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Roles of Motility and Flagellar Structure in Pathogenicity of *Vibrio cholerae*: Analysis of Motility Mutants in Three Animal Models

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Wild-type *Vibrio cholerae* of both El Tor and classical biotypes (strains N16961 and 395, respectively) and nonmotile mutant derivatives with and without flagellar structures were characterized in three different animal models: (i) the rabbit ileal loop, (ii) the removable intestinal tie adult rabbit diarrhea (RITARD) model, and (iii) the suckling mouse model. Both the wild-type strains and nonmotile mutants were toxinogenic in the rabbit ileal loop and the suckling mouse models. However, all of the nonmotile mutants produced significantly less fluid accumulation than did the wild-type parental strains. The two nonmotile mutants of strain N16961 did not adhere to rabbit ileal mucosa, but both nonmotile mutants derived from strain 395 exhibited adherence. In the RITARD model, the motile El Tor strains were more virulent than both the flagellate and aflagellate nonmotile mutants (all infected rabbits died within 18 h), while the nonmotile mutants, when fatalities occurred, required 78 to 105 h to produce a fatal outcome. Likewise, the motile classical parent 395 produced a fatal outcome within ca. 25 h, while nonmotile mutants required 69 to 96 h. The nonmotile flagellate strain KR31 was not significantly more virulent than the nonmotile aflagellate strain KR26. Of the two classical nonmotile mutants, KR1, which produces a coreless sheathlike structure, was clearly more virulent (5 of 10 rabbits died within 96 h), while KR3 (nonmotile, aflagellate) did not produce fatalities in any of the 10 rabbits tested. Similarly, no significant difference in diarrheagenicity or colonizing ability was detected between the two nonmotile mutants derived from the El Tor strain, but the classical nonmotile mutant with the coreless sheath caused significantly greater diarrhea and colonized for a longer time than did the isogenic nonmotile aflagellate strain, KR3. No significant differences between the nonmotile mutants were detected in competition studies done with suckling mice. Analysis of the wild-type and mutant strains in these three animal models clearly demonstrated a role for motility in *V. cholerae* pathogenicity, while analysis of only the nonmotile mutants derived from the classical parent suggested a role for flagellar structures.

Vibrio cholerae produces a potent exoenterotoxin, cholera toxin (CT), which is primarily responsible for the diarrheal syndrome associated with *V. cholerae* infection. While toxinogenicity is a predominate pathogenic factor, colonization is clearly a prerequisite. The organism must colonize the small bowel to elaborate and secrete CT. No diarrhea is seen when volunteers are fed strains of *V. cholerae* which colonize poorly or not at all (21). Although little is known about the mechanism of *V. cholerae* colonization, it is probably multifactorial. Hall et al. (17) have described three pilus types observed by transmission electron microscopy. Taylor and coworkers have isolated a transposon insertion mutant which no longer produces the TcpA pilin (31) and colonizes animal models and volunteers poorly (18). A transposon insertion mutant lacking the mannose-sensitive cell-associated hemagglutinin showed reduced colonization of rabbits (11). However, Teppema et al. (32) observed that *V. cholerae* strains with reduced mannose-sensitive hemagglutinating activity that were also nonmotile and aflagellate attached to rabbit intestinal epithelial cells in the ligated loop model. Expression of pili and hemagglutinins by *V. cholerae* varies considerably between pathogenic strains, yet they still colonize the intestine.

Motility, another potential factor involved in colonization, is expressed by virtually all *V. cholerae* strains. Several studies suggest that motility and/or flagellar structures are involved in colonization. Jones et al. (19) observed attachment of motile *V. cholerae* to isolated rabbit brush borders by phase-contrast microscopy, while in a second study nonmotile mutants exhibited reduced adherence even when impacted onto the brush borders by centrifugation (20). Motile revertants regained the ability to adhere. Guentzel et al., studying attachment to mouse intestinal segments in vitro (16) and in vivo (15), found that nonmotile *V. cholerae*, regardless of biotype or serotype, exhibited reduced adsorption.

Guentzel et al. (16) observed that nonmotile aflagellate mutants obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis were less virulent in vivo and did not penetrate the crypts of Lieberkühn to the same extent in suckling mice as did the motile parent strain. In rabbit ligated ileal loops, fluid accumulation (FA) and adherence to the ileal mucosa were reduced for the nonmotile mutants (36). None of these studies examined the role of the flagellar structure versus the role of motility in the colonization process.

The flagellum of *V. cholerae* is surrounded by a sheath which appears to be contiguous with the outer membrane (12). Eubanks et al. (10) found that offspring of mice vaccinated with crude flagella containing sheaths, flagellar cores, and probably outer membranes were protected against lethal challenge with virulent *V. cholerae* and fewer vibrios were associated with the mucosa. However, offspring of mice vaccinated with the commercial bivalent whole-cell vaccine were not protected. Several groups have analyzed the protection provided by a variety of vaccines and found that crude flagella alone were better than toxoid alone and the best vaccine included both (10, 16, 26, 37). In addition, rabbits could be protected passively and adsorption of the antiserum with aflagellate *V. cholerae* did not decrease protection (35), supporting a specific role for flagellar struc-

TABLE 1. *V. cholerae* strains used

Strain	Relevant characteristics	Source or reference
N16961	El Tor, Inaba, isolated from a patient	CVD[a] (6)
KR100	Spontaneous spectinomycin-resistant derivative of N16961	This paper
KR400	Spontaneous streptomycin-resistant derivative of N16961	This paper
KR26	Derivative of N16961, nonmotile aflagellate Tn*10* mini-Kan insertion mutant, Kan^r	27
KR126	Spontaneous spectinomycin-resistant derivative of KR26	This paper
KR31	Spontaneous nonmotile flagellate mutant derivative of N16961	27
KR131	Spontaneous spectinomycin-resistant derivative of KR31	This paper
KR517	Prototrophic, motile isolate with Tn*10* mini-Kan insertion, Kan^r	This paper
KR601	Spontaneous spectinomycin-resistant derivative of KR517	This paper
395	Classical, Ogawa, isolated from a patient	6
KR3	Derivative of 395, nonmotile aflagellate, produces a coreless sheath structure, Tn*5* insertion mutant, Kan^r	27
KR1	Derivative of 395, nonmotile aflagellate Tn*5* insertion mutant, Kan^r	27

[a] M. M. Levine, Center for Vaccine Development, University of Maryland, Baltimore.

tures in this immunity. All of these protective immunity studies can only suggest a role for the sheathed flagellum since the flagellar preparations were not pure and probably contaminated with outer membranes.

We have obtained, using transposon mutagenesis, *V. cholerae* motility and structural mutants which are (i) nonmotile and flagellate, (ii) nonmotile and aflagellate, and (iii) nonmotile and aflagellate but that form a sheathlike structure. Characterization of these mutants in three animal models, (i) the rabbit ileal loop, (ii) the removable intestinal tie adult rabbit diarrhea (RITARD) model, and (iii) suckling mice, demonstrated a predominant role for motility in colonization. The flagellar structure may play a role in the colonization of these animal models by classical strains.

