

## Alterations in *Vibrio cholerae* Motility Phenotypes Correlate with Changes in Virulence Factor Expression

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Motility is thought to contribute to the virulence of *Vibrio cholerae*, but the role it plays in pathogenesis is not completely understood. To investigate the influence of motility on virulence gene expression and intestinal colonization, we have isolated mutants with altered swarming abilities in soft agar medium. Both spontaneous hyperswarmer (exhibiting faster swarm rates) and spontaneous or transposon-induced nonmotile mutants of strain O395 were obtained. Surprisingly, we found that two of three classes of hyperswarmer mutants were defective in autoagglutination, a phenotype associated with expression of toxin-coregulated pili (TCP), an essential ToxR-regulated colonization factor of *V. cholerae*. In contrast, nonmotile mutants exhibited autoagglutination under growth conditions that normally repress this phenotype. Further characterization of mutant strains revealed differences in the expression of other virulence determinants. Class I hyperswarmer mutants were defective in production of TCP, cholera toxin, and a cell-associated hemolysin but showed increased levels of protease and fucose-sensitive hemagglutinin. All nonmotile mutants examined, including those with insertions in a sequence homologous to *motB*, exhibited increased expression of TCP pilin, cholera toxin, and cell-associated hemolysin but dramatically decreased levels of fucose-sensitive hemagglutinin and HEp-2 adhesins. In general, nonmotile mutants displayed few or no defects in intestinal colonization, while class I hyperswarmer mutants were highly defective in colonization. These results suggest that the motility phenotype of *V. cholerae* is tightly coupled to the expression of multiple ToxR-regulated and non-ToxR-regulated virulence determinants.

The causative agent of the intestinal disease cholera is *Vibrio cholerae*, a gram-negative motile bacterium with a single polar flagellum. Important steps in the disease process include ingestion of the organism together with contaminated food or water, passage of the vibrios through the gastric acid barrier of the stomach, adherence to and penetration of the mucus coat lining the epithelium of the small intestine, adherence to intestinal epithelial cells, multiplication, toxin production, and, finally, severe watery diarrhea (29). Studies of the bacterium have elucidated a number of products important for its virulence, including cholera toxin (CT), whose action is largely responsible for the host secretory response, and toxin-coregulated pili (TCP), which greatly enhances colonization of the intestinal epithelium (28, 56). Additional factors include other potential toxins, accessory colonization factors, outer membrane proteins, proteases, hemolysins, hemagglutinins (HAs), and, in some strains, a capsular polysaccharide, all of which may contribute to survival and multiplication of *V. cholerae* within the host (2, 17, 33, 44, 49, 58, 59).

A subset of these virulence factors are coordinately regulated by environmental conditions and are under the control of ToxR, an inner membrane transcriptional regulatory protein (45). ToxR activates the transcription of the *ctx* operon encoding CT and of the gene encoding ToxT, a second regulator that is responsible for subsequent activation of the *ctx* and *tcp* operons and many other genes associated with the ToxR regulon (15). Laboratory growth conditions that induce the synthesis of toxin and TCP in strain O395 have been elucidated (21, 44). However, the true in vivo environmental conditions that

modulate ToxR-regulated genes within the intestinal environment are not known. It is apparent that *V. cholerae* must either recognize external signals within the host environment that activate the expression of ToxR-regulated genes or, alternatively, may rely on some physiological internal signal of its own (e.g., growth phase, energy levels, etc.) that has yet to be defined experimentally.

*V. cholerae* is a highly motile organism and displays chemotactic behavior during its interaction with the intestinal mucosa (31). However, the importance of motility in virulence has been subject to somewhat discrepant conclusions. Early studies found that nonmotile (NM) strains exhibited a decrease in virulence as measured by rabbit ileal loop fluid responses, and although NM strains colonized ligated rabbit ileal loops at the same rate as wild-type strains, they were dramatically defective in adsorption to the intestinal wall (25, 31, 60). More recently, other investigators have found that there was no effect of motility mutations on the ability of *V. cholerae* to colonize adult rabbit ligated guts or adhere to rabbit intestinal sections (5, 57). NM mutants, including transposon insertion mutants, have been shown to be fully virulent in the suckling mouse, a model system that has accurately predicted the colonization properties of various live attenuated cholera vaccines (20, 28, 50). Finally, results from volunteer studies suggest that motility mutations can dramatically reduce reactogenicity of live, non-toxicogenic cholera vaccines without adversely affecting their ability to colonize the human intestine and induce protective immune responses (13, 34, 38, 55). Thus, the possibility exists that motility plays a yet-to-be-defined role in regulating some aspect of *V. cholerae* virulence.

In order to further explore the relationship between motility and virulence in *V. cholerae*, the expression of potential virulence determinants was examined in various mutants with alterations in their in vitro motility phenotypes. Our results indicate that the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<b>Strains</b>		
O395	Classical Ogawa <i>rpsL</i>	Laboratory collection (40)
O395N1	O395 $\Delta$ <i>ctxA</i>	Laboratory collection (41, 56)
O395NT	O395 $\Delta$ <i>ctxAB</i> ::Kan	Laboratory collection (41)
CG842	O395 $\Delta$ <i>lacZ</i>	This work
JJM16.28	O395N1 <i>ctxA</i> :: <i>TnphoA</i> (KP2-4)	Laboratory collection (48)
KP1.25	O395N1 <i>ctxA</i> :: <i>TnphoA</i>	Laboratory collection (49)
RT110.21	O395N1 <i>tcpA</i> :: <i>TnphoA</i>	Laboratory collection (56)
JJM43	O395N1 $\Delta$ <i>toxR</i>	Laboratory collection (28, 56)
JJM42	O395N1 $\Delta$ <i>toxS</i>	Laboratory collection (43)
JJM10.29	O395N1 $\Delta$ <i>toxR</i> $\Delta$ <i>toxS</i>	Laboratory collection (39)
NM39	O395N1 spontaneous NM	Laboratory collection (56)
NM51	O395N1 spontaneous NM	This work
NM CG996	CG842 <i>motB</i> :: <i>Tn5-lac</i> (NM)	This work
NM CG997	CG842 ?:: <i>Tn5-lac</i> (NM)	This work
NM CG1059	CG842 <i>motB</i> ::pCG1050	This work
HS CG379	O395N1 spontaneous Tox <sup>-</sup> Agg <sup>-</sup> class I HS	This work
HS CG380	O395N1 spontaneous Tox <sup>-</sup> Agg <sup>-</sup> class I HS	This work
HS30	CG842 spontaneous Tox <sup>+</sup> Agg <sup>+</sup> class III HS	This work
HS35	CG842 spontaneous Tox <sup>+</sup> Agg <sup>-</sup> class II HS	This work
SM10 $\lambda$ pir	<i>thi thr leu tonA lacY supE recA</i> ::RP4-2-Tc Mu $\lambda$ pir R6K	51
<b>Plasmids</b>		
pCG698	6-kb <i>Sau3A</i> insert including <i>V. cholerae lacZ</i> gene in BamHI site of pBR327	This work
pCG711	pCG698 with internal <i>HpaI</i> deletion within the <i>lacZ</i> gene	This work
pGP704	Mobilizable suicide vector requires Pir protein for replication	44
pCG1045	TA cloning vector::inverse PCR fragment with <i>motB</i> homology derived from NM CG996	This work
pCG1050	Homologous <i>motB</i> fragment from pCG1045 inserted into <i>EcoRI</i> cloning site of pGP704	This work
pBR322	Plasmid vector	8
pVM16	pBR322 with <i>toxR</i> gene	45
pLAFR2	Cosmid vector	15
pGJ2.3	<i>toxT</i> gene cloned on pLAFR2	15

