Vibrio cholerae Hemagglutinin/Protease, Colonial Variation, Virulence, and Detachment

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The structural gene, *hap*, for the secreted hemagglutinin/protease (HA/protease), a putative virulence factor of *Vibrio cholerae*, has recently been cloned and sequenced (C. C. Häse and R. A. Finkelstein, J. Bacteriol. 173:3311–3317, 1991). The availability of the null mutant, HAP-1, and HAP-1 complemented with pCH2 (which expresses HA/protease), enabled an examination of the role of HA/protease in the virulence of *V. cholerae* in an animal model. However, the mutants exhibited reversible colonial variation similar but not identical to that which was previously associated with dramatic changes in virulence of parental strain 3083. Regardless of colonial morphology, the mutants were found to be fully virulent in infant rabbits. Thus, the HA/protease is not a primary virulence factor (for infant rabbits). Observations using cultured human intestinal cells indicated, instead, that the HA/protease is responsible for detachment of the vibrios from the cultured cells by digestion of several putative receptors for *V. cholerae* adhesins.

The hemagglutinin/protease (HA/protease) of *Vibrio cholerae* (13), a reincarnation of the mucinase described earlier by Burnet (8), was discovered as a secreted or "soluble" hemagglutinin (12) and subsequently shown to be a zinc- and calcium-dependent protease (2). Although it is now recognized that the HA/protease is a member of a large family of such metalloproteases (22) produced by both nonpathogenic and pathogenic species (including the elastase of *Pseudomonas aeruginosa* [21]), its activity on potentially relevant substrates, i.e., mucin, fibronectin, lactoferrin, and the A subunit of cholera toxin and cholera toxin-related enterotoxins (3, 13), makes it an attractive candidate as a virulence factor in the pathogenesis and immunology of cholera. The structural gene, *hap*, for the HA/protease, Hap, of *V. cholerae* 3083 (El Tor biotype, Ogawa serotype) was recently cloned and sequenced in our laboratory (22). At the same time, a Hap⁻ mutant of strain 3083, HAP-1, was genetically engineered and complemented with plasmid pCH2 expressing the cloned structural gene (22). These mutants provided the opportunity of examining the role of the HA/protease in virulence of *V. cholerae* in an experimental animal model system.

However, before we could proceed with these experiments, a potential complicating factor had to be considered. It had previously been observed (23) and confirmed in this laboratory (1a) that strain 3083 spontaneously, reversibly, and at high frequency produced opaque (O) colonial variants which do not produce cholera enterotoxin and are avirulent in infant rabbits. If the engineered mutants HAP-1 and HAP-1(pCH2) were of the O CT⁻ phenotype, the results of virulence assays could be misleading.

The colonial morphology of the mutants, when they were examined by transmitted oblique illumination (14, 18), was found to be heterogeneous but distinct from the virulent, CT⁺ translucent (T) and the avirulent, CT⁻ O variants observed in parent strain 3083. Cloned sublines of the various colony phenotypes of the mutants were found to be

virulent like the T parent phenotype of strain 3083. Thus, we conclude that the *hap* gene product is not directly involved in virulence. This conclusion led us to consider whether the HA/protease, which had previously been shown to prevent attachment of cholera vibrios when used as a pretreatment (12), could be responsible for detachment of the vibrios. The results of this study support the conclusion that the HA/protease is a "detachase" which may act by destroying host cell receptors for several different putative *V. cholerae* adhesins.

MATERIALS AND METHODS

Bacterial cultures. The *V. cholerae* strains used and their relevant properties are summarized in Table 1. Wild-type strains used were lyophilized shortly after isolation. Working stocks of all strains were maintained at −70°C in 20% glycerol in tryptic soy broth (Difco Laboratories). For expression of the cell-associated mannose-sensitive hemagglutinin (20), El Tor biotype strains were cultured overnight at 37°C on meat extract agar (MEA). El Tor strains express a cell-associated mannose/fucose-resistant hemagglutinin when grown in tryptic soy broth, and classical biotype strains express a cell-associated fucose-sensitive hemagglutinin early during growth under the same conditions (4).