MATERIALS AND METHODS

Bacterial strains and media. The various strains used in this study are listed in Table 1 (see also Fig. 1). The strains were stored at −70°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) containing 50% glycerol (Difco). Strains were grown on L agar (23) or in L broth or BHI and incubated at 35°C.

Animals. Outbred New Zealand White female rabbits obtained from a local supplier and weighing 2 to 3 kg were used for the rabbit ileal loop assays and the RITARD model. Swiss Webster and CD-1 mice were used for the suckling mouse model and purchased as time-mated females from a local supplier and Charles River Breeding Laboratories, Inc., respectively.

Rabbit ligated ileal loop model. Ligated loops were done essentially as described by De and Chatterjee (7). Rabbits were fasted for 48 h prior to surgery and given water ad libitum. The rabbits were anesthetized as specified for the RITARD model. A laparotomy was performed, and the small intestine was tied off in alternating 10- and 2-cm segments proximally to the mesoappendix. *V. cholerae* strains were grown overnight at 35°C in BHI (Difco). The bacteria were washed, resuspended in fresh BHI, and diluted. The bacterial concentration was determined by plating dilutions on L-agar plates. An inoculum of 1 ml (containing 1×10^5 to 5×10^7 CFU) was introduced into the 10-cm-segment lumen with a 27-gauge needle. The intestine was returned to the peritoneal cavity, the incision was closed, and the rabbit was returned to a cage and given water ad libitum. Rabbits were sacrificed at 3, 6, 9, or 16 h, the peritoneal cavity was opened, and the small intestine was removed. The intervening loops were examined for fluid to determine whether the tie had held. At least four loops were done for each bacterial strain at each time point and inoculum. The results were evaluated by the Student-Newman-Keuls *t* test.

(i) FA ratio determination. The fluid in the loops and the length of the loops were measured, and the ratio of fluid (milliliters) to length (centimeters) was calculated.

(ii) Concentration of bacteria in fluid. The CFU of *V. cholerae* in recovered fluid was determined by plating dilutions of fluid on L-agar plates containing the appropriate antibiotics.

(iii) Adherence to the intestinal surface. A portion (ca. 0.5 to 1 cm) of intestine was cut from the center of each loop, rinsed three times with phosphate-buffered saline (PBS), weighed, and then homogenized for 45 s with an Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.) at maximum speed in 3 to 5 ml of cold 0.25× BHI. Dilutions of the homogenate were plated on L agar containing antibiotics. The CFU per gram of tissue was then calculated. The percentage of vibrios adhering to the intestinal surface was calculated in the following manner: percent adherence = 100 × [CFU associated with intestinal surface/(CFU associated with intestinal surface + CFU in accumulated fluid)], where CFU associated with the intestinal surface = length of loop (cm) × [weight of tissue homogenized (g)/0.4 g/cm of intestine] and CFU in accumulated fluid = CFU/ml of fluid accumulated × volume of fluid accumulated (ml). The value 0.4 g/cm of intestine is based on the average weight of eight different 1-cm segments of ileum.

CT production by strains grown in vitro and in vivo. CT production was determined for in vitro- and in vivo-grown *V. cholerae* strains by enzyme-linked immunosorbent assay (ELISA) essentially as described by Sack et al. (29) with ganglioside GM_1 (Supelco Inc. Bellefonte, Pa.)-coated 96-well microtiter plates (Falcon U-bottom, polystyrene; Becton Dickinson Co.). Antibody to CT (Sigma Chemical Co., St. Louis, Mo.) was obtained in New Zealand White rabbits. Secondary antibody, goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase, was obtained from Organon Technika-Cappel, West Chester, Pa. Optimum concentrations of GM_1 ganglioside and antibodies were determined by checkerboard reactions with known amounts of CT. For determination of in vitro production of CT, cultures were grown for 48 h in Casamino Acids-yeast extract broth (29) at 35°C with shaking, bacteria were removed by centrif-