expression of ToxR-regulated and other non-ToxR-regulated virulence traits can be dramatically influenced in reciprocal patterns by mutations which alter the motility phenotype of *V. cholerae*. These altered patterns of expression may explain the conflicting results obtained with different types of motility mutants in the past and, most importantly, suggest that the motility status of the cell may be an important signal sensed by the ToxR regulatory system.

#### MATERIALS AND METHODS

**Strains and plasmids.** All strains used in this study were of the classical biotype of *V. cholerae* and are described in Tables 1 and 2. Parental strain CG842 (O395  $\Delta$ *lacZ*) was constructed by crossing an internal deletion within the *V. cholerae lacZ* gene (encoded by plasmid pCG711, a derivative of pCG698) onto the *V. cholerae* chromosome (10).

**Media and growth conditions.** Luria-Bertani (LB) broth consists of 1% tryptone

(Difco), 0.5% yeast extract (Difco), and 0.5% NaCl. The starting pH of the medium was adjusted by addition of 2 N NaOH. LB agar plates contained 1.5% Bactoagar (Difco), while LB motility plates were made with 0.3 to 0.4% Bactoagar. Chemotaxis plates were prepared as 1% tryptone–0.5% NaCl–0.3 to 0.4% Bactoagar. Top agar is LB agar made with 0.75% Bactoagar. Antibiotics were added at the following concentrations: ampicillin, 50  $\mu$ g/ml; kanamycin, 30  $\mu$ g/ml; streptomycin, 100  $\mu$ g/ml; and tetracycline, 5  $\mu$ g/ml.

The permissive conditions for CT and TCP production by strain O395 are growth at 30°C on a roller incubator (30 rpm) in 16-mm-diameter culture tubes with 5 ml of LB or tryptone broth (80 to 150 mM NaCl [starting pH, 6.5]). The inoculum size was found to be critical for proper induction of toxin and pili, in that heavily inoculated cultures express toxin and pili poorly. To standardize inocula when different growth conditions were compared, a 10- $\mu$ l aliquot of a bacterial suspension with an optical density at 600 nm (OD<sub>600</sub>) of 0.01 was used to inoculate each 5-ml culture, ensuring an equal and low inoculum (21). The nonpermissive conditions for CT and TCP expression involved adjustment of LB broth to a starting pH of 8.5 with NaOH.

TABLE 2. HS mutant classes and phenotypes

Parental strain	Genotype	No. of HS mutants isolated	% of HS <sup>a</sup> :		
			Class I (Agg <sup>-</sup> Ctx <sup>-</sup> )	Class II (Agg <sup>-</sup> Ctx <sup>+</sup> )	Class III (Agg <sup>+</sup> Ctx <sup>+</sup> )
O395	Wild-type <i>rpsL</i>	6	83	17	0
O395N1	O395 $\Delta$ <i>ctxA</i>	28	64	29	7
CG842	O395 $\Delta$ <i>lacZ</i>	20	35	13	52
JJM16-28 <sup>b</sup>	O395N1 <i>ctxA</i> :: <i>TnphoA</i>	8	12.5	62.5	25
KP1.25 <sup>b</sup>	O395N1 <i>ctxA</i> :: <i>TnphoA</i>	9	78	0	22
Total		71	54.5	24.3	21.2

<sup>a</sup> Agg is the autoagglutination phenotype when grown under ToxR permissive conditions; Ctx indicates the ability to produce wild-type levels of CT B subunit.

<sup>b</sup> Carries a single *TnphoA* insertion in one of two copies of the *ctxAB* operon and is therefore positive for the CT B subunit in ELISAs.

**Selection for HS and NM mutants.** Hyperswarming (HS) mutants were obtained by stabbing motility agar with a pointed round toothpick or platinum needle, followed by incubation at 30°C or room temperature for 6 days. Arcs protruding from the edge of the swarms were stabbed and purified to single colonies on LB agar plates and then retested on LB motility agar plates with the parental strain inoculated on the same plate for comparison. Stable HS mutants consistently swarmed at rates at least 120% those of parental strains. Spontaneous NM mutants were found by plating dilutions of cultures in soft overlay agar over LB plates and then screening them for a loss of their ability to form spreading colonies. Transposon-induced NM mutants were obtained by screening a pool of *V. cholerae* O395  $\Delta$ lacZ cells mutagenized by Tn5-lac, (kindly provided by M. Waldor) for loss of swarming behavior when stabbed into chemotaxis agar (36).

**Quantitative swarm assays.** Bacterial colonies were stabbed into motility or chemotaxis plates as described above and incubated for 15 to 24 h at 23 or 30°C. The diameter of each swarm was measured and compared with the diameter of the parental swarm present on the same plate. The values of swarm size are presented as percentages of the parental strain scored on the same plate of motility or chemotaxis agar.

**HEp-2 cell and fucose-coated bead binding assays.** HEp-2 cell binding was induced as described previously (37), with minor modifications. Briefly, overnight bacterial cultures were subcultured by dilution at 1/100 into LB broth and incubated at 30°C with aeration for approximately 2 h until the bacteria reached mid-log phase. After washing of the bacterial cells two times in phosphate-buffered saline (PBS) to remove any toxin, the bacteria were added at a multiplicity of infection of 100 bacteria per HEp-2 cell. The HEp-2 cells were previously grown on small round glass coverslips seeded with approximately  $10^5$  HEp-2 cells in Iscove's modified Eagle's medium with 5% fetal bovine serum–2 mM glutamine and without antibiotics. Bacteria were spun down onto monolayers at 1,000 rpm in a Beckman desktop centrifuge for 10 min, followed by incubation at 30°C for 30 min. Tissue culture medium was removed by suction, and wells were washed four times with PBS. Cells were fixed by a 5-min incubation at room temperature in methanol and stained by incubation with Giemsa stain in a 1/12.5 dilution in H<sub>2</sub>O for 25 min. The stained coverslips were washed four times with H<sub>2</sub>O, air dried, and mounted onto glass slides for viewing by light microscopy.

