INFECTION AND IMMUNITY, Aug. 1996, p. 3369–3373 Vol. 64, No. 8
0019-9567/96/$04.00+0

Relative Significance of Mannose-Sensitive Hemagglutinin and Toxin-Coregulated Pili in Colonization of Infant Mice by *Vibrio cholerae* El Tor†

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Received 27 February 1996/Returned for modification 8 April 1996/Accepted 27 May 1996

A previously described in-frame deletion in *mshA*—the gene encoding the structural subunit of the mannose-sensitive hemagglutinin pilus—has been introduced into the chromosome of three El Tor O1 strains of *Vibrio cholerae*. None of the Δ*mshA* mutants showed significant attenuation or loss of colonization potential in the infant mouse cholera model. A second mutation, created by insertion of a kanamycin resistance cartridge into Δ*mshA*, also failed to affect in vivo behavior. In contrast, strains carrying mutations in *tcpA* (encoding the monomer of the toxin-coregulated pilus [TCP]) were markedly attenuated and showed dramatically impaired colonization. This result was in line with those of previous studies. Protection tests performed with antibodies to TCP and to MshA showed that only the former were able to confer immunity against El Tor O1 challenge in this model. Studies with mutants constructed from two O139 strains similarly suggest that TCP but not mannose-sensitive hemagglutinin pili are critical for colonization by strains of this serogroup.

Over the past decade, much research has focused on the colonization factors of *Vibrio cholerae* in the hope that these factors may be utilized for the development of improved cholera vaccines. Toxin-coregulated pili (TCP) were first described by Taylor et al. (15) and were shown to play a critical role in colonization in the infant mouse cholera model (IMCM) (15) and subsequently in human volunteers (4). Antibodies to TCP are sufficient to confer protection in the IMCM (11–14). These initial studies were performed with strains of the classical biotype, and until very recently, there was uncertainty as to whether TCP were expressed and similarly utilized by El Tor *V. cholerae*. However, the demonstration of surface TCP on El Tor strains (17) was quickly followed by reports that *tcpA* mutants of this biotype show dramatically impaired colonization of infant mice (1, 9, 18). Antibodies to El Tor TCP are sufficient to protect mice from challenge with both O1 and O139 serogroups of this biotype (18), illustrating the vaccine potential of these pili.

Other studies (5) have identified a second pilus produced by *V. cholerae* El Tor, the mannose-sensitive hemagglutinin (MSHA) pilus. Finn et al. (2) used transposon mutagenesis to derive *V. cholerae* strains which were unable to mediate hemagglutination. These mutants were subsequently shown to be defective in the *mshD* or *mshE* gene involved in the expression of MSHA (3). One such mutant showed dramatically reduced colonization in an adult rabbit model (2). Recently, Jonson et al. (6) reported the localization of the *mshA* gene within a cluster of type 4 pilin genes and also constructed an in-frame mutation in *mshA*. Although the in vivo behavior of *mshA* mutants has hitherto not been examined in the IMCM, antibodies to MSHA have been reported to be protective (7, 8), suggesting that these pili may also play a role in colonization of infant mice.

The aim of the present report is to determine the relative significance of TCP and MSHA in the pathogenesis of El Tor strains in the IMCM. Strains carrying mutations in *mshA* have been constructed and tested for residual colonization potential in comparison with *tcpA* mutants of the same strains. In addition, the protective efficacies of antibodies to MSHA and TCP have been compared. Our data suggest that MSHA pili are not necessary for colonization in this model and that antibodies to the MshA pilin subunit are not sufficient to mediate protection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *V. cholerae* strains used in these studies are shown in Table 1. Spontaneous streptomycin-resistant variants were selected from the wild type by plating bacteria onto medium containing the antibiotic at 200 μg/ml. As required, kanamycin at 50 μg/ml was used in agar plates.

Strains were cultured under a variety of growth conditions. For MSHA production, vibrios were grown in Trypticase soy broth without glucose (TSB-glu; Becton Dickinson Microbiology Systems, Cockeysville, Md.) at 37°C as described before (7). For expression of TCP, strains were grown in CO_2-gassed AKI medium at 30°C as previously described (17). Oxoid nutrient broth was used in some experiments.