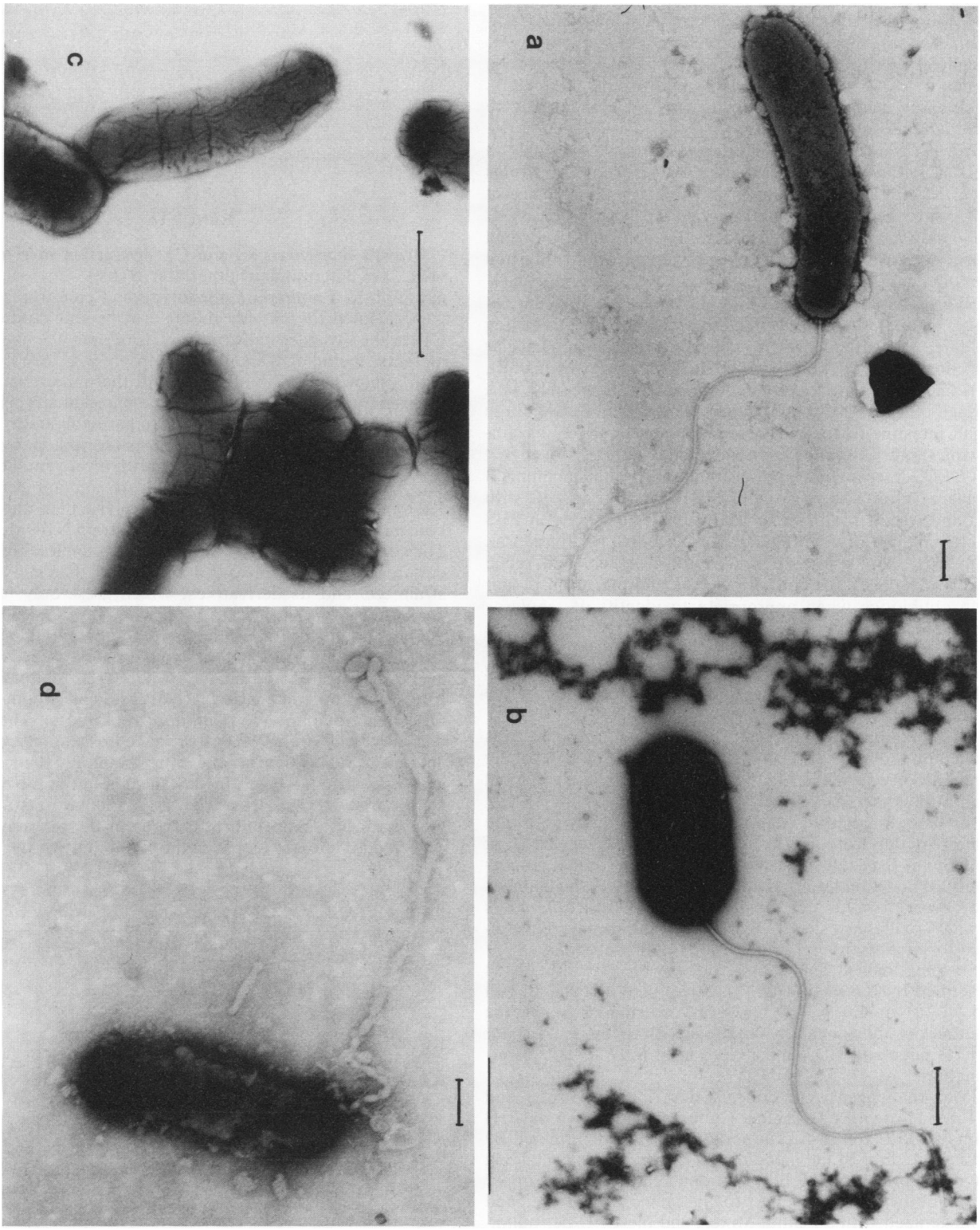

FIG. 1. Transmission electron micrograph of motile, flagellate, wild-type *V. cholerae* N16961 (a) and 395 (b) and nonmotile aflagellate mutant KR3 (c) negatively stained with uranyl acetate and nonmotile mutant KR1 with a coreless sheath negatively stained with phosphotungstic acid (d). Original magnifications of negative, ×10,000 for N16961, ×25,000 for 395, ×19,000 for KR3, and ×14,000 for KR1. Bars, 500 nm.

ugation at 10,000 × *g* for 20 min, the culture supernatant was filtered through a 0.22-μm-pore-size membrane (Schleicher & Schuell, Keene, N.H.), and the sterile culture supernatant was assayed. For in vivo CT production, fluid contents from 16-h loops were assayed. To determine nonspecific background, assays were done with wells not coated with GM_1 ganglioside. The amount of CT was calculated based on standard curves obtained by using known amounts of CT. Each assay was done in duplicate, and CT standards were included on each plate.

In vitro growth curves. Cultures were grown overnight in BHI at 35°C with shaking, diluted 1:100 for El Tor strains and 1:50 for classical strains in fresh BHI, and incubated at 35°C with shaking. At time intervals the optical density at 550 nm (OD_{550}) was determined. To determine the final density the strains attained, cultures were grown for 14 h and the OD_{550}s of dilutions of the cultures were determined.

RITARD model. For the RITARD model, the procedure of Spira et al. (30) was essentially used with the following modifications. Rabbits were anesthetized by intramuscular injection of a cocktail containing ketamine (60 mg/ml), xylazine (6 mg/ml), and acepromazine (1.2 mg/ml) at a dosage of 1 ml/kg of body weight. The slipknot closing the small intestine at the mesoappendix was removed 2 h following surgery. Rabbits were inoculated with 1×10^7 to 8×10^9 CFU, determined by plating dilutions of the inoculum on L agar. Cultures were grown in BHI for 18 h at 35°C with shaking, washed with fresh BHI, and resuspended in PBS containing 0.1% (wt/vol) gelatin, as specified by Spira et al. (30). The rabbits were observed for diarrhea, and rectal swabs were obtained daily for 5 days and plated on L agar with antibiotics when appropriate to identify *V. cholerae* being shed. Rabbits which died during the 5 days were autopsied. At the end of 5 days all rabbits were sacrificed and autopsied. For each strain and dosage tested four to five rabbits were infected. The data were evaluated by the Student-Newmann-Keuls *t* test.

(i) Evaluation of diarrhea. The diarrheal response was graded on the basis of the consistency of the stool. A totally liquid stool was graded as +4, while a completely normal stool was graded as +1.

(ii) Determination of colonization. The rectal swabs were plated in duplicate on L agar containing 5 or 25 U of polymyxin B per ml for classical or El Tor strains, respectively. When doubt existed about the identity of the colonies they were assayed for oxidase activity (*V. cholerae* is strongly oxidase positive). *V. cholerae* colonies were examined for motility by light microscopy with wet mounts.

Suckling mouse assay. FA ratios were determined as described by Baselski et al. (3). Mice were inoculated with 2.5×10^6 to 6.0×10^8 CFU, as determined by plating dilutions of the inoculum on L agar. Both Swiss Webster (CFW) mice and CD-1 mice were used for FA ratio determination.

Dual challenges were done as described by Freter and O'Brien (14) with CD-1 mice. Cultures were grown on BHI slants at 35°C for 18 h. The input ratio was determined by plating dilutions of the inoculum on L-agar plates containing the appropriate antibiotic. For dual challenge the upper halves of the bowels from six to eight mice were pooled and homogenized with an Omni-Mixer, and the CFU per milliliter for each strain was determined by plating dilutions on appropriate antibiotic-containing L-agar plates. For determination of in vivo growth the entire intestine was homogenized. The output ratio was then calculated.

TABLE 2. Culture density of *V. cholerae* isolates grown for 14 h in BHI[a]

Strain	OD_{550}
N16961	5.0
KR517	7.4
KR26	6.6
KR31	6.6
395	3.7
KR3	3.5
KR1	3.4

[a] Cultures were grown at 35°C with shaking.

RESULTS

Growth characteristics and CT production in vivo and in vitro. To determine whether the transposon insertion or mutation to a nonmotile phenotype had pleiotropic effects, we examined the growth characteristics and production of CT for the two parental strains, N16961 and 395, and various mutants, including a motile prototroph of N16961, KR517, with a mini-Tn*10*-Kan insertion. All the mutants grew with the same kinetics as their wild-type parental strains both in vivo and in vitro. However, the two parental strains differed from each other in growth characteristics both in vivo and in vitro. While all strains grew logarithmically at about the same rate, classical strain 395 and its mutant derivatives KR3 and KR1 exhibited a longer lag phase than did the wild type and mutant derivatives of El Tor strain N16961. Also El Tor strain N16961 and its derivatives attained a higher final culture density of 5.0 to 7.4 at 550 nm than did classical strain 395 and its derivatives, which grew only to a final density of 3 to 4 (Table 2).

The amounts of CT produced by the parental and mutant strains grown in vitro and in vivo were determined by using an ELISA system and binding to GM_1 (22). Parental strain N16961 and the mutant derivatives tested all produced more CT when grown in vivo than in vitro (Table 3). The mutant derivatives of strain N16961 produced essentially the same amount of CT as that produced by the parental strain when grown in vitro; however, in vivo the amount of CT produced by the mutants was less. The large standard errors of the means reflect significant differences in the amounts of CT detected in different loops and not variability in the ELISA.

TABLE 3. CT production in vitro and in vivo

Strain	Amt of CT (ng/ml)		No. of loops	No. of rabbits
	Culture supernatant[a]	Ileal loop fluid[b]		
N16961	46 ± 5[c]	1,474 ± 1,576	6	3
KR100	31 ± 15	479 ± 311	5	1
KR26	52 ± 24	99	2	1
KR126	62 ± 15	993	1	1
KR31	41 ± 16	873 ± 241	5	3
395	2,569 ± 315	110 ± 96	11	3
KR10	90 ± 37	171 ± 84	3	2
KR12	85 ± 38	291 ± 341	3	1

[a] CT was assayed in supernatants of cultures grown for 24 h in yeast extract-Casamino Acids broth. Data are the means ± standard errors of the means for three separate cultures and ELISAs done in duplicate for each culture.