For observation of colonial morphology, cultures were streaked for isolation on MEA, incubated overnight at 37°C, and examined by using a stereoscope with transmitted oblique illumination (18). Photographs were taken by using a Leitz Diavert inverted tissue culture microscope fitted with a Wild MP5 515 automated camera and illuminated by a series 180 Fiber Lite high-intensity illuminator (Dolan Jenner Industries, Inc., Markson Science, Phoenix, Ariz.). The microscope stage was removed, inverted, and reinserted upside down. This enabled the open petri plate to be inverted, i.e., colony side down, in a circular depression in what was formerly the underside of the stage, thus placing the colonies within focal distance of the 2.5× lens. The fiber optic light source was directed at an approximately 45° angle through the bottom of the petri dish toward the lens for maximal

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TABLE 1. *V. cholerae* strains used

Strain(s)	Characteristics
3083-T	El Tor biotype, Ogawa serotype; virulent, hypertoxinogenic, T colony type; isolated by R.A.F. from cholera patient in Saigon, Vietnam, in 1964 (19); parent of A^-B^+ candidate vaccine strain TS-SR (7, 24)
3083-O	Spontaneous and reversible O nontoxigenic, avirulent variant of 3083-T
HAP-1-T	T HA/protease-neagative derivative of 3083 (21)
HAP-1-O	Hypervariably O HA/protease-negative derivative of 3083 (21)
HAP-1(pCH2)	HAP-1 electroporated with pCH2, which contains the 3.2-kb *Hind*III fragment encoding *hap* in pACYC184 (21)
HAP-1(pACYC184)	HAP-1 electroporated with pACYC184 (21)
CA 401	Classical biotype, Inaba serotype; isolated from cholera patient in Calcutta, India, by C. E. Lankford in 1953 (26)
C6707 and C6709	El Tor biotype, Inaba serotype; isolated in Peru in January 1991 and provided by I. K. Wachsmuth

contrast. Subtle differences in colonial opacity or translucency can be reliably observed only when the different colony types are cross-streaked on the same plate such that the differing colonies are adjacent to each other or in the same microscopic field.

Virulence assays. Virulence was assessed by intraintestinal inoculation of 6-day-old infant rabbits (approximately 80 to 120 g) with 0.25 ml of live overnight cultures harvested from confluently streaked plates of MEA and diluted in sterile 0.1% peptone (Difco) in normal saline according to a modification (17) of the method introduced by Dutta and Habbu (10). Mean choleragenic scores (16) were determined for groups of three or more rabbits at 24 h and for survivors at 48 h. The maximum response, a mean choleragenic score of 10, indicates that all of the rabbits had succumbed to diarrhea, their ventral surfaces were wet, and the bowels were full of clear fluid. Mean scores of less than 10 indicate that some animals were still alive and exhibited various degrees of ventral wetness and fluid in the gut. Samples of intestinal fluid were streaked for isolation on MEA to examine the colonial morphology of the microflora. In practically every case, the cultures were virtually pure *V. cholerae*.

Adherence assays. Adherence of *V. cholerae* to cultured human intestinal epithelial cells (Intestine 407, CCL6, Amer-
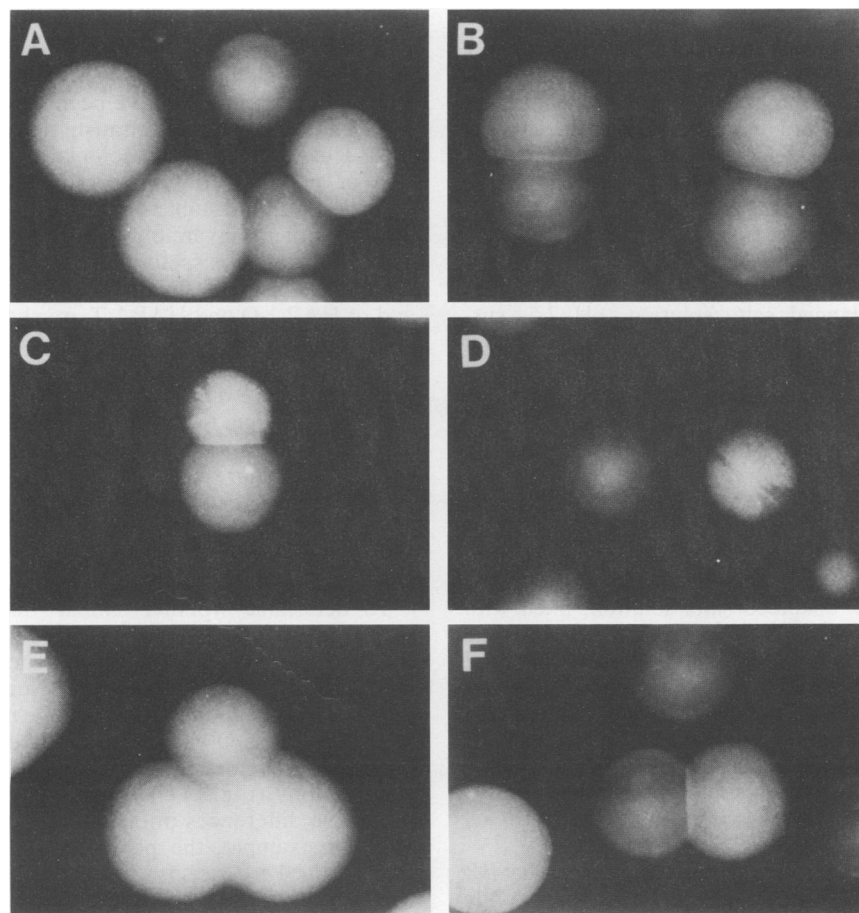