Construction of *mshA* mutants. The construction of a 171-bp in-frame deletion in *mshA* has been detailed elsewhere (6). This mutation was transferred to the chromosome of several wild-type strains by using either of two suicide vectors, pCACTUS (6) or pCVD442 (18). The former was introduced into *V. cholerae* by electroporation, and the latter was introduced by conjugation from an *Escherichia coli* λ*pir* donor (18). Both vectors carry the *sacB* gene to facilitate mutant screening; after chromosomal integration of the vector construct, growth in the presence of sucrose selects against bacteria which have retained the vector in the chromosome but allows growth of vibrios for which resolution of the vector has occurred.

Subsequently, a kanamycin resistance (Km^r) cartridge was inserted into the *Bgl*II site introduced during construction of Δ*mshA* (6). This cartridge has a stop codon at the end of the gene that specifies antibiotic resistance and is followed by a stem-loop structure and so was considered likely to have polar effects on the expression of downstream genes. The Δ*mshA*::Km^r mutation was introduced into the chromosome with pCACTUS.

Construction of *tcpA* mutants. Previous reports have described strategies for

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† We dedicate this report to the memory of our friend and colleague, Gunhild Jonson.

‡ Deceased.

TABLE 1. *V. cholerae* strains

Serogroup and strain	Genotype	Phenotype[a]		MshA[b]	TcpA[b]
		MSHA	FSHA		
V. cholerae O1 El Tor					
Phil 6973	Wild type	+	+	+	+
Phil 6973 Sm^r	Spontaneous Sm^r variant of wild type	+	+	+	+
Phil 6973 Δ*mshA*	171-bp in-frame deletion in *mshA*	−	+	−	+
Phil 6973 Δ*mshA*::Km^r	Km^r cartridge insertion in Δ*mshA*	−	+	−	+
Phil 6973 Sm^r Δ*tcpA*	612-bp in-frame deletion in *tcpA*	+	+	+	−
174	Wild type	+	−	+	+
174 Sm^r	Spontaneous Sm^r variant of wild type	+	−	+	+
174 Sm^r Δ*mshA*	171-bp in-frame deletion in *mshA*	−	−	−	+
174 Sm^r *tcpA*::Km^r	Km^r cartridge insertion in *tcpA*	+	NT[c]	NT	−
H1	Wild type	+	−	+	+
H1 Sm^r	Spontaneous Sm^r variant of wild type	+	−	+	+
H1 Δ*mshA*	171-bp in-frame deletion in *mshA*	−	−	−	+
H1 Δ*mshA*::Km^r	Km^r cartridge insertion in Δ*mshA*	−	−	−	+
H1 Sm^r Δ*tcpA*	612-bp in-frame deletion in *tcpA*	+	−	+	−
O17	Wild type	+	+	+	ND[d]
O17 *tcpA*::Km^r	Km^r cartridge insertion in *tcpA*	+	+	+	ND
O17 *tcpA*::Km^r Δ*mshA*	As above, with 171-bp in-frame deletion in *mshA*	−	+	−	ND
V. cholerae O139					
4260B	Wild type	+	−	+	+
4260B Δ*mshA*::Km^r	Km^r cartridge insertion in Δ*mshA*	−	−	−	NT
AI-1838	Wild type	+	−	+	+
AI-1838 *tcpA*::Km^r	Km^r cartridge insertion in *tcpA*	NT	NT	NT	−

[a] Hemagglutination was tested with chicken and/or human (group O) erythrocytes. MSHA, D-mannose-sensitive HA; FSHA, L-fucose-sensitive HA.
[b] Detection by Western blotting with anti-MshA monoclonal antibody and anti-TCP antiserum, respectively.
[c] NT, not tested.
[d] ND, not detectable. Unlike the other wild-type strains listed but like about 50% of the El Tor strains examined, O17 does not produce TcpA during growth in AKI medium (17).

the construction of mutants carrying an in-frame deletion in *tcpA* (18) or a *tcpA* gene inactivated by the insertion of a Km^r cartridge (1).

Characterization of mutants. Constructs involving the Δ*mshA* or Δ*mshA*::Km^r mutations were confirmed by PCR of chromosomal DNA with primers for *mshA* flanking sequences as described before (6). In addition, Southern hybridization with digoxigenin-labelled, PCR-amplified *mshA* was performed to probe chromosomal DNA cut with *Sal*I, *Bgl*II, and *Eco*RI. The *Bgl*II site is diagnostic of the Δ*mshA* mutation (6). Mutants carrying Δ*tcpA* or *tcpA*::Km^r were verified by Southern blotting with probes specific for *tcpA* or the Km^r cartridge as described elsewhere (1, 18).