[b] CT was determined in ileal loop fluid from the FA ratio experiment by GM_1 ELISA in duplicate for each loop.

[c] When three or more loops were assayed the means ± standard errors of the means are given.

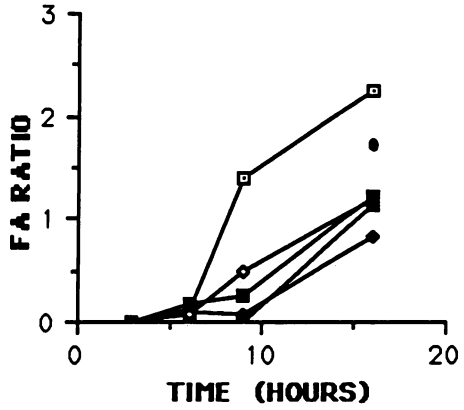

FIG. 2. FA ratio produced at various times in rabbit ileal loops by *V. cholerae* isolates. Ileal loops were inoculated, and at 3, 6, 9, and 16 h rabbits were sacrificed and the FA ratio (milliliters of fluid per centimeter of length of loop) was determined. At least four loops were used for each *V. cholerae* isolate, except for strain 395, which was examined only at 16 h in two loops. Shown are results for strains N16961 (⊡), KR31 (◆), KR26 (◘), KR10 (◇), KR12 (■), and 395 (●).

Multiple separate analyses produced the same result for the contents from each loop. Classical strain 395 produced substantially more CT than the mutant derivatives when grown in vitro. Interestingly, when strain 395 and its mutant derivatives, KR3 and KR1, were grown in vivo they all produced about the same amount of CT. Except for strain 395, production of CT was greater for both biotypes and the mutants when they were grown in vivo.

Evaluation in the rabbit ileal loop. Both FA ratios and adherence of the wild type and motility mutants were determined at 3, 6, 9, and 16 h following inoculation, except for strain 395, which was examined at 9 and 16 h. Significant fluid began to accumulate 9 h after inoculation (Fig. 2). While the nonmotile mutants clearly produced significant FA regardless of the type of nonmotile mutant or biotype, it was significantly less ($P < 0.01$) than that produced by N16961 and 395, whose FA ratios were 0.57 ± 0.10 at 9 h and 1.75 ± 0.30 at 16 h. No significant difference ($P < 0.05$) in FA ratios was observed between the nonmotile mutants from the same parental strain. At 9 and 16 h all the strains exhibited net growth in the loops.

The association of vibrios with the ileal surface in the ligated loops was determined in the 9- and 16-h loops, in which significant FA occurred. The nonmotile derivatives of El Tor strain N16961, the nonmotile flagellated mutant, KR31, and the nonmotile aflagellate mutant, KR26, clearly adhered less well than did the parental strain (Table 4) at both 9 and 16 h. In contrast, the nonmotile derivatives of classical strain 395 adhered as well as the parental strain at 16 h with a low inoculum. However, at high doses the mutants adhered better than the parental strain, although the percent associated with the mucosa for all strains was significantly less. This reduced percent association probably reflects the high dose and saturation of the tissue receptors or adherence sites. At 9 h, 90% of the classical nonmotile mutant vibrios were associated with the mucosa while about 15% of the parental strain were associated with the mucosa.

395 is a classical biotype strain known to spontaneously mutate to the nonmotile phenotype at a high frequency in vitro (25). We examined in soft agar the motility of 17 to 24 colonies obtained from the plates used to determine CFU associated with the mucosa and in the loop fluid. Six 9-h loops and six 18-h loops were examined. For these experiments the inoculum contained 26% motile vibrios. The mean percentages of motile vibrios associated with the mucosa were 93% ± 12% at 9 h and 98% ± 3% at 18 h. In the fluid the mean percentages of motile vibrios were 59% ± 1% at 9 h and 88% ± 5% at 18 h.

Evaluation in the RITARD model. Three different parameters were examined in the RITARD model: (i) virulence, as measured by the time to death; (ii) diarrheagenicity, as measured by the period of time during which diarrheal stools were obtained; and (iii) colonization, as measured by the period of time vibrios could be recovered from stools or rectal swabs after infection. Control strain KR517 was included in these experiments.

Parental strains 395 and N16961 and control strain KR517 were clearly more virulent than their nonmotile derivatives, producing fatal diarrhea in all of the rabbits tested (Table 5). KR31 (Mot$^-$ Fla$^+$) at a log dose of 7.0 caused fatal diarrhea in two of five rabbits, while in an earlier challenge at a 500-fold higher dose it produced no fatalities. Mutant KR26 (Mot$^-$ Fla$^-$) produced fatal diarrhea in three of five rabbits tested. There was no significant difference between the Mot$^-$ Fla$^-$ strain KR26 and the Mot$^-$ Fla$^+$ strain KR31 by these measures ($P < 0.05$). Thus, nonmotile mutants of

TABLE 4. Association of vibrios with rabbit ileal mucosa

Strain	Phenotype	9 h		16 h	
		Inoculum[a]	Adherence[b]	Inoculum	Adherence
N16961	Wild type, Mot$^+$	6.78–7.00	56 ± 14	5.78–6.30	61 ± 51
KR31	Mot$^-$ Fla$^+$	6.63–7.70	13 ± 10	6.90–7.11	6.4 ± 6.7
KR26	Mot$^-$ Fla$^-$	7.14	5.8 ± 1.0	7.15	6.6 ± 0.6
				9.60	3.0 ± 3.3
395	Wild type, Mot$^+$	9.40–9.91	14.6 ± 13.2	5.40	30.4
				9.40–9.91	5.4 ± 4.2
KR3	Mot$^-$ Fla$^-$	6.60–7.60	92 ± 15	5.23–6.30	26 ± 19
				9.43	13 ± 4.6
KR1	Mot$^-$ Fla$^-$ Sheath^{+c}	3.00–6.48	96 ± 3	3.48–6.00	27 ± 29
				9.67	22 ± 19

[a] The inoculum is given as the log value.

[b] Adherence values are the percent of the total CFU in the loop that associated with the ileal mucosa. See Materials and Methods for calculations used. The values are the means ± standard errors of the means for four to six loops in two or three rabbits, except for strain 395 with a log inoculum of 5.40, for which the value is the average of two loops in one rabbit.

[c] This mutant produces a sheathlike flagellar structure without the flagellar filament.