FIG. 1. Comparison of colonial variants of *V. cholerae* 3083 and mutant HAP-1 cross-streaked on MEA and examined with transmitted oblique illumination after 24 h at 37°C. Magnification, ×17. (A) 3083-O versus 3083-T; (B) HAP-1-T (upper colonies) versus 3083-T; (C) HAP-1-O (upper colony with sectors) versus 3083-O; (D) HAP-1-O (with sectors) versus HAP-1-T; (E) HAP-1-T (upper colony of three; although not clear from this photo, the two colony types are distinguishable) versus 3083-O; (F) HAP-1-O versus 3083-T.
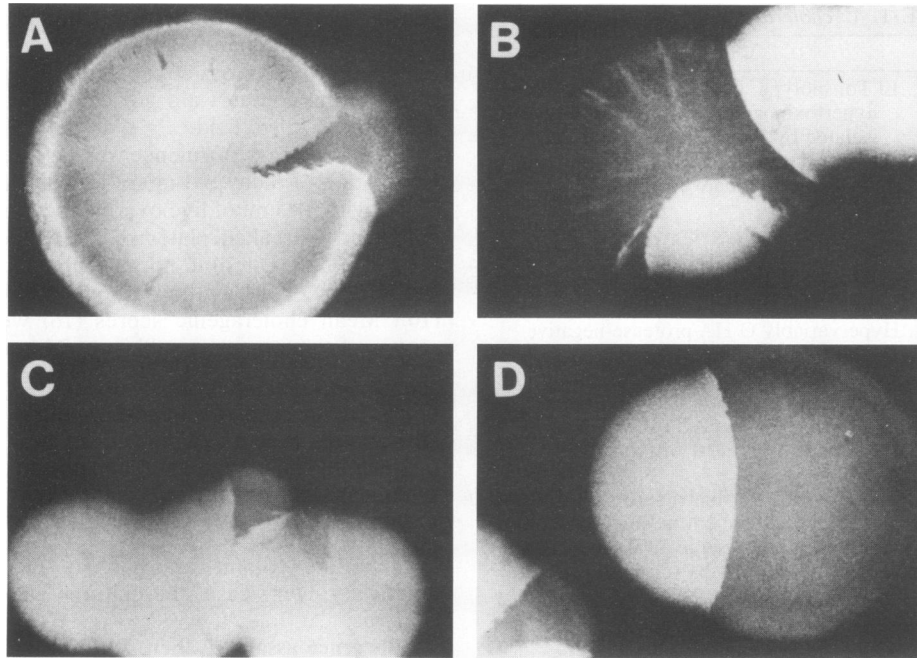
FIG. 2. Colonial variants of Peru strains on MEA at 37°C for 48 h. (A, B, and C) Strain C6707 O variants reverting to T wild-type colonies; (D) same for strain C6709. Magnification, ×17.