All mutant strains were checked for production of cholera toxin during growth in AKI medium at 30°C. Culture supernatants were stored at −20°C until they were assayed by GM1 enzyme-linked immunosorbent assay (ELISA) as previously described (17). Hemagglutination assays were performed in the presence and absence of D-mannose and L-fucose with chicken or human group O erythrocytes being used as described previously (6). Immunoblotting studies (17) were performed to confirm that mutant strains no longer produced the appropriate pilin subunit. Synthesis of MshA following growth in TSB-glu was checked with monoclonal antibody 17:10 directed against this protein (5). TcpA production in CO_2-gassed AKI cultures was assessed with an absorbed polyclonal anti-TCP serum (17).

Studies with the IMCM. (i) Competition experiments. These experiments were performed to evaluate the in vivo consequences of mutations in *mshA* or *tcpA*. As was described elsewhere (1), mixed inocula comprising approximately equal numbers of wild-type and mutant bacteria were administered to (Swiss outbred) infant mice. The concentrations of the two strains were adjusted so that the dose of each corresponded to 10 to 20 50% lethal doses (LD_{50}) of the wild-type strain; the precise input ratio was determined retrospectively by plating dilutions of the suspension on nutrient and antibiotic (streptomycin or kanamycin) media. Unless stated otherwise, mice were sacrificed at 24 h and their small intestines were placed in tubes containing 2 ml of phosphate-buffered saline (PBS; pH 7.4). After homogenization (Ultra-Turrax tissue homogenizer [Janke and Kunkel, Staufen, Germany], setting 6 for 15 s), the suspensions were diluted in PBS and spread onto nutrient and selective agars for determination of the output ratio for the two strains.

(ii) Virulence assays. The virulence of the wild-type and mutant strains was compared by the (simultaneous) determination of 48-h LD_{50} as previously described (1). For studies with *mshA* mutants, strains were cultured in TSB-glu, washed, and suspended in PBS prior to administration; for comparisons involving *tcpA* mutants, bacteria were grown in nutrient broth and suspended in 0.1% (wt/vol) Proteose Peptone (Difco) in saline.

(iii) Protection tests. The protective efficacies of antibodies to MshA or TCP were assessed essentially as described elsewhere (7, 18). Strains were grown under conditions conducive to production of the appropriate pilus type, and challenge suspensions were prepared so that each mouse received 10 to 20 LD_{50}. For assays using the 17:10 anti-MshA monoclonal antibody, bacteria were grown in TSB-glu, washed, and resuspended in PBS; one aliquot of this suspension was pretreated with antibody (a 1-in-20 dilution of ascitic fluid), while another was left untreated and was fed to control mice. The protective efficacy of an absorbed

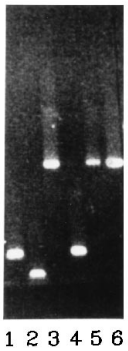

FIG. 1. PCR of *V. cholerae* Δ*mshA* mutants. Chromosomal DNA was amplified by PCR with primers flanking the *mshA* gene. Lanes: 1, H1; 2, H1 Δ*mshA*; 3, H1 Δ*mshA*::Km^r; 4, 4260B; 5, 4260B Δ*mshA*::Km^r; 6, control DNA from the pCACTUS Δ*mshA*::Km^r construct. The positions of the bands are in accord with their predicted sizes of 437, 602, and 1,537 bp.

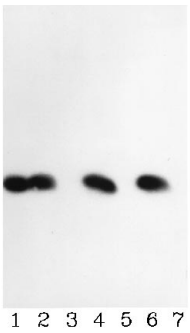

FIG. 2. Western blotting (immunoblotting) of *tcpA* mutants. Bacteria were cultured in AKI medium, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with an absorbed polyclonal serum specific for TCP. Lanes: 1, EV37 (positive control [17]); 2, Phil 6973; 3, Phil 6973 Δ*tcpA*; 4, 174; 5, 174 *tcpA*::Km^r; 6, AI-1838; 7, AI-1838 *tcpA*::Km^r.

anti-TCP serum (anti-H1 TcpA [18]) was assayed with vibrios cultured in AKI medium at 30°C. The bacteria were resuspended and diluted in fresh AKI medium, and aliquots were pretreated with dilutions of the test serum at 30°C for 20 min. Protective endpoints were expressed as the serum dilution able to protect 50% of the mice challenged, as described before (18).