TABLE 5. Characteristics of *V. cholerae* isolates in RITARD model

Biotype and strain	Phenotype	Log challenge dose	Time to death (h)[a]	Duration of diarrhea (h)[a]	% Time colonized[b]	No. of rabbits
El Tor						
N16961	Wild type, Mot$^+$	7.6	12 ± 8 (5)[c]	7 ± 11	100	5
KR517	Mot$^+$ Tn*10*::mini-Kan	7.3	18 ± 4 (5)	0[d]	100	5
KR26	Mot$^-$ Fla$^-$	7.5	78 ± 38 (3)	52 ± 17	87	5
KR31	Mot$^-$ Fla$^+$	7.0	105 ± 26 (2)	27 ± 24	52	5
KR31	Mot$^-$ Fla$^+$	9.7	NA[e]	80 ± 11	73	4
Classical						
395	Wild type, Mot$^+$	8.9	16 ± 9 (4)	0	100	4
		9.9	25 ± 9 (5)	0	100	5
KR3	Mot$^-$ Fla$^-$	7.0	NA	0	0	5
KR3	Mot$^-$ Fla$^-$	9.9	NA	25 ± 33	45	4
KR1	Mot$^-$ Fla$^-$ Sheath$^+$	8.0	96 ± 23 (3)	41 ± 44	45	5
KR1	Mot$^-$ Fla$^-$ Sheath$^+$	9.9	69 (2)	28 ± 27	54	5

[a] Values are means ± standard errors of the means.
[b] Calculated as the ratio of the length of time the rabbits lived to the length of time *V. cholerae* organisms were excreted.
[c] Values in parentheses indicate number of rabbits that died.
[d] No diarrhea was observed. Rabbits died prior to exhibiting diarrhea.
[e] NA, not applicable; no deaths occurred.

strain N16961 regardless of flagellar structure were equally virulent. In contrast, nonmotile mutant derivatives of strain 395 did exhibit a significant difference; KR3 (Mot$^-$ Fla$^-$) did not produce fatal diarrhea in any of the rabbits tested at log doses of 7.0 and 9.9, while KR1 (Mot$^-$ Sheath$^+$) produced fatal diarrhea in three of five rabbits at a log dose of 8.0 and in two of five rabbits at a log dose of 9.9, suggesting that the presence of a sheath enhanced virulence.

No significant diarrhea was observed in rabbits infected with strain N16961 or 395 or control strain KR517 since the rabbits died in a short time. The two nonmotile mutant derivatives of N16961 produced significant diarrhea; however, no significant difference between these two mutants was observed. Among the classical nonmotile mutants, the coreless sheath mutant, KR1, produced diarrhea at log doses of 8.0 and 9.9, while the nonmotile mutant without any flagellar structure, KR3, produced diarrhea only at a log dose of 9.9 and not at a log dose of 7.0.

Both of the El Tor nonmotile mutants colonized infected rabbits. No significant difference could be observed between these two mutants. For the classical nonmotile mutants, the coreless sheath mutant, KR1, colonized the rabbits, while KR3 colonized only at the higher dose of log 9.9.

To check for reversion of the nonmotile mutants to the motile phenotype in these in vivo studies, all positive cultures from the colonization studies were examined for motility by phase-contrast microscopy. No revertants to the motile phenotype were observed.

Suckling mouse assays. Diarrheagenicity was determined by examining FA in the intestine. Two strains of mice, Swiss Webster and CD-1, were compared for sensitivity (Table 6). Except for strain KR31, FA ratios were generally greater in CD-1 mice than in Swiss Webster mice, although statistically there was only a marginal difference. On the basis of these results CD-1 mice were then used for studies of competition between various parental and mutant strains. All of the strains, parental and nonmotile mutants, produced sufficient FA in the mice to be characterized as virulent. No significant difference from parental strain N16961 was detected by the Student-Newman-Keuls test.

To analyze the growth characteristics of the various strains relative to each other, the input and output ratios for pairs of strains were determined with the entire intestine (Table 7). The suckling mice do not excrete anything by 16 h as shown by the presence of a blue dye marker visible in the stomach and lower part of the bowel. Except for the pairs KR26/KR131 and KR31/KR126, all the strains exhibited net growth. Input and output ratios did not differ by more than sixfold. For the dual challenge with KR31 and KR126, strain KR31 exhibited a decrease in CFU in the intestine. A sixfold difference is the minimum required to assign a difference (14). KR26 and the spectinomycin-resistant derivative KR126 appear to grow faster than KR31 and the spectinomycin-resistant derivative KR131 (Table 7).

For the dual competition studies it was necessary to isolate spontaneous mutants with antibiotic resistance markers so that the two populations could be differentiated by plating on antibiotic-containing plates. Control mixtures were examined for competition to determine whether acquisition of an antibiotic resistance marker would alter colonization. Initial experiments were done with spectinomycin-resistant derivatives. These derivatives colonized less well than the parental strain, giving ratios of N16961 to spectino-

TABLE 6. FA ratios for El Tor isolates obtained in CD-1 and Swiss Webster mice

Strain	Log challenge dose	FA ratio (mean ± SEM)	
		CD-1 mice	Swiss Webster mice
N16961	7.57	0.0827 ± 0.006 (6)[a]	
	7.67		0.0745 ± 0.0119 (5)
KR100	7.18	0.0751 ± 0.0077 (6)	
	8.69		0.0732 ± 0.0081 (7)
KR26	6.74	0.0818 ± 0.00996 (6)	
	8.70		0.0818 ± 0.0207 (7)
KR31	6.88	0.0697 ± 0.0126 (6)	
	8.68		0.0757 ± 0.0146 (6)
KR3	6.93	0.0792 ± 0.00731 (7)	
	8.78		0.0658 ± 0.00942 (6)
KR1	6.40	0.0811 ± 0.0113 (5)	
	7.62		0.0668 ± 0.00856 (6)

[a] Values in parentheses are the numbers of mice used to determine FA ratios.

TABLE 7. In vivo growth of dual cultures in CD-1 suckling mice[a]

Strains	Characteristics	Input inoculum[b]	Input ratio	Output ratio	Change
Control mixtures					
N16961/KR100	Mot$^+$/Mot$^+$; wild type/Spec^r	7.87/7.64	1.69	6.93	4-fold increase
		6.70/6.51	1.58	2.78	2-fold increase
		7.21/7.24	0.92	1.34	No change
KR517/KR100	Mot$^+$ Tn*10*/Mot$^+$; Kan^r/Spec^r	5.74/5.60	1.38	0.529	3-fold decrease
KR517/KR601	Mot$^+$ Tn*10*/Mot$^+$ Tn*10*; Kan^r/Kan^r Spec^r	5.16/5.04	1.36	1.00	No change
Test mixtures					
KR26/KR100	Mot$^-$ Fla$^-$/Mot$^+$; Kan^r/Spec^r	5.95/5.90	1.1	1.58	No change
		6.89/6.51	2.45	5.79	2.4-fold increase
		7.54/7.30	1.72	0.694	2.5-fold decrease
KR26/KR601	Mot$^-$ Fla$^-$/Mot$^+$ Tn*10*; Kan^r/Kan^r Spec^r	8.25/7.82	2.65	32.0	12-fold increase
		7.48/7.05	1.00	0.222	5-fold decrease
KR31/KR100	Mot$^-$ Fla$^+$/Mot$^+$; no resistance/Spec^r	8.35/7.64	5.09	1.00	5-fold decrease
		6.78/6.61	1.88	0.375	5-fold decrease
		7.41/7.18	1.72	0.818	2-fold decrease
KR31/KR601	Mot$^-$ Fla$^+$/Mot$^+$ Tn*10*; no resistance/Kan^r Spec^r	7.48/7.05	2.69	3.10	No change
		6.23/5.89	2.18	1.07	2-fold decrease
		7.95/7.65	2.00	6.33	3-fold increase
		6.65/6.35	2.00	0.667	3-fold decrease
KR26/KR131	Mot$^-$ Fla$^-$/Mot$^-$ Fla$^+$; Kan^r/Spec^r	8.25/8.05	1.57	10.6	7-fold increase
		7.48/7.39	1.23	24.2	20-fold increase
		6.89/6.85	1.11	69.7	63-fold increase
KR31/KR126	Mot$^-$ Fla$^+$/Mot$^-$ Fla$^-$; no resistance/Kan^r Spec^r	8.08/7.67	2.59	0.0097	267-fold decrease
		7.54/7.42	1.30	0.167	8-fold decrease

[a] The contents of the entire intestine were assayed at 16 h postinoculation. Each set of value is from an experiment in which five to eight mice were pooled for assay.

