. When the growth tube was not stirred, the parent and the nonchemotactic mutant were both displaced at comparable rates by a filamentous mutant which settled to the bottom of the tube and thereby escaped removal from the culture via the effluent. It is apparent, then, that parent and mutant showed equal competence (or fitness) for growth under these conditions. Although determinations of competition (or the lack of it) among bacteria under a given set of culture conditions cannot prove that similar results would also be obtained at different culture conditions or *in vivo*, the continuous flow culture was chosen here because it had been found in several earlier studies to simulate bacterial com-

petition in the gut of disassociated mice (8, 18). This may give the above data some added significance.

DISCUSSION

The mutants described in this paper were selected without the use of mutagenesis, a precaution which minimizes the probability of encountering multiple mutations in a single isolate. Consistent with this are the findings, described above, that nonchemotactic mutants and the parent did not differ in their fitness for growth in continuous flow cultures, in their ability to utilize various carbohydrates as sole carbon sources in minimal medium, and in their ability to adhere to isolated brush border membranes. Additional data showing identical behavior of nonchemotactic mutant and parent strains in situations where chemotaxis cannot be expected to confer an ecological advantage are presented in the accompanying papers (9, 11, 12). In addition, a chemotactic revertant of one of the mutants is shown to react exactly like the parent strain (9, 12). This rather extensive body of diverse observations strongly supports the assumption that the mutant and parent strains differ only with respect to chemotactic reactivity and that the observed *in vivo* and *in vitro* differences are not the results of pleiotropic phenotypic effects of single or multiple mutations—assumptions that are, of course, *sine qua non* to the validity of the studies reported in the accompanying papers. The genetic aspects of chemotaxis in *V. cholerae* are unknown. By analogy with *E. coli* and *Salmonella* one must suspect that a number of genes are involved (2). Since all of the mutants in this study were generally nonchemotactic (i.e., did not respond to any taxin tested) one may assume that the defect was in one of the functions involving sensory response coupling. The mutants are therefore less likely to have alterations in their surface properties than would be the case with specific nonchemotactic mutants which are likely to have defects in one of several surface receptors for specific taxins (2).

Adler (2) has reviewed evidence showing that chemotactic reactivity of a given substance does not necessarily parallel its ability to serve as a nutritional substrate. This was also true for the vibrio strain tested in the present study. Chemotaxis of this vibrio strain differed profoundly from that of *E. coli* in that there was no negative or neutral chemotaxis to amino acids, but rather a uniform strong attraction. It is possible that this phenomenon may relate to the ability of the vibrio strain to associate quickly with intestinal mucus gel. Consistent with this idea is the find-

ing of taxin activity in all but one of the carbohydrates tested, most of which are commonly found as components of intestinal mucopolysaccharides. The finding that L-fucose had no taxin activity and could not be utilized as a carbon source by the parent vibrio was somewhat surprising, however, because this strain had been shown earlier to possess fucose-sensitive adhesins for erythrocytes and intestinal brush border membranes (16). The significance of this finding, if any, remains obscure.

In view of the strong attraction of the parent vibrio to intestinal mucosa *in vitro* and *in vivo* (9, 11), it is noteworthy that a mucosal extract was a strong chemotactic attractant for this strain. The maximum number of vibrios entering a capillary containing this material was as high as that attracted by tryptone or by some of the more highly active single amino acids, i.e., much higher than that which could be attracted by the single carbohydrates tested. This does not rule out the possibility, however, that the carbohydrate moieties of intestinal mucus may also function as taxins *in vivo*, because carbohydrates and amino acids were equally effective attractants at high dilutions.

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