ican Type Culture Collection, Rockville, Md.), originally used by Bergman et al. (1) to measure attachment of *Escherichia coli*, was performed by using eight-chamber tissue culture slides (Lab Tek 4808; Nunc, Inc., Naperville, Ill.) as described previously (4). Cells, which were grown to near confluency in Dulbecco's modified Eagle's medium (Mediatech, Washington, D.C.) with added glutamine (2 mM), 15% heat-inactivated calf serum, and antibiotics (4) in plastic T-75 flasks, were harvested with EDTA buffer (10 mM EDTA, 0.14 M NaCl, 10 mM Tris, pH 7.4). The cells were washed in Hanks' balanced salts solution, pH 7.5, suspended in Dulbecco's modified Eagle's medium without antibiotics, and adjusted to 10^4 cells per ml. Cell suspensions (0.3 ml; i.e., 3×10^3 cells) were added to each chamber and incubated overnight at 37°C in 5% CO_2. Just before use, the culture medium was replaced with 0.3 ml of serum- and antibiotic-free medium. *V. cholerae* organisms grown under various conditions were suspended, washed, and diluted to 5 × 10^8 vibrios per ml in 0.1% peptone–saline with or without 0.5% D-mannose and/or L-fucose before 100 μl was added to the cells. At various intervals, the chambers were washed vigorously with Hanks' balanced salts solution, fixed with 85% methanol, and stained with safranin. The average number of vibrios per cell, plus and minus the standard error, was determined after various periods of incubation by observing 50 cells in 10 different microscopic fields selected randomly. In some instances, the cells were pretreated with purified HA/protease (15) prior to addition of vibrios.

RESULTS

Colonial variation. As it was previously known that *V. cholerae* 3083, the parent of the HA/protease mutant HAP-1, exhibited reversible (at relatively high frequency) phenotypic changes in colonial morphology which were associated with dramatic alterations in virulence, it was appropriate to examine the colonial appearance of the mutants before proceeding to assay virulence. That is, if by chance an avirulent O colonial variant had been selected, the results of virulence assays could have been confounded.

When examined with transmitted oblique illumination, HAP-1 exhibited marked colonial heterogeneity. By selection of colonies, it was possible to establish relatively homogeneous and stable sublines of the T colony type, but O colony variants invariably generated T forms, which were usually apparent as sectors in virtually every O colony. The differences between the appearances of 3083-O and 3083-T and of HAP-1-O and HAP-1-T are shown in Fig. 1A and D, respectively. When the sublines established from HAP-1 were compared directly with the O and T sublines of 3083, differences were observed (Fig. 1). HAP-1-T was considerably more opaque than 3083-T (Fig. 1B) and was practically as opaque as 3083-O (Fig. 1E). HAP-1-O was, however, considerably more opaque than 3083-O (Fig. 1C) and was easily distinguished from 3083-T (Fig. 1F). Similar results (not shown) were obtained with HAP-1(pCH2), indicating that these colonial variations were unrelated to the presence or absence of HA/protease.

To determine whether similar variations could be observed with fresh isolates, strains C6707 and C6709, recently isolated in Peru, were examined. These strains were much more homogeneous. However, by careful examination, O sectors could be found, usually in the more crowded areas of the plate. These O variants were, like HAP-1-O, extremely unstable, and more T sectors could be found in virtually every colony (Fig. 2). It should be emphasized, however, that these differences in opacity and translucency are relative: for example, the presumed wild-type "translucent" form of the Peru strains appears more opaque than 3083-O when the two are compared side by side (not shown) but would still be considered translucent in comparison with other components of the intestinal microflora (14).

Virulence of colonial variants and mutants. Results of virulence assays of the various colonial forms and mutants in

TABLE 2. Virulence of T and O colonial variants of *V. cholerae* 3083 and Peru strains C6707 and C6709 in infant rabbits

Strain	Inoculum (viable bacteria)	Mean choleragenic score[a] at:	
		24 h	48 h
3083-T	10^8	10.0 (3)	
3083-O		0.3 (3)	10.0 (3)
3083-T	10^6	9.8 (6)	
3083-O		0.0 (6)	6.8 (6)
3083-T	10^4	8.7 (3)	10.0 (3)
3083-O		0.0 (3)	1.0 (3)
HAP-1-T	10^6	9.3 (6)	
HAP-1-O		9.5 (6)	
HAP-1(pCH2)	10^6	8.5 (11)	
HAP-1(pACYC184)	10^6	8.0 (3)	
C6707-Wt[b]	10^6	8.3 (3)	
C6707-O		10.0 (3)	
C6709-Wt[b]	10^6	10.0 (3)	
C6709-O		8.0 (3)	

[a] The maximum response, a mean choleragenic score of 10, means that all the animals had succumbed to diarrhea, the ventral surfaces were wet, and the bowels were full of clear liquid. Mean scores of less than 10 indicate that some animals were still alive and exhibited various degrees of ventral wetness and fluid in the gut. Numbers in parentheses are numbers of animals.