[b] Log of the number of CFU used as inoculum.

mycin-resistant N16961 of 35 to 93. The same result was obtained when streptomycin-resistant derivatives were examined. In fact, the streptomycin-resistant derivatives colonized less well than did the spectinomycin-resistant mutants (Table 8). Simple inspection of the data obtained for the competition pairs of KR26 and KR31 showed no obvious differences. Statistical analysis by the Wilcoxon signed-rank test also revealed no significant differences.

DISCUSSION

The bacterial flagellar structure can contribute by two mechanisms to colonization and hence pathogenicity of enteric pathogens. First, motility hypothetically increases the number of potential interactions between the bacterium and the epithelial mucosal layer. Motility combined with a chemotactic response could enhance the probability of interactions with specific regions of the intestinal mucosa and resistance to elimination by peristalsis. Secondly, the flagellar structure might contain factors involved in adherence to the intestinal mucosa. *V. cholerae* has a sheathed flagellum, which could contain adherence factors (1). Interestingly, given these potential contributions to pathogenesis not all enteric pathogens require motility for virulence. *Shigella* spp. are by definition nonmotile organisms (28), as are *Escherichia coli* enteric pathogens of rabbits (4). Originally *Salmonella typhimurium* was believed to require motility for virulence; however, Carsiotis et al. (5) demonstrated that a virulence gene is located adjacent to flagellar genes. Lockman and Curtiss (22) showed that nonmotile *S. typhimurium* mutants remained virulent in BALB/c mice regardless of whether a flagellar structure was present. However for other enteric pathogens motility appears to play a role in pathogenesis. *Campylobacter jejuni* nonmotile mutants with or without flagellar structures were observed by Morooka et al. (24) to exhibit reduced virulence in a mouse model compared with that of the parental motile strain. Drake and Montie (9) have observed a role for motility in the pathogenicity of *P. aeruginosa* in the burned mouse model. While paralyzed mutants with the flagellar structure were no more virulent than the aflagellate mutants, mice could be passively protected with antiserum to the flagellar filament (8).

Several studies of nonmotile *V. cholerae* mutants in animal models have strongly suggested that motility is required for virulence in these models (14, 15, 20). The mutants used in these studies were either isolated as spontaneous mutants or obtained following chemical mutagenesis. In some cases it is not clear whether flagellar structures were present. Analysis of our motility mutants in the three animal models clearly shows that motility is a major contributing factor to *V. cholerae* pathogenicity and colonization in these animal models, while the flagellar structure appears to be less important. A clear difference between motile and nonmotile El Tor strains was observed in the RITARD model for virulence as measured in time to death, diarrheagenicity, and the ability to colonize. In the ileal loop the FA ratios reflected the same difference. No significant difference was observed between the nonmotile flagellated mutant KR31 and the nonmotile aflagellate mutants; however, clearly nonmotile organisms produced lower FA ratios. These lower FA ratios might be due to decreased CT production, since all the derivative strains tested produced somewhat less toxin than the parental strain in vivo as detected by ELISA.

The results with the classical strains also show a clear role for motility as well as suggesting a role for the flagellar structure in colonization. Nonmotile mutants produced less FA in the rabbit ileal loop than did the motile parent strain 395, even though they produced about the same amount of

TABLE 8. Dual infection competition assay in CD-1 mice[a]

Strains	Characteristics	Input inoculum[b]	Input ratio	Output ratio	Change
Control mixtures					
N16961/KR100	Mot$^+$/Mot$^+$; no resistance/Spec^r	6.95/6.88	1.17	2.38	2-fold increase
		6.76/6.74	1.05	35.7	35-fold increase
N16961/KR400	Mot$^+$/Mot$^+$; no resistance/Str^r	6.98/6.86	1.30	221	170-fold increase
		7.15/7.04	1.27	174	137-fold increase
KR517/KR601	Mot$^+$::Tn*10*/Mot$^+$ Tn*10*; Kan^r/ Kan^r Spec^r	7.04/7.15	0.786	72.7	93-fold increase
		6.95/7.15	0.629	2.75	4-fold increase
Test mixtures					
KR26/N16961	Mot$^-$ Fla$^-$::Tn*10*/Mot$^+$; Kan^r/no resistance	7.26/6.98	1.89	0.0877	22-fold decrease
		7.18/7.15	1.07	0.0674	16-fold decrease
KR26/KR100	Mot$^-$ Fla$^-$::Tn*10*/Mot$^+$; Kan^r/ Spec^r	7.15/6.88	1.87	8.67	5-fold increase
		6.36/6.74	0.418	0.444	No change
KR26/KR601	Mot$^-$ Fla$^-$::Tn*10*/Mot$^+$::Tn*10*; Kan^r/Kan^r Spec^r	7.15/6.98	1.47	3.74	2.5-fold increase
		6.36/6.95	0.256	0.333	No change
KR31/KR100	Mot$^-$ Fla$^+$/Mot$^+$; no resistance/ Spec^r	7.15/6.88	1.87	0.147	13-fold decrease
		6.93/6.74	1.55	1.50	No change
KR31/KR400	Mot$^-$ Fla$^+$/Mot$^+$; no resistance/ Str^r	7.26/6.86	2.47	34.6	14-fold increase
		7.32/7.04	1.91	0.0357	54-fold decrease
KR31/KR601	Mot$^-$ Fla$^+$/Mot$^+$::Tn*10*; no resistance/Kan^r Spec^r	8.75/8.58	1.47	0.100	15-fold decrease
		6.93/6.95	0.944	0.400	2.4-fold decrease
KR26/KR31	Mot$^-$ Fla$^-$/Mot$^-$ Fla$^+$; Kan^r/no resistance	7.26/7.26	1.00	1.30	No change
		7.18/7.32	0.714	4.17	6-fold increase
KR26/KR131	Mot$^-$ Fla$^-$/Mot$^-$ Fla$^+$; Kan^r/Spec^r	8.74/8.76	0.933	11.2	12-fold increase
		6.36/7.15	0.164	1.88	12-fold increase
KR31/KR126	Mot$^-$ Fla$^+$/Mot$^-$ Fla$^-$; no resistance/Kan^r Spec^r	8.75/8.61	1.4	0.267	5-fold decrease

[a] The upper half of the intestine was assayed at 16 h postinoculation. Each set of values is for five to eight mice pooled for assay.
[b] Log of the number of CFU used as inoculum.

