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Toxin-Coregulated Pilus, but Not Mannose-Sensitive Hemagglutinin, Is Required for Colonization by *Vibrio cholerae* O1 El Tor Biotype and O139 Strains

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The relative contributions of toxin-coregulated pilus (TCP) and cell-associated mannose-sensitive hemagglutinin (MSHA) to the colonization ability of *Vibrio cholerae* O1 El Tor biotype strains and O139 Bengal strains was determined by using isogenic parental and in-frame deletion mutant pairs in the infant mouse cholera model. Both the El Tor and O139 *tcpA* mutant strains showed a dramatic defect in colonization as indicated by their competitive indices, whereas deletion of *mshA* had a negligible effect on colonization in either background.

The acute diarrheal disease cholera has historically been caused by *Vibrio cholerae* O1 of the classical and El Tor biotypes, with El Tor strains predominating in the current, seventh pandemic (36). In late 1992 a newly identified *V. cholerae* serogroup capable of causing clinical, epidemic cholera was identified in regions of India and Bangladesh (28). This serogroup, designated O139, has a number of properties in common with El Tor strains and is likely to be derived from them (16, 24, 36). Currently, O139 and El Tor strains appear to coexist in Asia, whereas El Tor strains are prevalent in Africa and have been responsible for epidemic cholera in parts of the Western Hemisphere, initiating in South America (36).

In order for *V. cholerae* to cause disease, the organism must attach to the intestinal epithelium, where it colonizes and secretes a potent exotoxin which is ultimately responsible for the massive fluid efflux characteristic of cholera illness. Toxin-coregulated pilus (TCP) has been clearly established as a major determinant of classical-biotype colonization (12, 27, 30, 31, 34), whereas its role in El Tor and O139 colonization is less clear (1, 17, 25, 26, 30). The more stringent conditions required to elicit TCP expression by El Tor strains has led to inconsistent observations regarding the existence of TCP in this biotype (5, 19, 25, 30, 35). Furthermore, antibodies raised against classical-biotype TCP, or TcpA, exhibit strong protection against challenge by strains of the same biotype but provide weaker protection, or none at all, against El Tor strains (25, 27, 30). The recent isolation and characterization of the *tcpA* gene from El Tor and O139 strains has helped to clarify some of these issues. The *tcpA* sequence from El Tor strain N16961 is identical to that of O139 strain MO3 (26) but shows significant deviation from the classical-biotype gene, especially in the portion encoding the C-terminal region of the pilin, where epitopes recognized by protective monoclonal antibodies map (14, 26, 31). Knowledge of the sequence has also facilitated the construction of El Tor *tcpA* insertion mutants. As in the case of classical-biotype strains, such mutants exhibit a marked decrease in their ability to colonize (1, 26). However, it has not been ruled out that in these constructions, polarity caused by the insertion might decrease the level of ToxT protein, the gene for which is located downstream of *tcpA* within the *tcp* operon (2). ToxT is a regulatory protein responsible for direct activation of many of the genes in the ToxR virulence regulon (4). Thus, before the colonization defect in El Tor strains can be attributed specifically to a lack of TCP, nonpolar mutations within *tcpA* need to be constructed and assessed. Such a nonpolar deletion in classical-biotype strain O395 shows loss of colonizing ability (3). Little is known about the role of TCP in colonization by O139 serogroup strains. No *tcpA* mutants have been constructed and assessed for colonization properties. It is known, however, that expression of *tcpA* by O139 strains requires ToxR and is maximized by the conditions used for El Tor strains (37). When *toxR* is disrupted in O139 strain MO3 or MO10, expression of *tcpA* is eliminated and colonization is greatly diminished, thus implying a role for TCP in colonization by these strains (37).