[b] Wild type.

infant rabbits are summarized in Table 2. As had been demonstrated previously (23), strain 3083-T was highly virulent; i.e., doses as low as 10^4 live vibrios caused experimental cholera within 24 h, whereas higher doses of 3083-O produced few or no symptoms until the next day. When those animals were examined at 48 h, T colonies could invariably be isolated at high frequency from the liquid intestinal contents.

Each of the HAP-1 sublines, O and T, HAP-1(pCH2), and HAP-1(pACYC184), respectively, was comparable in virulence to 3083-T in the infant rabbit model; i.e., like 3083-T, doses of 10^6 live vibrios caused fatal choleraic diarrhea in most of the animals by 24 h. Similarly, both T (i.e., wild-type) and O variants of the Peru strains were virulent. While the results with O colony types of HAP-1 and the Peru O strains could be confounded by their high frequency of switching to T types, the results with HAP-1, HAP-1(pCH2), and HAP-1(pACYC184) indicate that the presence or absence of HA/protease has no direct bearing on virulence (for infant rabbits).

Attachment and detachment of V. cholerae. If such an attractive putative virulence factor as the HA/protease is not involved in virulence, what does it do? Studies of the abilities of the strains to attach to and detach from cultured human intestinal epithelial cells revealed (Fig. 3) that all strains adhered to the cells rapidly, peaking at 15 min (as had been described previously [4]). However, strains expressing HA/protease, i.e., 3083 and HAP-1(pCH2), then detached; strains lacking the HA/protease, i.e., HAP-1 and HAP-1(pACYC184), remained attached.

Pretreatment with purified HA/protease inhibited attachment (Fig. 4) in a time- and dose-related fashion. Further, pretreatment with HA/protease inhibited attachment of
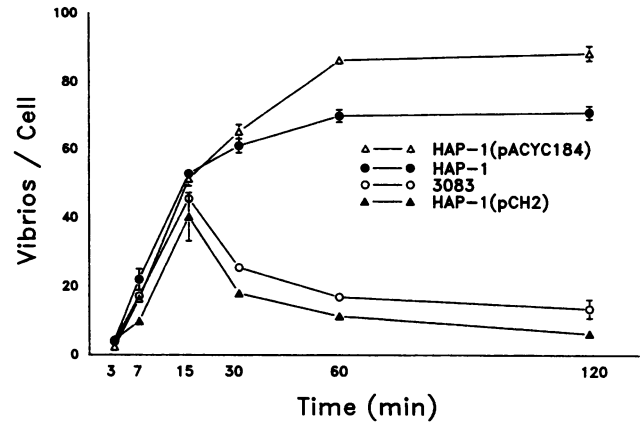

FIG. 3. Attachment and detachment of mutant and wild-type *V. cholerae* on cultured human intestinal epithelial cells. Values are the average number of vibrios per cell ± standard error.

vibrios which were cultivated under conditions to stimulate expression of mannose-sensitive (Fig. 5A), mannose/fucose-resistant (Fig. 5B), and fucose-sensitive (Fig. 5C) hemagglutinin/adhesins (4).

DISCUSSION

The original intention of the present work was to examine the role of the *V. cholerae* HA/protease in the virulence of *V. cholerae* in the infant rabbit experimental model system by using previously engineered mutants (22) which express or do not express the HA/protease. Our original expectation was that mutants genetically lacking HA/protease would be demonstrably less virulent than wild-type or null mutants complemented with HA/protease because the HA/protease, by virtue of its many potentially relevant proteolytic activities (3, 5, 6, 13), is certainly an attractive candidate virulence factor.