CT in vivo. In addition, more spontaneous nonmotile mutants of strain 395 were observed in the ileal fluid than associated with the mucosa. Surprisingly, no difference in adherence to mucosa was observed in 16-h ligated loops inoculated at lower doses, while at a high dose the mutants adhered better than the motile parent. However, at the high dosage all adherence was reduced. This may reflect the increased inoculum size and accumulation of toxic products or saturation of adherence sites on the mucosa. Additionally, analysis of the classical strains in the RITARD model suggests a role for the flagellar structure. While all nonmotile mutants were less virulent than the wild-type motile parent, the nonmotile mutant with a sheathlike structure, KR1, was more virulent than the aflegellate mutant KR3. KR1 caused death, while KR3 did not. KR1 produced diarrhea and colonized at both doses, while KR3 produced diarrhea and colonized only at the higher dose.

Classical *V. cholerae* strains differ from El Tor vibrios in a number of characteristics (2), including virulence in human volunteers (6). We observed that mutant strains of the classical biotype adhered better to the rabbit ileal mucosa than did the parental or mutant El Tor isolates at 9 h. While further examination of these classical mutants as well as an additional flagellated nonmotile mutant (25) is required, these results further support the concept that classical *V. cholerae* strains are more virulent than El Tor vibrios. This increased virulence may be due to the greater ability of classical vibrios to colonize or adhere, since the classical vibrios examined grew more slowly and attained a lower final density in vivo and in vitro than did the El Tor strains.

We could not demonstrate significant adherence of El Tor, motile or nonmotile, to the rabbit ileum at 16 h. Some difference between the motile wild-type strain and the mutants could be observed, but the standard deviation for all strains was so large that the difference was not significant. Yancy et al. (36) using ^{35}S-labeled vibrios and high concentrations observed a difference between motile and nonmotile aflagellate strains. Freter and Jones (13) using isolated brush border membranes and tissue slices from rabbit intestine also observed a difference between motile and nonmotile aflagellate strains. The use of ^{35}S-labeled vibrios may increase the sensitivity of the adherence assay, and the use of isolated tissue, no longer physiologically active, may explain the difference observed by these investigators. Common to all rabbit models used for studies of *V. cholerae* pathogenesis is the fact that colonization is compromised by obstructing the small bowel or slowing down peristalsis with drugs. The rabbit small intestine may provide few adherence sites for *V. cholerae*. In fact, Yamamoto et al. (33, 34) have observed by scanning electron microscopy that *V. cholerae* organisms attach to rabbit ileum very poorly compared with their adherence to human ileum.

The suckling mouse assays for FA did not reveal any significant differences between the El Tor parent and isogenic mutants. FA ratios produced by the mutants in suckling mice showed very little difference between the wild-type and mutant strains, unlike the rabbit ileal loop FA ratios, for which a distinct difference was observed between motile and nonmotile organisms. These results suggest that the rabbit model may be more sensitive than the mouse model for FA ratio determination. The structural arrangement of the rabbit ileum and villi may be different from that of suckling mice, requiring bacterial motility for the epithelial cells to be efficiently exposed to toxin. Or the mucus layer may be more extensive in rabbits than in suckling mice. Neither FA model detected any difference between nonmotile mutants with and without flagellar structures. This observation is quite reasonable since the rabbit ligated loop is a closed system and

potential attachment factors associated with the flagellum may not be required. The suckling mouse model is an open system and hence theoretically might have detected a difference. However, since no difference was detected between motile and nonmotile mutants in mice it is not surprising that no difference was detected between flagellate and aflagellate nonmotile mutants.

No conclusions can be reached concerning the role of motility or the flagellar structure by using the dual infection competition assay of infant mice. Control assays in which the ratio of control and test mixtures were determined for the entire intestine demonstrated that net growth occurred, and except for dual infection by KR31 and KR126, the same output ratio as input ratio was obtained. These data indicate that both strains in the dual infections are growing at the same rate, since no secretion of vibrios had yet occurred. Yet, subsequent analysis of test strains in the upper intestine did not yield statistically significant data. Control mixtures exhibited larger changes of input ratios. Motile spectinomycin- and streptomycin-resistant derivatives of strain N16961 exhibited decreased capacity to colonize the upper small bowel, resulting in large output ratios. Since no change in output ratio occurred when the entire intestine was assayed it appears that the antibiotic-resistant strains exhibited decreased colonizing capacity and not a decreased growth rate. In *E. coli* both of these antibiotic resistances are known to involve alterations of different ribosomal proteins. It is possible that in these *V. cholerae* isolates antibiotic resistance and altered ribosomal proteins have led to inefficient transcription of genes involved in colonization. Results for many of these dual challenges exhibited large standard deviations. Freter and O'Brien (14) examined nonchemotactic *V. cholerae* in this model and also observed large standard deviations between individual experiments.

The results for each animal model and set of nonmotile mutants did not correlate closely. Differences were observed between motile and nonmotile mutants when FA ratios were determined in ligated rabbit loops, but no differences were observed in suckling mice. While the RITARD model suggests a role for the sheathed flagellum, the other open system, suckling mouse dual infections, was not able to demonstrate a difference between motile and nonmotile *V. cholerae*. Freter and O'Brien (14) observed that nonchemotactic mutants adhered less well to rabbit and mouse intestines in vivo and in vitro but that in dual infection studies in suckling mice they appeared to be more virulent than the chemotactic parent strains. On the basis of correlate analysis they suggested that chemotactic strains were killed by some mechanism located near the epithelial cell surface. We did not observe any killing of vibrios. Many explanations are possible, including the different physiology of the two animals, their different developmental ages, potentially different distribution of colonization factor receptors or toxin receptors, or the distribution, thickness, and viscosity of the mucus layer.

We have demonstrated, using two rabbit models and defined mutants with transposon insertions and paralyzed mutants, that motility and not flagellar structures are of primary importance for *V. cholerae* pathogenicity in these animal models. The RITARD model alone suggests a minor role for the sheathed flagellum of classical vibrios in colonization. It is quite possible that these animal models are not sufficiently sensitive to detect the contribution of minor colonization factors. It is possible that human tissue contains more receptors pertinent to *V. cholerae* colonization than do small bowels of mice or rabbits, animals which are not naturally susceptible to *V. cholerae* infection.