Adherence of *V. cholerae* cells to fucose-coated beads was induced as described previously, with minor modifications (32). Exponentially growing bacteria were washed in modified Krebs-Ringer solution (KRT [described below]) and mixed with washed agarose beads chemically coupled with L-fucose (Sigma F-7379). Beads were sedimented, washed with KRT three times, and then observed under the light microscope.

**Immunoassays.** CT antigen was measured by GM1-enzyme-linked immunosorbent assay (ELISA) as described previously (21). For Western blot (immunoblot) analysis, cells recovered from overnight cultures were resuspended in protein sample buffer, heated for 10 min at 100°C, and then analyzed after electrophoresis in 12.5% polyacrylamide gels with anti-ToxR and anti-TcpA rabbit antibodies as described previously (47, 49).

**Protease assay.** Casein plates were prepared by mixing freshly autoclaved 3% instant milk in water with an equal volume of warm 1% tryptone (Difco), 0.5% yeast extract (Difco), 1% NaCl, and 3% Bactoagar (Difco). Plates were streaked with purified bacterial strains, incubated overnight at 30°C, and observed for zones of clearing around individual colonies. Diameters of zones were measured and compared with those of the parental strain on the same plate.

**Hemagglutination and CAH assays.** Strains were grown in LB broth with aeration at 30°C, and assays were performed with cells derived from either stationary-phase or exponentially growing cultures. Bacterial cells were recovered by centrifugation, washed, and resuspended in KRT, consisting of 7.5 g of NaCl, 0.383 g of KCl, 0.318 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, and 0.305 g of CaCl<sub>2</sub> in 1 liter of 10 mM Tris hydrochloride (pH 7.4). Initial concentrations of approximately  $10^{10}$  bacteria were serially diluted in U-bottomed wells of 96-sample microtiter dishes, each well containing 100  $\mu$ l of 2% (vol/vol) washed chicken erythrocyte or 5% human type O erythrocyte suspension in KRT. Dishes were incubated at room temperature for approximately 20 to 30 min and scored for hemagglutination. The titer was recorded as the reciprocal of dilution factor of the sample still showing hemagglutination.

Assays to measure hemolysin activity associated with washed bacterial cells (cell-associated hemolysin [CAH]) were performed in two ways: microtiter and tube formats. The microtiter format was identical to the hemagglutination assay described above, except that after hemagglutination titers were recorded, the microtiter dishes were covered and further incubated overnight at room temperature. Lysis of erythrocytes was scored by visualization of the endpoints of hemoglobin release. For the tube format, reagents were scaled up to 1 ml of total volume, serially diluted washed bacterial cells were added, and the reaction was allowed to proceed in 1.5-ml microcentrifuge tubes at room temperature for approximately 18 h. Centrifugation was used to remove cell debris, and the OD<sub>540</sub> (wavelength of maximum hemoglobin absorbance) of the supernatant fluid was measured with a spectrophotometer.

**Intestinal colonization assay.** A competition assay for intestinal colonization in 4- to 5-day-old CD-1 suckling mice was performed basically as described previously (20, 56). Briefly, mixtures of mutant and parental strains, grown overnight under ToxR-permissive conditions, were intragastrically administered

( $10^6$  bacteria) to groups of four to six anesthetized mice. After 18 to 24 h, the small intestine was removed, homogenized, and plated on appropriate selective or differential media to allow separate enumeration of the mutant and parental cells recovered. The in vivo competitive indices were calculated by dividing the small intestine output ratios by the inoculum input ratio of mutant to parental strains. The in vitro competitive index was calculated in a similar way, except the same input inoculum was grown for 18 h in LB broth without antibiotics at 37°C, and then the cells were plated on the appropriate selective or differential media.

**Construction of motB mutations by plasmid integration.** Inverse PCR was used to clone a fragment of chromosomal DNA adjacent to the *motB::Tnlac* insertion carried by strain NM CG996 essentially as previously described (35). Briefly, 2  $\mu$ g of chromosomal DNA prepared from the Tn5-lac insertion mutant was digested with *TaqI*, extracted with phenol and chloroform, ethanol precipitated, and then subjected to overnight ligation under dilute conditions (53). The ligation reaction mixture was phenol and chloroform extracted, and the DNA was resuspended in 10  $\mu$ l of water, half of which was subjected to PCR amplification for 35 cycles of 95°C for 1 min, 50°C for 2 min, and 73°C for 2 min with the primers for Tn5-lac H1 (5' CCATCTCATCAGAGGGTAGT 3') and H2 (5' ACCATGTTAGGAGGTCACAT 3') (kindly provided by H. Kimsey). PCR products were visualized after electrophoresis in agarose gels, and a 440-bp fragment was recovered for DNA sequencing. The PCR product was subcloned into the TA cloning vector (Invitrogen Corporation, San Diego, Calif.), giving pCG1045, and then further subcloned as an *EcoRI* fragment into suicide vector pGP704, giving plasmid pCG1050. Plasmid pCG1050 was mobilized into *V. cholerae* strains by conjugation from *Escherichia coli*, and correct integration of the plasmid by homologous recombination into the chromosomal *motB* locus was confirmed by Southern blot hybridization (44).

## RESULTS AND DISCUSSION

**Selection of *V. cholerae* mutants altered in motility phenotypes.** To evaluate the effects of motility on the virulence properties of *V. cholerae*, we isolated mutants with altered swarming behaviors. HS mutants displayed an increase relative to respective parental strains in their rate of penetration of soft motility agar, while NM mutants remained embedded in the soft agar at the point of inoculation. From five parental strains, 71 HS mutants were obtained (Table 2). The swarm diameters of HS mutants ranged from approximately 120% to over 141% of those displayed by respective parental strains in our motility agar assay (Table 3). The swarming capability of one HS mutant on motility agar is shown in Fig. 1.

NM mutants were obtained by screening for mutants unable to penetrate motility agar. Transposon Tn5-lac-induced NM mutants were isolated from the classical strain O395 *lacZ*. Of 1,137 transposon-insertion mutants analyzed, 44 were found to show no apparent motility. These 44 were subsequently divided into various classes on the basis of their expression of flagella and the results of Southern blot analysis with transposon-derived probes (data not shown). Two flagellated Tn5-lac-induced mutants, NM CG996 and NM CG997, were included in our analyses, and sequence analysis of chromosomal DNA adjacent to one of these (NM CG996) revealed it to have an insertion in a gene homologous to the *motB* locus of *E. coli* as discussed below. NM mutants have been reported to occur spontaneously, and we too have observed the appearance of NM mutants of derivatives of strain O395 occurring at a frequency of approximately  $1 \times 10^{-4}$  (46). Two such spontaneous NM strains selected for our analysis included NM39, a non-flagellated strain, and NM51, a flagellated strain.