One feature which distinguishes the El Tor from the classical biotype is the expression of a cell-associated mannose-sensitive hemagglutinin (MSHA) (7). This hemagglutinin has been associated with the expression of a pilus (18) and is proposed to be a colonization factor for El Tor strains (18, 25). The gene encoding the structural subunit of MSHA was recently sequenced and found to encode a type 4 pilin (20). The gene is located near the distal end of a large operon involved in MSHA secretion and assembly (11, 23). Although disruption of the *mshA* gene has been shown to result in loss of all MSHA activity (20, 23), the effect of such a deletion on colonization by El Tor strains has yet to be determined. Furthermore, no information regarding O139 *mshA* mutant strains is available, and it is not known whether the capsule elaborated by O139 strains has the potential to influence colonization properties attributable to TCP or MSHA.

The purpose of the present study was to more decisively determine the relative roles of both TCP and MSHA in colonization by O1 El Tor biotype strain C6706 and O139 strains MO3 and CVD112 (Table 1). This was accomplished by constructing in-frame chromosomal deletions of *tcpA* and *mshA* in the above-described backgrounds and then assessing the ability of the mutants to colonize using the infant mouse cholera model. CVD112 is an O139 vaccine candidate strain suitable for future analysis of the *tcpA* and *mshA* deletions in humans.

To construct in-frame deletions within the *mshA* and *tcpA* genes, primer pairs were designed to amplify portions of the chromosome by PCR immediately upstream and downstream of the region to be deleted. Restriction enzyme sites (underlined in the sequences below) were included near the 5′ end of each primer so that the resulting products could be digested and directionally ligated together into the suicide allelic ex-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
V. cholerae		
C6706	O1 El Tor isolate from Peru	CDC[a]
C6706str2	C6706, spontaneous Sm^r	This study
KSK262	C6706 *str2* Δ*lacZ3*	K. Skorupski
KHT46	C6706str2 Δ*mshA1*	This study
KHT52	C6706str2 Δ*tcpA10*	This study
MO3	O139 isolate from Madras, India	37
KHT75	MO3 Δ*lacZ3*	This study
KHT73	MO3 Δ*mshA1*	This study
KHT74	MO3 Δ*tcpA10*	This study
AI1837	O139 isolate from Dacca, Bangladesh	24
CVD112	AI1837 Δ*ace* Δ*zot* Δ*cep* Δ*ctxAB* *hlyA*::*ctxB*$^+$ *mer*$^+$	32
KHT76	CVD112 Δ*lacZ3*	This study
KHT37	CVD112 Δ*mshA1*	This study
KHT47	CVD112 Δ*tcpA10*	This study
E. coli		
SM10λpir	*thi thr leu tonA lacY supE recA* (RP4-2-Tc::Mu) λpirR6K Km^r π$^+$	33
KHT4	SM10λpir(pHT1)	This study
KHT42	SM10λpir(pHT3)	This study
KHT68	SM10λpir(pHT5)	This study
Plasmids		
pCVD442	*oriR6K mobRP4 sacB* Ap^r	6
pKAS48	*oriR6K mobRP4 rpsL* Δ*lacZ3* Ap^r Km^r	29
pHT1	pCVD442 Δ*mshA1*	This study
pHT3	pCVD442 Δ*tcpA10*	This study
pHT5	pCVD442 Δ*lacZ3*	This study

[a] CDC, Centers for Disease Control and Prevention.