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REFERENCES

1. **Attridge, S. R., and D. Rowley.** 1983. The role of the flagellum in the adherence of *Vibrio cholerae*. J. Infect. Dis. **147**:864–872.
2. **Barua, D.** 1974. Laboratory diagnosis of cholera, p. 85–128. *In* D. Barua and W. Burrows (ed.), Cholera. The W. B. Saunders Co., Philadelphia.
3. **Baselski, V., R. Briggs, and C. Parker.** 1977. Intestinal fluid accumulation induced by oral challenge with *Vibrio cholerae* or cholera toxin in infant mice. Infect. Immun. **15**:704–712.
4. **Cantey, J. R., and R. K. Blake.** 1977. Diarrhea due to *Escherichia coli* in the rabbit: a novel mechanism. J. Infect. Dis. **135**:454–462.
5. **Carsiotis, M., B. A. D. Stocker, D. L. Weinstein, and A. D. O'Brien.** 1989. A *Salmonella typhimurium* virulence gene linked to *flg*. Infect. Immun. **57**:3276–3280.
6. **Clements, M. L., M. M. Levine, C. R. Young, R. E. Black, Y. L. Lin, R. M. Robins-Browne, and J. P. Craig.** 1982. Magnitude, kinetics, and duration of vibriocidal antibody responses in North Americans after ingestion of *Vibrio cholerae*. J. Infect. Dis. **145**:465–473.
7. **De, S. H., and D. N. Chatterjee.** 1953. An experimental study of the mechanisms of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Pathol. Bacteriol. **46**:559–562.
8. **Drake, D., and T. C. Montie.** 1987. Protection against *Psesudomonas aeruginosa* infection by passive transfer of anti-flagellar serum. Can. J. Microbiol. **33**:755–763.
9. **Drake, D., and T. C. Montie.** 1988. Flagella, motility and invasive virulence of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **134**:43–52.
10. **Eubanks, E. R., M. N. Guentzel, and L. J. Berry.** 1977. Evaluation of surface components of *Vibrio cholerae* as protective immunogens. Infect. Immun. **15**:533–538.
11. **Finn, T. M., J. Reiser, R. Germanier, and S. J. Cryz, Jr.** 1987. Cell-associated hemagglutinin-deficient mutant of *Vibrio cholerae*. Infect. Immun. **55**:942–946.
12. **Follett, E. A. C., and J. Gordon.** 1963. An electron microscope study of Vibrio flagella. J. Gen. Microbiol. **32**:235–239.
13. **Freter, R., and G. W. Jones.** 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with intact mucosal surfaces. Infect. Immun. **14**:246–256.
14. **Freter, R., and P. C. M. O'Brien.** 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: fitness and virulence of nonchemotactic *Vibrio cholerae* mutants in infant mice. Infect. Immun. **34**:222–233.
15. **Guentzel, M. N., and L. J. Berry.** 1975. Motility as a virulence factor for *Vibrio cholerae*. Infect. Immun. **11**:890–897.
16. **Guentzel, M. N., L. H. Field, E. R. Eubanks, and L. J. Berry.** 1977. Use of fluorescent antibody in studies of immunity to cholera in infant mice. Infect. Immun. **15**:539–548.
17. **Hall, R. H., P. A. Vial, J. B. Kaper, J. J. Mekalanos, and M. M. Levine.** 1988. Morphological studies on fimbriae expressed by *Vibrio cholerae* O1. Microb. Pathog. **4**:257–265.
18. **Herrington, D. A., R. H. Hall, G. Losonsky, J. J. Mekalanos, R. K. Taylor, and M. M. Levine.** 1988. Toxin, toxin-coregulated pili and the *tox*R regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med. **168**:1487–1492.
19. **Jones, G. W., G. D. Abrams, and R. Freter.** 1976. Adhesive properties of *Vibrio cholerae*: adhesion to isolated rabbit brush border membranes and hemagglutinating activity. Infect. Immun. **14**:232–239.

20. **Jones, G. W., and R. Freter.** 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect. Immun. **14**:240–245.
21. **Levine, M. M., R. E. Black, M. L. Clements, L. Cisneros, A. Saah, D. R. Nalin, D. M. Gill, J. P. Craig, C. R. Young, and P. Ristaino.** 1982. The pathogenicity of nonenterotoxinogenic *Vibrio cholerae* El Tor isolated from sewage water in Brazil. J. Infect. Dis. **145**:296–299.
22. **Lockman, H. A., and R. Curtiss III.** 1990. *Salmonella typhimurium* mutants lacking flagella or motility remain virulent in BALB/c mice. Infect. Immun. **58**:137–143.
23. **Miller, J. H.** 1982. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Morooka, T., A. Umeda, and K. Amako.** 1985. Motility as an intestinal colonization factor for *Campylobacter jejuni*. J. Gen. Microbiol. **131**:1973–1980.
25. **Mostow, P., and K. Richardson.** 1990. High-frequency spontaneous mutation of classical *Vibrio cholerae* to a nonmotile phenotype. Infect. Immun. **58**:3633–3639.
26. **Resnick, I. G., C. R. Ford, G. M. Shackleford, and L. J. Berry.** 1980. Improved protection against cholera in adult rabbits with a combined flagellar-toxoid vaccine. Infect. Immun. **30**:375–380.
27. **Richardson, K., L. Nixon, P. Mostow, J. B. Kaper, and J. Michalski.** 1990. Transposon-induced non-motile mutants of *Vibrio cholerae*. J. Gen. Microbiol. **136**:717–725.
28. **Rowe, B., and R. J. Gross.** 1984. Genus II. *Shigella* Castellani and Chalmers 1919, 936^{AL}, p. 423–427. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
29. **Sack, D. A., S. Huda, P. K. B. Neogi, R. R. Daniel, and W. M. Spira.** 1980. Microtiter ganglioside enzyme-linked immunosorbent assay for vibrio and *Escherichia coli* heat-labile enterotoxins and antitoxin. J. Clin. Microbiol. **11**:35–40.
30. **Spira, W. M., R. B. Sack, and J. L. Froehlich.** 1981. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *Escherichia coli* diarrhea. Infect. Immun. **32**:739–747.
31. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.
32. **Teppema, J. S., P. A. M. Guinee, A. A. Ibrahim, M. Paques, and E. J. Ruitenberg.** 1987. In vivo adherence and colonization of *Vibrio cholerae* strains that differ in hemagglutinating activity and motility. Infect. Immun. **55**:2093–2102.
33. **Yamamoto, T., T. Kamano, M. Uchimwa, M. Iwanaga, and T. Yokota.** 1988. *Vibrio cholerae* O1 adherence to villi and lymphoid follicle epithelium: in vitro model using Formalin-treated human small intestine and correlation between adherence and cell-associated hemagglutinin levels. Infect. Immun. **56**:3241–3250.
34. **Yamamoto, T., and T. Yokota.** 1988. Electron microscopic study of *Vibrio cholerae* O1 adherence to the mucus coat and villus surface in the human small intestine. Infect. Immun. **56**:2753–2759.
35. **Yancey, R. J., and L. J. Berry.** 1978. Motility of the pathogen and intestinal immunity of the host in experimental cholera, p. 447–455. *In* J. R. McGhee, J. Mestecky, and J. L. Babb (ed.), Secretory immunity and infection. Plenum Publishing Corp., New York.
36. **Yancey, R. J., D. L. Willis, and L. J. Berry.** 1978. Role of motility in experimental cholera in adult rabbits. Infect. Immun. **22**:387–392.
37. **Yancey, R. J., D. L. Willis, and L. J. Berry.** 1979. Flagella-induced immunity against experimental cholera in adult rabbits. Infect. Immun. **25**:220–228.