RESULTS

Mutant construction and characterization. Construction of a 171-bp in-frame deletion in *mshA*, the gene encoding the MSHA pilin subunit, has been described elsewhere (6). This deletion was introduced into the chromosomes of strains of interest with either of two suicide vectors, pCACTUS or pCVD442. Subsequently, a Km^r cartridge was inserted into the *Bgl*II site within Δ*mshA*, and this potentially polar mutation was transferred into the chromosome with pCACTUS. The various *mshA* mutants were verified by PCR with primers flanking *mshA* (Fig. 1) and also by Southern blotting with PCR-amplified *mshA* labelled with digoxigenin being used as a probe (data not shown). Immunoblotting confirmed that none of the *mshA* mutants produced the pilin subunit in vitro (Table 1), although all showed normal production of cholera toxin as determined by GM1 ELISA.

Previous reports have described the construction of the *tcpA*::Km^r and Δ*tcpA* mutations and the integration of these into the chromosome (1, 18). For the present studies, similar mutants were constructed with strains Phil 6973, 174, and AI-1838. These mutants were confirmed by Southern hybridization (data not shown) and immunoblotting (Fig. 2; Table 1). None showed abnormal production of cholera toxin.

Competition experiments with *mshA* mutants. The in vivo consequences of the Δ*mshA* and Δ*mshA*::Km^r mutations were assessed in mixed-infection competition experiments. In the case of the Δ*mshA* mutants, it was necessary to introduce an identifying marker into one member of each strain pair; this was achieved by selecting spontaneous, streptomycin-resistant (Sm^r) variants of the wild-type strains. Preliminary experiments confirmed that introduction of the Sm^r marker did not significantly affect in vivo behavior (data not shown).

Subsequently, the Sm^r variants of wild-type strains were compared in competition experiments with their corresponding (Sm^s) Δ*mshA* mutants. For each of three strain pairs, the ratios of mutant and wild-type bacteria recovered in gut homogenates were very similar to the ratios present in the challenge inocula (Fig. 3). The Δ*mshA*::Km^r mutation was introduced into one of the O1 El Tor strains and also into a strain of the O139 serogroup. When the resulting mutants were compared with the wild type in vivo, there was again no detectable change in the levels of gut colonization (Fig. 3). Similar results were obtained regardless of the culture conditions used. In various experiments, competing strains were grown and resuspended in nutrient broth or grown in TSB-glu to promote MSHA production. In the latter case, the bacteria were either resuspended in fresh TSB-glu for feeding or washed and suspended in PBS as described previously (7).

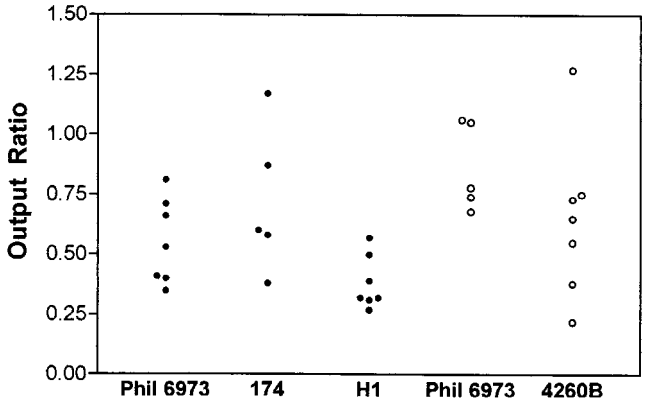

FIG. 3. Effects of Δ*mshA* (●) and *mshA*::Km^r (○) mutations on colonization by various El Tor strains. Mice were fed mixed suspensions of wild-type and mutant bacteria; input ratios (wild type to mutant) were 0.65:1 (Phil 6973 Δ*mshA*), 0.74:1 (174), 0.63:1 (H1), 1.02:1 (Phil 6973 *mshA*::Km^r), and 0.93:1 (4260B). The symbols indicate the wild type:mutant output ratios for bacteria recovered from individual mice (n = 5 to 7).

Other competition experiments with lower challenge doses were performed to allow sampling of gut contents at a later time point. However, neither Phil 6973 Δ*mshA*::Km^r (mutant: wild type input ratio = 2.1; median output ratio = 1.8; n = 7) nor H1 Δ*mshA* (input ratio = 0.7; output ratio = 10.4; n = 7) showed impaired persistence when intestinal populations were recovered 45 h after feeding. Collectively, these data suggest that MSHA does not play a critical role in the colonization of the infant mouse gut by O1 El Tor strains; indeed, Δ*mshA* mutants of Phil 6973, 174, and H1 retained full virulence (Table 2).