**TCP pilin and CT expression in HS mutants.** When classical strain O395 expresses CT and TCP when grown in liquid media under permissive conditions, bacterial cells clump together, a phenomenon known as autoagglutination, or the Agg<sup>+</sup> phenotype (44). After overnight growth under ToxR-permissive conditions, a striking number of HS strains (78.8%) were deficient in autoagglutination, a phenotype known to be specifically associated with production of TCP by strain O395 (Table 2). The marked reduction in production of TCP by spontaneous Agg<sup>-</sup> HS mutants was confirmed by Western analysis with antibody against TcpA, the major subunit of TCP (Fig. 2).

TABLE 3. Class I HS and regulatory mutant phenotypes

Strain	Description	% of wild-type:		Agg phenotype <sup>a</sup>	µg of cholera toxin/OD <sub>600</sub> unit	% of wild-type protease diam
		Motility	Chemotaxis			
O395N1	Parental strain	100	100	++++	3.7	100
HS CG379	Class I HS	142	141	-	<0.005	500
HS CG380	Class I HS	144	145	-	<0.005	400
HS35	Class II HS	194	204	-	0.4	900
HS30	Class III HS	140	149	++++	4.0	100
JJM43	O395N1 $\Delta$ <i>toxR</i>	135	74	-	<0.005	200
JJM42	O395N1 $\Delta$ <i>toxS</i>	132	130	-/+	8.3	100
JJM10-29	O395N1 $\Delta$ <i>toxR</i> $\Delta$ <i>toxS</i>	142	76	-	<0.005	200
JJM43(pVM16)	pVM16 carries <i>toxR</i>	119	106	+++	2.3	150
HS CG379(pVM16)		145	172	-	0.09	350
HS CG380(pVM16)		164	195	-	0.09	400

<sup>a</sup> Relative agglutination from none (-) to high (++++). Each + represents approximately 10 bacteria per bead observed by light microscopy. -/+ indicates most beads were bare, and those with adherent bacteria had fewer than four bacteria.

Since TcpA and CT are coregulated, CT levels of the Agg<sup>-</sup> HS mutants were assayed. The majority of mutants unable to autoagglutinate did not produce TcpA or CT (Ctx<sup>-</sup>; less than 5 ng of CT per OD<sub>600</sub> unit) (Table 3 and Fig. 2). Mutants displaying this Agg<sup>-</sup> Ctx<sup>-</sup> phenotype were designated class I (represented by strains HS CG379 and HS CG380). However, some Agg<sup>-</sup> HS mutants synthesized toxin at wild-type toxin levels, and accordingly these Agg<sup>-</sup> Ctx<sup>+</sup> HS mutants were designated class II and are represented by strain HS35. Finally, class III HS mutants are Agg<sup>+</sup>, express wild-type levels of CT and TcpA pilin, and are represented by HS30.

The TCP<sup>-</sup> phenotype of class I and class II HS mutants suggested that the increased swarming ability of these *V. cholerae* strains might be due to the loss of mechanical hindrance caused by TCP appendages. We reasoned that if this were true, then mutants unable to synthesize TCP, such as mutants with deletions or insertions in the *tcp* operon, would be expected to swarm at a faster rate than wild-type strains. However, none of

several different strains with mutations in *tcp* tested were found to exhibit a HS phenotype on motility agar, and the diameters of swarms of *tcpA* mutants were identical to those of parental strains (Table 4). Thus, the absence of TCP does not increase the swarming ability of *V. cholerae*, and therefore the HS phenotype in the class I and class II TCP<sup>-</sup> mutants is not due to a reduction of mechanical interference by TCP filaments or decreased TCP-mediated autoagglutination.

***toxR* mutants display an HS phenotype, but HS mutants apparently do not carry mutations in *toxR*.** Class I HS mutants are similar to ToxR regulatory mutants in that they are unable to synthesize TCP and CT. Thus, we asked whether mutants carrying known mutations in ToxR exhibited increased swarming ability. Given that other regulatory gene products, including ToxS and ToxT, act in concert with ToxR to control TCP and CT expression, we also examined the swarm plate phenotypes of strains carrying mutations in these regulators (14, 15, 43). The swarm diameter of the *toxT* mutant VJ675 (kindly provided by V. DiRita) was equivalent to the swarm diameter of the wild-type strains (data not shown) (9, 11). In contrast, mutants with deletions in either *toxR* or *toxS* or both *toxR* and *toxS* showed increased swarming ability on motility agar (Table

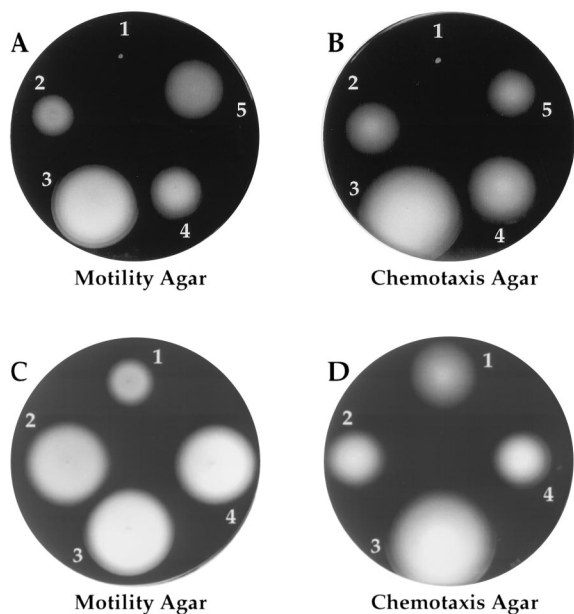


FIG. 1. Swarming behavior of *Vibrio* strains on motility agar or chemotaxis agar. (A and B) Strains: 1, NM CG996; 2, O395N1 (wild type); 3, HS CG380; 4, O395 (wild type); 5, JJM43 ( $\Delta$ *toxR* mutant). (C and D) Strains: 1 O395N1 (parental); 2, JJM43 ( $\Delta$ *toxR*); 3, JJM42 ( $\Delta$ *toxS*); and 4, JJM10.29 ( $\Delta$ *toxR*  $\Delta$ *toxS*).