However, it was previously observed (23) that the parent strain, 3083, exhibited reversible T-to-O variations in colo-
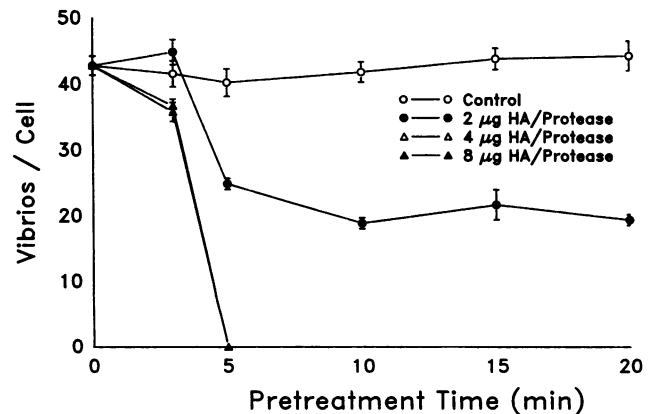

FIG. 4. Effect of pretreatment with purified HA/protease on attachment of *V. cholerae* 3083 grown overnight on MEA at 37°C to cultured human intestinal epithelial cells. Pretreatment time and dose are as indicated, and attachment was determined at 15 min after addition of the vibrios.
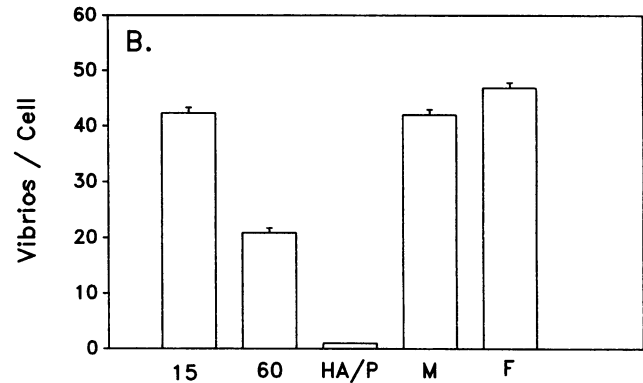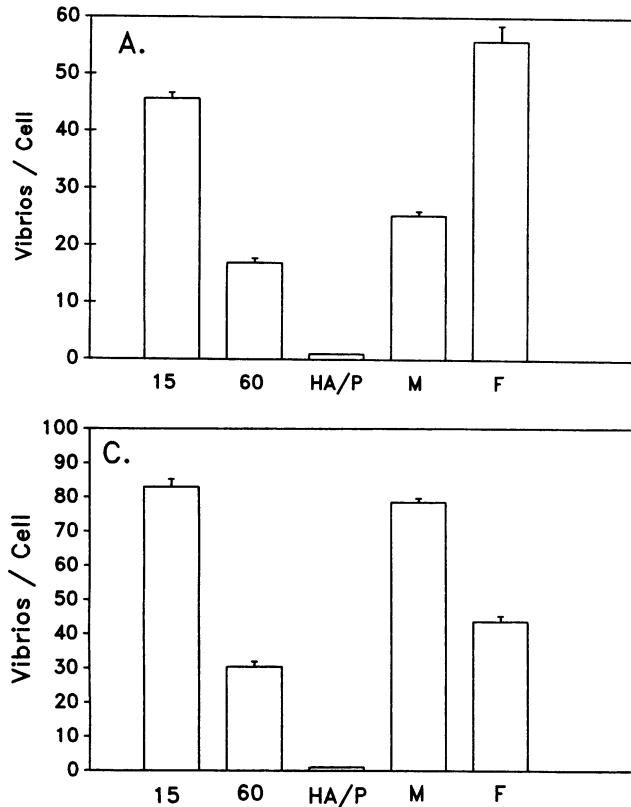
FIG. 5. Effect of pretreatment with 4 μg of purified HA/protease (HA/P) on attachment of vibrios grown under conditions to enhance expression of different putative adhesins. The attached bacteria were counted after 15 (or 60 where indicated) min of incubation. HA/P indicates pretreatment with HA/protease for 15 min before vibrios were added. M or F indicates the presence of 0.5% D-mannose or L-fucose, respectively. (A) 3083 after 18 h on MEA; (B) 3083 after 4 h in tryptic soy broth; (C) CA 401 after 4 h in tryptic soy broth.

nial morphology which were associated with loss of virulence. Thus, virulence analyses could be confounded by marked colonial heterogeneity exhibited by the mutants. However, the colonial variations of the mutants were not identical with those of the parent strain, and the virulence analyses revealed that, regardless of colonial phenotype and HA/protease genotype, the Hap mutants were equally virulent in the infant rabbit model. Thus, we conclude that the HA/protease is not a primary virulence factor (in this model system), nor is it responsible or related to the colonial variations observed. The reasons for the differences in colonial appearance between the mutant and parent sublines are not clear. They could relate to the genetic manipulations employed (e.g., electroporation), or they could have arisen spontaneously, as has been noted previously in V. cholerae stock cultures (26).