Competition experiments with *tcpA* mutants. Previous studies (1, 9, 18) have shown that *tcpA* mutants are virtually avirulent and show dramatically reduced colonization in the IMCM. Figure 4 shows that *tcpA* mutants constructed for the Phil 6973 and 174 strains display a similar colonization defect (similar data for H1 *tcpA* mutants have been reported elsewhere [1, 18]). Introduction of the *tcpA*::Km^r mutation into a strain (AI-1838) of the O139 serogroup had a similar effect (Fig. 4). All three *tcpA* mutants showed at least 1,000-fold higher LD_{50} than did the wild type (data not shown).

In an earlier report, the O17 strain appeared somewhat unusual in that it retained a slight colonization capacity (ca.

TABLE 2. Virulence of *mshA* mutants in the IMCM

Strain	48-h LD_{50}	
	Wild type	*mshA* mutant[a]
174	1.2×10^5	1.3×10^5
Phil 6973	1.6×10^4	8.6×10^4
H1	1.7×10^3	1.3×10^3
4260B	$<10^3$	$<10^3$

[a] The mutants carry Δ*mshA* (174, Phil 6973, or H1) or Δ*mshA*::Km^r (4260B).

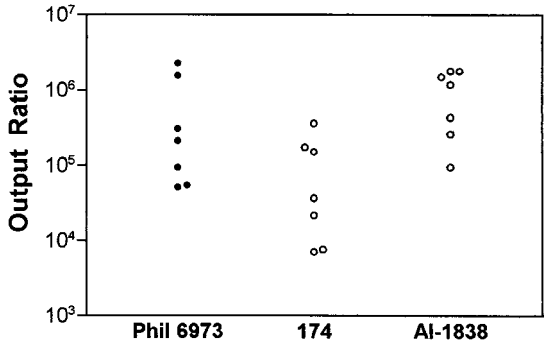

FIG. 4. Effects of Δ*tcpA* (●) and *tcpA*::Km^r (○) mutations on colonization by various El Tor strains. Mice were fed mixed suspensions of wild-type and mutant bacteria, with input ratios of 0.72 to 0.84:1 for the three experiments. The symbols indicate wild type:mutant output ratios for individual mice (n = 7).

3% of that of the wild type) after the insertional inactivation of *tcpA* (1). To ascertain whether this residual colonization was mediated by MSHA, an O17 *tcpA*::Km^r Δ*mshA* double mutant was constructed and compared with its O17 *tcpA*::Km^r antecedent. Bacteria recovered from gut homogenates were patched and identified by colony blotting with monoclonal antibody 17:10, which is specific for MshA. The Δ*mshA* mutation did not affect the in vivo persistence of the *tcpA* mutant (single mutant:double mutant input ratio = 0.67; median output ratio = 0.91; n = 7).

Protection tests. Antibodies to MSHA and TCP were compared for protective activity in the IMCM, using wild-type O1 El Tor challenge strains grown under conditions conducive to the production of either pilus type being used. As can be seen from Table 3, ascitic fluid containing monoclonal antibodies to MshA was unable to confer protection in this model. In contrast, a highly absorbed, TCP-specific serum was protective against each of the strains tested.

TABLE 3. Protective efficacies of antibodies to MshA and TCP

Strain	Titer[a]	
	Anti-MSHA	Anti-TCP
174	<20	690
Phil 6973	<20	1,200
H1	<20	3,500

[a] The protective titers of monoclonal antibody 17:10 to MshA and (absorbed) polyclonal serum to El Tor TCP are shown. The figures represent the means of two determinations. Challenge bacteria were cultured under conditions appropriate for the production of either pilus type.

DISCUSSION

An in-frame deletion in *mshA* was constructed and introduced into three O1 El Tor strains. *V. cholerae* 174 was chosen because earlier reports (7, 8) indicated that antibodies to MSHA could protect mice from challenge with this strain, suggesting an important role for these pili in the process of colonization. Phil 6973 is the strain that was used recently to clone *mshA* and localize this gene within a cluster of type 4 pilin genes (6). H1 was of interest because previous experiments revealed that TCP were critically important for the in vivo persistence of this strain (1, 18). None of the Δ*mshA* mutants showed altered in vivo behavior; each retained the virulence and colonization potential of its wild-type parent (Table 2; Fig. 3). Similarly, introduction of Δ*mshA* into O17 *tcpA*::Km^r did not further reduce the residual (TCP-independent) colonization potential of this mutant.