change plasmid pCVD442 (6) for recombination into the chromosome. Sources for sequence design were GenBank entries X74730 (24a) for the *tcp* region and X77217 (20) for the *msh* region. In-frame deletion Δ*mshA1* was constructed by using the primer pair msh1 (5′-AAAAGTCGACAGCGAAAGCG AATAGTGG-3′) and msh2 (5′-AAAAGGATCCATTGCAC CAGCAACTGCACC-3′) to amplify the region upstream of the deletion and the primer pair msh3 (5′-AAAAGGATCCT GCAACGGTTGCTATGC-3′) and msh4 (5′-AAAAGCATG CGTGGTTACCACCGCAAAGG-3′) to amplify the region downstream of the deletion. Codons for residues G-48 through N-149 of the 169-residue mature pilin are deleted in this construct. The Δ*tcpA10* mutation was designed to be analogous to the nonpolar *tcpA* deletion previously constructed in the classical-biotype O395 derivative strain BGD4 (2, 4). The primer pair tcp5 (5′-AAAAGTCGACATAGTGATAAGAGTCTTA CCC-3′) and tcp2 (5′-AAAAAGATCTGCACCTTCTTTCA CGTTGAT-3′) were used to amplify the region upstream of the deletion and the primer pair tcp3 (5′-AAAAAGATCTAAC TAATATCACGCATGTTG-3′) and tcp6 (5′-AAAAGCATGC CAGAGTTCTATCTTTCGTC-3′) were used to amplify the region downstream of the deletion. In this construct, codons for residues F-141 through N-175 of the 199-residue mature pilin are deleted. The resulting plasmids, pHT1 carrying Δ*mshA1* and pHT3 carrying Δ*tcpA10*, were transformed into *Escherichia coli* donor strain SM10λpir and mated into *V. cholerae* recipients as described previously (21). For recipient strain C6706str2, exconjugants were selected on agar containing ampicillin (100 μg/ml) and streptomycin (100 μg/ml). MO3 or CVD112 exconjugants were selected on ampicillin and trimethoprim (50 μg/ml). Subsequent loss of the integrated vector to enrich for allelic exchange was performed by a modification of the procedure previously described (6). Purified colonies were incubated on Luria-Bertani (LB) agar lacking NaCl and containing 6 to 10% sucrose for 24 to 30 h at 30 to 37°C and then for 1 to 2 additional days at room temperature. Resulting colonies were then purified on LB agar. The successful incorporation of Δ*tcpA10* and Δ*mshA1* into the chromosomes of the various Ap^s strains was assessed by using colony PCR (21) with primers flanking the site of the deletion. The results of such an analysis for the El Tor C6706str2 constructions are shown in Fig. 1. Amplification of the *mshA* region produced an 857-bp product from the Δ*lacZ3* parental strain KSK262 (see below) and a 551-bp product from the Δ*mshA1* mutant strain KHT46 (Fig. 1, lanes A and B). Amplification of the *tcpA* region produced a product of 1,747 bp from strain KSK262 and a 1,645-bp product from the Δ*tcpA10* mutant strain KHT52 (Fig. 1, lanes C and D). The same results were obtained for the corresponding derivatives of strains MO3 and CVD112 (data not shown).

In order to easily distinguish between colonies of the parental strains and those of the deletion mutants in the colonization assays, the wild-type *lacZ* gene in each parental strain was replaced with the Δ*lacZ3* mutation. The resulting color difference on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates allowed the input and output ratios for the infant mouse colonization studies to be easily enumerated and permitted small numbers of blue mutant colonies to be detected among large numbers of parental white colonies. The *lacZ3* deletion was introduced into the C6706str2 parent strain by using the pKAS48 *rpsL* allelic exchange plasmid as previously described (29). C6706str2 exconjugants were isolated on LB agar containing polymyxin B (50 IU/ml) and kanamycin (45 μg/ml); this was followed by selection for loss of the integrated plasmid on LB agar containing 2.5 mg of streptomycin per ml and X-Gal. To construct the *lacZ3* MO3 and CVD112 deletion strains, a 1.5-kb restriction fragment carrying the *lacZ3* deletion flanked by an *Xba*I and a filled-in *Eco*RI site was inserted into pCVD442 at the *Xba*I and *Sma*I sites. The deletion was

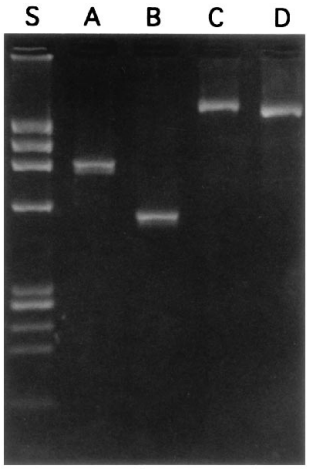

FIG. 1. Colony PCR analysis of the *mshA1* and *tcpA10* chromosomal deletions in El Tor C6706str2. Parental strain KSK262 DNA (lane A) and Δ*mshA1* strain KHT46 DNA (lane B) were amplified with the primer pair msh1 and msh4. Parental strain KSK262 DNA (lane C) and Δ*tcpA10* strain KHT52 DNA (lane D) were amplified with the primer pair tcp5 and tcp6. Lane S, ϕX174 replicative-form DNA *Hae*III standard. Sizes (in base pairs): 1,353, 1,078, 872, 603, 310, 281/271, 234, 194, and 118.