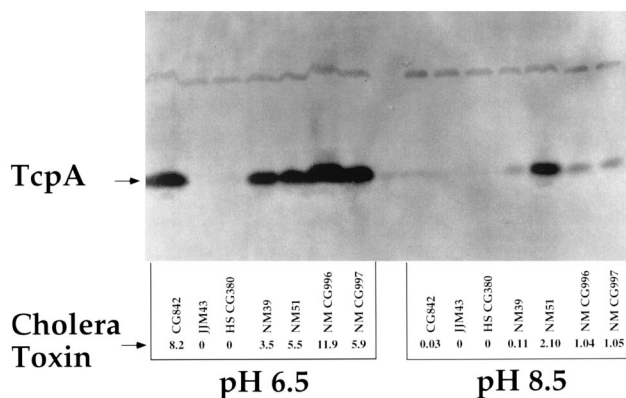


FIG. 2. Western analysis of TcpA pilin and CT values. CT expression and Western analysis of TcpA pilin production in *Vibrio* strains grown under permissive and nonpermissive conditions are shown. Strains are grown overnight at 30°C in LB broth with a starting pH of 6.5 or 8.5. Results are shown for the following strains: CG842 (wild type) JJM43 ( $\Delta$ *toxR*), HS CG380, NM39 (spontaneous NM mutant), NM51 (spontaneous NM mutant), NM CG996 (transposon-induced NM mutant), and NM CG997 (transposon-induced NM mutant). The protein band above TcpA is an unknown peptide that cross-reacts with the rabbit anti-TcpA antibody. Toxin values are in micrograms of CT per OD<sub>600</sub> unit.

TABLE 4. HEP-2 cell adherence

Strain	Description	% of parental value <sup>a</sup>		Adherence	Mean (SD) no. of bacteria per HEP-2 cell <sup>b</sup>
		Motility	Chemotaxis		
	Parental strain	100	100	100	15.0 (6.7)
HS CG379	HS class I	155	156	102	15.3 (5.0)
HS CG380	HS class I	140	145	131	19.7 (6.2)
NM39	NM (nonflagellated)	NM	NM	<0.5	0.07 (0.23)
NM51	NM (flagellated)	NM	NM	<0.1	0.014 (0.12)
JJM43	Regulatory mutant ( <i>toxR</i> )	145	67	297	44.6 (6.3)
JJM42	Regulatory mutant ( <i>toxS</i> )	125	140	94	14.1 (7.7)
JJM10.29	Regulatory mutant ( <i>toxR toxS</i> )	145	74	248	37.2 (10.4)
RT110.21 ( <i>tcpA</i> )	Nonpiliated mutant	94	98	122	18.3 (15.9)
CG842	Parental strain	100	100	100	20.7 (9.3)
NM CG997	NM	NM	NM	<0.3	0.06 (0.24)
NM CG996	NM <i>motB</i>	NM	NM	<0.2	0.03 (0.27)
NM CG1059	NM <i>motB</i>	NM	NM	0.05	0.01 (0.1)

<sup>a</sup> Number reported is the mean of at least six swarm assays in which the ability of the mutant is compared with that of the parental strain on the same agar plate. NM, nonmotile.

<sup>b</sup> Number reported is the mean of adherent bacteria on 10 to 400 HEP-2 cells, with the standard deviation (SD) reported in parentheses.

3 and Fig. 1). Analysis of toxin production confirmed that all mutants with a defect in *toxR* produced no CT, but the *toxS* mutant JJM42 produced wild-type or slightly elevated levels of CT under these assay conditions (Table 3). Thus, it is unlikely that HS mutants are *toxT* mutants, but it is possible that some class I HS mutants may have mutations in either *toxR* or *toxS*.

To investigate the possibility that class I HS mutants might have mutations in *toxR*, a number of different analyses were performed. Western blot analysis with anti-ToxR antibody showed that several class I and class II HS mutants produced normal levels of ToxR protein that was electrophoretically identical to wild-type ToxR (data not shown). Additionally, neither plasmid pVM16 (which carries wild-type *toxR*) nor plasmid pGJ2.3 (which carries wild-type *toxT*) was found to fully complement HS mutants CG379 and CG380 for their defects in CT production (Table 3) or TCP production (data not shown). Although pVM16 did complement these mutants partially for CT production, we did not observe a corresponding decrease in the motility of these HS mutants, which would have suggested true complementation of the HS phenotype. Together, these results suggest that the class I HS mutants probably do not carry null mutations in *toxR*, *toxS*, or *toxT*.

Further analysis of *toxR*, *toxS*, *toxT*, and HS mutants revealed another difference between these various types of mutants when grown on 1% tryptone chemotaxis medium (4). On chemotaxis medium, a *toxR* deletion mutant swarms at a rate approximately 74% of that of the parental strains, while most spontaneous HS mutants swarm at a rate approximately 140 to 150% of that of the parental strains (Fig. 1 and Table 3). The impaired chemotaxis observed in the *toxR* mutant was not seen for strain JJM42 carrying just a deletion in *toxS* (which swarms at a rate approximately 130% of that of the parental strain on chemotaxis medium) nor was it seen for strain VJ675 carrying a *toxT* deletion (which exhibits parental swarm rates). Furthermore, introduction of the wild-type *toxR* gene on plasmid pVM16 alleviates the swarming defect of the *toxR* mutant JJM43 on chemotaxis agar and restores autoagglutination and CT production (Table 3). These data support our conclusion that class I HS mutants are unlikely to carry null mutations in *toxR*, *toxS*, or *toxT*. One possible explanation for this impaired chemotaxis phenotype observed in all *toxR* mutants is a recent observation that the ToxR-activated genes *acfB* and *tcpI* encode proteins with high similarity to methyl-accepting chemoreceptors of *E. coli*, and thus a *toxR* mutant would be deficient in at least these putative chemoreceptors (16, 26).

In order to further define the HS class I mutations, we attempted to clone genes from wild-type *V. cholerae* by complementation of the CT production defect present in class I HS mutants. Both plasmid and cosmid libraries were introduced into strains CG379 and CG380 and then screened for clones that produced detectable CT by ELISA. However, the positive clones obtained all carried the structural genes for CT as determined by Southern blot analysis (data not shown). Class I HS strains harboring a multicopy cosmid encoding CT still produced 2,000-fold lower levels of toxin than the wild-type strains (data not shown). Formally, these data suggest that HS mutants may carry gain-of-function mutations which, for example, send a dominant negative signal to ToxR, ToxS, or ToxT. Thus, hypermotility may be directly sensed by one or more of these virulence regulatory factors. Interestingly, two of these (ToxR and ToxS) are membrane proteins that might be positioned appropriately to obtain information directly from the motility apparatus localized within the cell envelope.

**NM mutants autoagglutinate and produce CT under nonpermissive conditions.** We reasoned that if hypermotility was sensed by the ToxR regulatory system as a negative signal, then loss of motility might alter the expression of ToxR-regulated gene products in a positive way. Indeed, we found that both spontaneous and transposon-induced NM mutants derived from strain O395 produced more CT and TCP than their corresponding parental strains under nonpermissive conditions of growth. For example, NM mutants grown in LB broth at pH 8.5 exhibit autoagglutination, whereas parental strains do not, and this autoagglutination observed for NM strains correlates with the overproduction of TcpA and CT compared with production by isogenic strain CG842, which displays wild-type motility (Fig. 2). When grown in a high-pH medium, CG842 made only 0.03  $\mu$ g of CT per OD<sub>600</sub> unit and a barely detectable amount of TcpA protein, while all four NM strains examined produced higher levels of TcpA and dramatically higher levels of CT. In contrast, both JJM43, the *toxR* mutant, and CG380, a class I hypermotile mutant, produced no detectable TcpA or CT under either ToxR permissive or nonpermissive growth conditions (Fig. 2). Thus, a variety of different NM mutants of *V. cholerae* O395 display at least a partially constitutive phenotype in their ability to express ToxR-activated genes under environmental conditions that wild-type strains usually find nonpermissive for such expression.