Adherence and detachment studies with cultured human intestinal epithelial cells demonstrated that whereas all of the strains and mutants tested attached with equal facility, those which did not express HA/protease did not subsequently detach. Further experiments revealed that pretreatment of the cultured intestinal epithelial cells with purified HA/protease prevented attachment of vibrios cultivated under conditions in which they expressed mannose-sensitive, fucose-sensitive, or mannose/fucose-resistant hemagglutinins/adhesins. We conclude that it is likely that the V. cholerae HA/protease is a "detachase" which enables the vibrios to free themselves from the intestinal epithelium, thus enabling them to be discharged into the environment to find another human host. V. cholerae in nature is exclusively a pathogen of humans and (like typhoid) depends ultimately on human-to-human transmission, although it is known that it can be maintained at least temporarily in

shellfish and plankton in aquatic environments in areas in which cholera is endemic. The present observations complement those of an earlier study (29) in which it was demonstrated that V. cholerae does detach following colonization in a patent intestinal tract experimental animal model system. Jones et al. (25) also noted that V. cholerae attached, with a peak at 15 min, and then detached from rabbit brush borders in vitro.

Although the present study suggests that the HA/protease does not play a primary role in virulence, the limitations of the experimental model system do not permit us to exclude the possibility that the HA/protease may serve as an accessory virulence factor by enhancing the activity of the cholera enterotoxin and/or by directly stimulating secretion of mucus from goblet cells. Crowther et al. (9) recently noted that a Zincov-inhibitable metalloprotease in V. cholerae culture filtrates had these properties. It should also be mentioned that the activity of the elastase of Pseudomonas aeruginosa, which is remarkably similar to the HA/protease (21), can be enhanced by accessory factors such as the LasA protein of P. aeruginosa (30), although such a phenomenon has yet to be demonstrated with the V. cholerae product.

V. cholerae has long been recognized as mercurial in its colonial variability. Robert Koch, in 1884, was the first to observe that the colonial appearance of cholera vibrios on gelatin and, later, agar medium was distinct from that of (all) other bacterial species (p. 546 in reference 31), and Kolle and Gotschlich soon thereafter noted (p. 124 in reference 31) that in all fresh isolates from Egypt that had been subcultivated once or several times, one could invariably find colonies which might be called O and T. Lankford subsequently (26) associated differences in colonial morphology with differences in virulence (for embryonated hen's eggs), and it was noted previously (11) that T colonies of El Tor biotype strains isolated in Teheran were hemolytic, whereas O colonies were not. The present work reveals that the situation is more complex: certain O-T phase variations are dramatically associated with changes in virulence, whereas others are not. It is noteworthy that parent strain 3083,

originally isolated from a cholera patient in Vietnam, was previously considered to be avirulent when it failed to cause cholera in four control volunteers given 10^6 live vibrios (27). As we were already aware of the T → O variation of strain 3083, we were careful to provide the virulent T subline for that study, but isolates from the stools of volunteers contained a large proportion of O colonies (1a).

The mechanisms and reasons for colonial variation in *V. cholerae* are clearly deserving of further study. In preliminary work in this laboratory, Y.-M. Yang (unpublished data) identified an antigenic 50-kDa outer membrane protein in 3083-T which was not present in 3083-O, and M. Sasser (personal communication) has observed differences in fatty acid compositions of O and T sublines provided by this laboratory.

Finally, the role of the HA/protease in detachment merits further consideration from the standpoint of vaccine development (a vibrio which adheres longer is more likely to stimulate an effective immune response) and for its role in the ecology of *V. cholerae* (a vibrio which is prevented from detaching is less likely to cause devastating epidemics). Further, a recent study (28) indicated that pretreatment of rabbits with an enteric-coated protease preparation prevented intestinal colonization by *E. coli*. The HA/protease might similarly be useful as a prophylactic agent in preventing colonization by *V. cholerae*.

ACKNOWLEDGMENTS

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