The *mshA* gene is the second of a set of four contiguous genes which each encode type 4 pre-pilin-like polypeptides (6). To ascertain whether a polar mutation in *mshA* would affect in vivo behavior, a Km^r cartridge was inserted into the *Bgl*II site introduced during the construction of Δ*mshA*. This cartridge has a stop codon at the end of the antibiotic resistance gene and a stem-loop structure which has the potential to reduce transcription of downstream genes. However, the Δ*mshA*::Km^r mutation also failed to affect colonization potential in the IMCM (Fig. 3).

It seemed possible that the product of the *mshA* gene might play a role much later in infection. To examine this possibility, other competition experiments were performed with lower doses of bacteria so that recovery of intestinal organisms could be delayed until 45 h. Still, however, *mshA* mutants showed unimpaired persistence in vivo.

In contrast to these findings, the introduction of *tcpA* mutations into 174 and Phil 6973 virtually abolished infant mouse colonization (Fig. 4) and markedly attenuated both strains. This result brings to six the number of O1 El Tor strains shown to be critically dependent upon TCP for pathogenesis in the IMCM (1, 18). Experience with *mshA* and *tcpA* mutants of two O139 strains also suggests that TCP is of much greater significance for colonization by strains of this serogroup (Fig. 3 and 4).

The results of protection tests were consistent with the findings of the competition experiments. Given the pivotal role of TCP in colonization by El Tor strains (1, 9, 18) (Fig. 4), it was not surprising that antibodies to these pili were sufficient to mediate protection against the strains used here (Table 3). This result is consistent with our recent report that antibodies to El Tor TCP are sufficient to protect infant mice from challenge with El Tor strains of the O1 or O139 serogroup (18). In contrast, MSHA pili are not required for colonization in this model (Fig. 3) and a monoclonal antibody to MshA did not confer immunity, even at a high concentration (a 1-in-20 dilution of ascitic fluid) (Table 3).

The latter finding conflicts with data from earlier reports (7, 8) in which the same anti-MSHA ascitic fluid showed some protective effect in Swiss infant mice (protective efficacy = 64% at a 1-in-30 dilution). The difference in the two sets of experiments is paradoxical, given that the earlier studies in Göteborg used a much higher challenge dose of the 174 strain (5×10^8) than that used here (ca. 4×10^5, i.e., about 20 LD_{50}). The higher dose was necessary to ensure consistent killing of the infant mice (6a), but even then several groups of control animals showed a survival rate of >30% (7, 8). This result suggests that the mice used in these studies were comparatively resistant to choleraic infection, in which case a relatively modest protective effect associated with the antibody to MshA might be more likely to affect survival rates. In contrast, it appears that the Swiss infant mice used in the present experiments are more sensitive to choleraic infection, reducing the likelihood that any marginal benefit provided by anti-MshA will appear significant. In Adelaide, it has not been possible to detect any protective activity of the 17:10 monoclonal antibody against any of the three El Tor O1 strains used in this report, even when the 174 strain and the methodology described previously were used (7).

Our data indicate that at least in this cholera model, colonization by El Tor O1 and O139 strains is critically dependent upon a functional *tcpA* gene but is independent of *mshA* function. As a corollary, antibodies to TCP but not to MshA are

sufficient to mediate protection. These and other recent results (18) have implications for the continued development of more effective cholera vaccines. For example, one highly promising vaccine comprises killed vibrios together with the isolated B subunit of cholera toxin. This formulation protects older recipients (>5 years) from cholera caused by *V. cholerae* of either biotype (10, 16), but in an extensive field trial, its efficacy in younger recipients (2 to 5 years) was restricted to disease caused by classical strains. The basis for this differential protective effect in young children remains unclear, and approaches to improve the vaccine's efficacy against El Tor cholera have been recently discussed (16). While earlier experiments suggested that such an improvement might be achieved by the incorporation of MSHA antigen into the vaccine (8), our present studies indicate that TCP might represent a more relevant addition to the formulation. No firm conclusion can be reached, however, until findings with the IMCM have been tested in clinical trials. Volunteer studies with isogenic *tcpA* and *mshA* mutants will be necessary to determine the relative importance of these pilus types in the establishment of human infection by strains of the El Tor biotype.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Health and Medical Research Council of Australia. G.J. was supported by the Wenner-Gren Institute, Stockholm.

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Editor: P. E. Orndorff