**NM mutant CG996 has an insertion in a region homologous to *motB*.** It is possible that the ToxR constitutive phenotype of

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Vibrio: 148 IPEKGAFFEGSAFLQPKFRPLVRQIAELVKDIPGKVRVTGNTDQKLDSELYRSS 312
      F GSA ++P R ++R IA ++ IP ++ ++G+TD+
E. coli: 161 FRTGSADVEPYMRDILRAIAPVLNGIPNRIISLSGHTDD 198
      + E+ F GA + K P ++ L IP ++V G+TD+ ++ Y
B. mega: 102 LQEQVLPETGQADILKKGTPFLDELGRLEFSTIPNDIKVBEHTDNRPIHTYAY 153
      + E F GA + L+ QIA L++ IP ++V G+TD+ ++ Y S+
B. subt: 124 LQEAFLFDTGEAKVLKNAEYLLHQIYAVLLQTIENDIQVEGHTDSRNISTYRVPSN 178

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FIG. 3. Sequence adjacent to NM CG996 transposon insertion. Amino acid sequence comparison with the sequence derived from the insertion junction of NM CG996 and its homologs. Shown at top is the *Vibrio* sequence and below is the *E. coli* MotB sequence (52), followed by the *B. megaterium* (*B. mega*) MotB homolog and the *B. subtilis* (*B. subt*) MotB homolog (24, 30). The amino acid code noted above each alignment designates identical residues present in the *Vibrio* sequence. The + symbols represent amino acids scored as similar (3, 23).

NM strains is associated with a secondary mutation rather than directly with the mutations causing the NM phenotype. To address this, we have characterized one of the NM mutants carrying a Tn5-*lac* insertion and reconstructed new NM mutants by allelic exchange. Inverse PCR was used to synthesize a 440-bp fragment of DNA corresponding to the junction between the *V. cholerae* chromosome and that of the Tn5-*lac* insertion carried by strain CG996. As shown in Fig. 3, this DNA fragment carries an open reading frame (ORF-1) that shows a high degree of similarity to the deduced amino acid sequences of the products of several different prokaryotic *motB* genes. The top three entries in the Brookhaven and Swiss protein data banks that show the most similarity to ORF-1 are the MotB protein of *E. coli* (36% identity and 68% similarity over 38 amino acids), the MotB homolog of *Bacillus megaterium* (28% identity and 51% similarity over 52 amino acids), and the MotB homolog of *Bacillus subtilis* (30% identity and 53% similarity over 52 amino acids) (24, 30, 52). The MotB protein is one of the membrane components of the flagellar motor, and *motB* mutants are unable to swim but still produce morphologically normal flagella (7). Consistent with ORF-1 being a portion of the *V. cholerae motB* gene, the NM mutant CG996 is unable to penetrate soft agar or swim but produces a normal sheathed flagellum when viewed by electron microscopy (data not shown).

The 440-bp PCR fragment derived from CG996 was cloned into suicide vector pGP704 to produce pCG105 (44). This plasmid was transferred to *V. cholerae* by conjugation, and all ampicillin-resistant colonies obtained were NM. Southern blot analysis of one of these NM strains (CG1059) confirmed the expected integration of pCG1050 in the *motB* locus (data not shown). Strain CG1059 was found to be flagellated but NM and additionally had exactly the same phenotype as the original NM Tn5-*lac* insertion mutant CG996 when analyzed for TCP and CT expression under permissive and nonpermissive conditions (data not shown). Thus, null mutations of the *V. cholerae motB* homolog produce the same ToxR constitutive phenotype that is seen with other less-characterized spontaneous and transposon-induced NM flagellated and aflagellated mutants.

**Protease production is increased in HS strains.** *V. cholerae* secretes a number of extracellular proteases which have been implicated in various steps in the infection cycle (17). The activity of these proteases can be conveniently detected on casein medium plates by a zone of clearing under and surrounding the colonies. To determine if motility mutants secreted protease at levels similar to those of the parental strain, we examined colonies on casein plates. Surprisingly, class I and class II HS mutants exhibited a striking increase in the zone of clearing, whereas NM mutants appear similar to the parental strain (Fig. 4 and Table 3). Mutants with defective *toxR* alleles



FIG. 4. Extracellular protease production by *Vibrio* class I HS mutants. A casein plate with bacterial streaks is shown. O395N1 is the parental strain.

showed a slight increase in zone of clearing compared with that of parental strains, consistent with their HS phenotype.

The fact that class I HS mutants are hyperproducers of protease suggested that degradation by proteases could account for the observed reduction in CT and TCP levels expressed by these strains. However, in experiments in which class I HS strains were mixed with parental strains and they were grown together overnight, there was no reduction in the level of toxin or TCP expression by the parental strains (data not shown). Thus, the hyperproduction of proteases by HS mutants does not explain the reduced expression of CT or TCP in these strains.

**Fucose-sensitive HA is increased in HS strains.** Because alterations in the hemagglutination properties of NM mutants had been previously reported by other investigators, we measured the HA activity of our mutants (31). Both human type O and chicken erythrocytes were observed to agglutinate in the presence of washed bacterial cells of parental strain O395. Class I HS mutants exhibited more hemagglutination than the

TABLE 5. Hemagglutination and hemolysis of chicken erythrocytes by bacteria at different growth stages<sup>a</sup>

Strain	Hemagglutination		Hemolysis	
	Stationary phase	Exponential phase	Stationary phase	Exponential phase
CG 842	6	12 (4) <sup>b</sup>	3	ND <sup>c</sup>
NM CG997	ND	ND	12	>4,096
NM CG1059	ND	ND	768	>4,096
O395N1	8	2	4	ND
HS CG379	8	48 (ND)	2	ND
HS CG380	8	48 (ND)	2	ND
JJM43	6	16	2	ND
JJM42	6	16	2	ND
JJM10.29	6	16 (ND)	4	ND
RT110.21	6	16	ND	2
NM39	2	2	>1,536	256
NM51	2	2	>1,536	256

<sup>a</sup> Values represent the reciprocal of the dilution in which hemagglutination and hemolysis can be observed.

<sup>b</sup> Values in parentheses represent assay results when performed in the presence of 1% L-fucose.

<sup>c</sup> ND, not detectable.

TABLE 6. Hemolysis and hemagglutination of human type O erythrocytes and adherence to fucose-coated beads by exponential-phase bacteria

Strains	Hemagglutination <sup>a</sup>	Hemolysis <sup>a</sup>	Fucose-coated bead adherence <sup>b</sup>
O395N1	16	4	+
HS CG379	256	2	+++++
HS CG380	256	2	+++++
NM 39	8	>4,096	-/+
NM 51	8	>4,096	-

<sup>a</sup> Values represent the reciprocal of the dilution in which hemagglutination and hemolysis can be observed.

<sup>b</sup> Each + represents approximately 10 bacteria per bead observed by light microscopy; -/+ indicates most beads were bare, and those with adherent bacteria had fewer than four bacteria; - indicates no bacteria were found.

parental strain with both chicken and human blood cells, while NM mutants showed reduced hemagglutination (Tables 5 and 6). Addition of 1% L-fucose in the assay inhibited hemagglutination with all HA-positive strains, consistent with the results of other investigators studying classical strains of *V. cholerae* (18). We were concerned that the increased hemagglutination (15-fold higher levels) seen with HS mutants might be attributable to their increased protease production, given that at least one extracellularly secreted protease of *V. cholerae* has hemagglutinating activity (17, 27). However, this HA-protease is not inhibited by L-fucose and is generally not produced by classical strains of *V. cholerae*. Moreover, no hemagglutination was observed when cell culture supernatant fluids from wild-type O395 or HS mutants were used in HA assays (data not shown). Thus, strain O395 expresses a fucose-sensitive HA (FSHA) that is tightly cell associated, and it is the expression or activity of this HA that is modulated by the motility phenotype. Consistent with this conclusion, we also observed that HS mutants exhibited a dramatic increase in adherence to L-fucose-coated Sepharose beads compared with parental strains (Table 6). NM strains adhered to the beads at a level clearly well below that of parental strains.

**NM mutants produce high levels of CAH.** During the course of performing hemagglutination assays, it became apparent that our NM strains elaborated a hemolytic activity that was not highly expressed by parental strains or HS mutants. This hemolytic activity was also observed for NM *TnphoA* insertion mutants of strain O395 (48). This type of hemolytic activity was demonstrable after incubation of erythrocyte-bacterium mixtures at room temperature and could be quantitated visually in microtiter plates on the basis of the amount of hemoglobin released (Table 5). The hemolytic activity elaborated by NM mutants was cell associated and was not present in supernatant fluids (data not shown). Therefore, the hemolytic activity associated with NM mutant cells is not likely to be related to the product of the *HlyA* locus of *V. cholerae* (a secreted, fast-acting hemolysin produced by *V. cholerae* strains of the El Tor biotype) (2). Although the cell-associated hemolysin (hereafter designated the CAH) was slowly acting, its titer and potency were impressive. The NM strain diluted greater than  $2 \times 10^5$ -fold exhibited hemolysis similar to that of the wild-type strain when hemoglobin release was measured quantitatively in an assay done in microcentrifuge tube format (data not shown). Thus, NM mutants are deficient in hemagglutination but show a dramatic increase in CAH levels, while HS mutants have increased levels of hemagglutination but are deficient in CAH production.

**NM strains are deficient in adherence to HEp-2 cells.** In order to correlate our hemagglutination results with adherence to other mammalian cells, we examined the interaction of our mutants with cultured monolayers of HEp-2 cells. Differences in adherence were calculated by determining the average number of bacteria adhering to HEp-2 cells (Table 4). Approximately 15 O395 vibrios adhered per HEp-2 cell. The spontaneous HS strains were indistinguishable from their parental strains in the ability to adhere to HEp-2 cells, suggesting that the overproduction of FSHA observed for HS mutants was not advantageous in HEp-2 binding. Consistent with this result, the addition of L-fucose (or D-mannose) had no detectable effect on the adherence of various strains to HEp-2 cells (data not shown). In sharp contrast, all NM strains (including the flagellated *motB* mutant CG996) showed a dramatic decrease in adherence to HEp-2 cells (0.01 to 0.06 bacteria per HEp-2 cell) (Table 4). Given that NM strains are deficient in FSHA, these results suggest that FSHA may act as a HEp-2 adhesin of *V. cholerae* O395 derivatives. If FSHA is the only O395 adhesin expressed in vitro, then it is apparent, on the basis of the inability of L-fucose to inhibit HEp-2 adherence of wild-type and HS mutants, that the affinity of FSHA for HEp-2 receptors is probably greater than its affinity for the erythrocyte receptor. Alternatively, it has been reported that *V. cholerae* does express multiple HAs and thus the expression of more than one (i.e., both the FSHA and the HEp-2 adhesin) is adversely affected by loss of bacterial motility (33).

We have observed that adherence to HEp-2 cells is maximized if mid-log bacterial cultures are used. Because the TCP are optimally expressed during the late log phase of these cultures, it seems likely that TCP-associated adhesins are not required for *V. cholerae* to bind to HEp-2 cells. Consistent with this observation, a *toxR* mutant and a *tcpA* mutant (both unable to synthesize TCP) show no defect in adherence to HEp-2 cells. In fact, the *toxR* mutant showed increased HEp-2 cell binding, indicating that ToxR-dependent gene products (e.g., OmpU, TCP, and Acf proteins) are probably not involved in HEp-2 cell binding (Table 4 and Fig. 5) (44, 56).

**Motility mutants in the infant mouse model of intestinal colonization.** The fact that *toxR* mutants of O395 display a HS phenotype and increased HEp-2 adherence prompted us to question the in vivo relevance of this adherence phenotype, because it was known that O395 *toxR* mutants are dramatically deficient in intestinal colonization in both suckling mice and volunteer subjects (28, 56). Accordingly, in vivo competition assays in suckling mice were used to measure the intestinal colonization capacity of various HS and NM mutants. Consistent with the importance of TCP in colonization, all HS mutants lacking TCP showed a distinct disadvantage compared with the wild type in the suckling mouse model, regardless of other associated phenotypes (i.e., hyperexpression of FSHA, protease, or HEp-2 adherence factors) (Table 7) (12, 49, 56). A class III HS strain (HS30) that was able to synthesize toxin and TCP showed no colonization defect, indicating that the ability to swarm faster is not itself detrimental to the colonization process. NM mutant strains showed either a small defect or a slight gain in output numbers, suggesting that the array of secondary phenotypes associated with these NM mutations (e.g., enhanced ToxR-activated gene expression and decreased levels of FSHA and HEp-2 adhesins) do not adversely affect colonization in this animal model. Interestingly, our studies revealed an inverse correlation between HEp-2 adherence and the ability to colonize infant mice. NM mutants exhibit a striking defect in HEp-2 cell binding, yet are unimpaired in infant mouse colonization (Fig. 6 and Tables 4 and 7). Conversely, a *toxR* mutant and other chemotaxis mutants show a marked

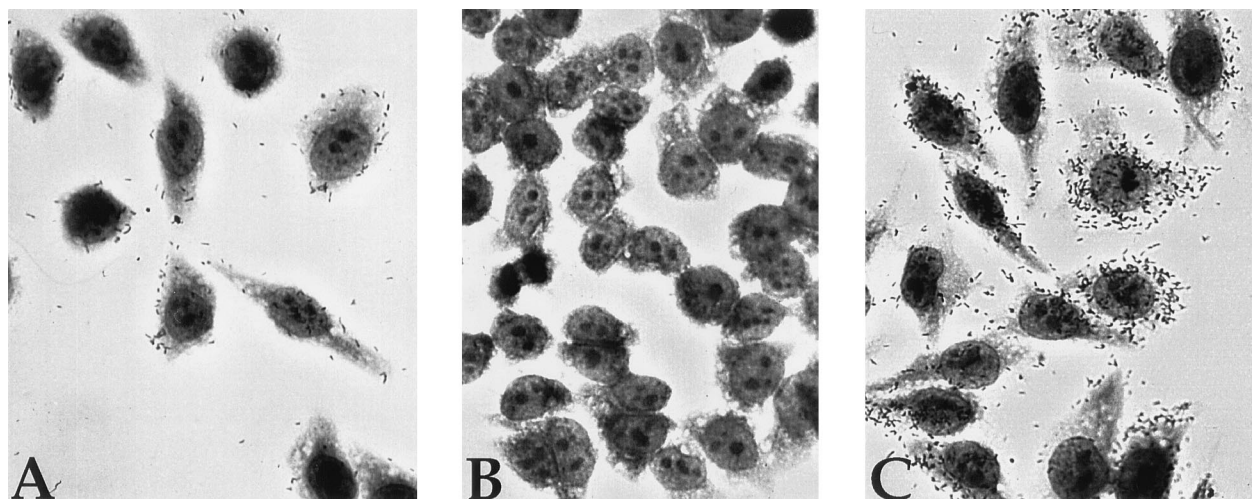


FIG. 5. Adherence to HEp-2 monolayers. HEp-2 cells after incubation with *V. cholerae* strains. (A) CG842 (parental wild type). (B) NM CG997 (transposon-induced NM mutant). (C) JJM43 ( $\Delta toxR$  mutant).

increase in adherence to HEp-2 cells, while exhibiting a dramatic colonization defect in the infant mouse model (22).

**Relationship between motility and virulence factor regulation.** Previous research on the virulence of NM strains led to conflicting results about the importance of motility in virulence (5, 19, 20, 25, 31, 50, 57, 60). Because NM strains show decreased expression of some traits (HAS and HEp-2 adhesins) and increased expression of others (CT, CAH, and TCP), it is possible that some of the discrepancies in earlier studies may indicate the biases of various model systems for particular virulence traits. It is apparent from the phenotype of *motB* mutants of *V. cholerae* that simply the lack of rotation of the flagellum is a sufficient signal for the cell to produce toxin and pili under nonpermissive growth conditions, reduce expression of FSHA and HEp-2 adhesins, and, finally, increase expression of CAH. A nearly opposite pattern of expression was observed for these potential virulence factors in hypermotile mutants.

These results are reminiscent of other virulence regulatory systems such as the BvgAS system of *Bordetella* cells or the PhoPQ system of *Salmonella* cells, for which in both cases there exist groups of reciprocally regulated virulence genes (6, 42, 54). It is particularly interesting to note that in *Bordetella bronchiseptica*, motility is a BvgAS-regulated property that is down-regulated when known BvgAS-activated virulence gene expression is up-regulated in vivo (1). A notable difference in the case of *V. cholerae* is the fact that mutations altering motility per se (i.e., either HS or NM mutations) seem to directly feed back to the ToxR regulatory system.

The observed pattern of virulence factor expression in *V. cholerae* HS and NM mutants might provide some insights into complex patterns of expression occurring in vivo. It is conceivable that the HS and NM states might reflect distinct stages in the infection cycle of *V. cholerae*. The HS state may correspond to an early stage in the human infection cycle in which the coordinately expressed phenotypes of motility, FSHA, and protease may be required to interact with and degrade the mucus gel and glycocalyx, thereby facilitating penetration to the underlying epithelium, where HEp-2 adhesins would act. Consistent with this proposal are recent studies examining the interaction of *V. cholerae* with human small intestinal tissue, which demonstrate that the bacteria bind to the mucus overlying the intestinal epithelia and that this adherence is fucose sensitive (59). The NM state might logically correspond to a stage after mucus gel penetration (for example, when the vibrios are attached to epithelial cells underlying the mucus coat) and it is during this sedentary stage that they might optimally express ToxR-activated genes encoding CT and TCP. Since NM bacteria optimally express CAH, it follows that the target for this hemolysin may be epithelial cells (i.e., the target of CT).

Recent clinical studies involving volunteers who have ingested various live, attenuated, nontoxic vaccine derivatives that were either motility proficient or deficient have uncovered the correlation between reactogenicity (i.e., adverse symptoms such as diarrhea, cramps, and fever) and the motile state (13, 34, 38, 55). Interestingly, motility-deficient vaccine derivatives are nonreactogenic but colonize subjects well and induce excellent immune responses. Clearly, a better understanding of the role that motility plays in *V. cholerae* patho-

TABLE 7. Infant mouse competition assays

Mutant strain	Description	Competing parental strain	Competitive index	
			In vitro (in vitro output <sup>a</sup> /in vivo input <sup>b</sup> )	In vivo (in vivo output <sup>c</sup> /in vivo input <sup>b</sup> )
HS CG379	HS class I	CG842	0.6	0.0015
HS CG380	HS class I	CG842	1.41	0.001
HS35 (Tox <sup>+</sup> Agg <sup>-</sup> )	HS class II	O395	0.09	0.054
HS30 (Tox <sup>+</sup> Agg <sup>+</sup> )	HS class III	O395	0.3	1.34
NM39	NM	CG842	0.22	2.0
NM51	NM	CG842	0.27	0.74
NM CG997	NM	O395	0.41	0.1675
NM CG996	NM O395 <i>motB</i>	O395	0.53	0.092
NM CG1059	NM O395 <i>motB</i>	O395	1.8	0.8

<sup>a</sup> In vitro output is calculated as the mutant/parental strain ratio after growth of the input inoculum in media at 37°C.

<sup>b</sup> In vivo input is calculated as the mutant/parental strain ratio at the time of inoculation of mice or in vitro grown cultures.

<sup>c</sup> In vivo output is calculated as a mean average of bacteria (mutant/parental strain) recovered from intestinal homogenates ( $n = 5$  to 8 mice per strain).



genesis may provide important and practical knowledge in the field of vaccinology.

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