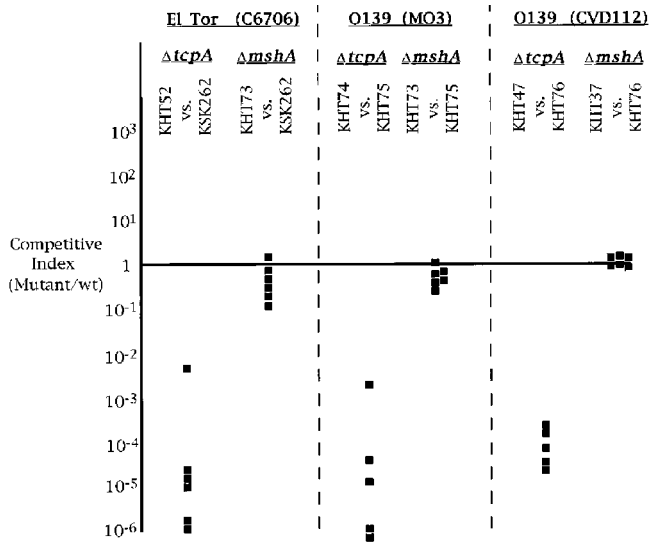

FIG. 2. Competitive indices of Δ*tcpA10* and Δ*mshA1* mutant derivatives compared with their isogenic Δ*lacZ3* parental strains, plotted for individual infant mice. The CVD112 strains colonized approximately 10- to 100-fold less than the other derivatives. In this case no blue colonies were detected for the KHT47-versus-KHT76 competition, so the datum points for this pair indicate the limit of detection, with the index actually being less than the values indicated. wt, wild type.

incorporated into the chromosome by the sucrose procedure as described above except that X-Gal was included in the plates.

The parental and mutant strains were subsequently tested for their expected phenotypes. The hemagglutination titer obtained with mouse CD-1 erythrocytes (8) was 64 to 128 in two independent experiments for each of the Δ*lacZ3* parental strains and less than 2 for the Δ*mshA1* mutants. TcpA pilin was detected by Western immunoblot using TcpA specific antibodies (31). The Δ*tcpA10* mutants did not produce the 20.5-kDa full-length pilin, whereas the Δ*lacZ3* and Δ*mshA1* strains produced TcpA pilin of the expected size (data not shown).

To assess the colonization properties of the *tcpA* and *mshA* deletion mutants, the O1 El Tor C6706 and the O139 MO3 and CVD112 mutant strains were paired with the corresponding parental Δ*lacZ3* strains for competition studies in the infant mouse cholera model (9, 34). For each pair, the relative viability was first compared by mixing the strains at equal ratios and performing in vitro competition by growth for 20 h in LB broth. In all cases, the in vitro competitive index was close to 1 (data not shown), indicating no general growth defect resulting from deleting the putative colonization factor genes or *lacZ*. Next, the ability of the *mshA* and *tcpA* deletion strains to compete in vivo against the *lacZ* deletion parental strains was assessed in infant mice. Strains grown overnight in LB broth were mixed equally and diluted 1:100, and 30 μl was fed orally to 4- to 7-day-old CD-1 mice (Charles River) obtained from mixed litters. After 20 h at 30°C the mice were euthanized, and dilutions of intestinal homogenates were plated on agar containing selective antibiotic and X-Gal. Five or more mice were used for each strain pair. The results for each mouse are plotted graphically in Fig. 2. Consistent with previous findings for *tcpA*::*kan*^{r} mutant El Tor strains (1, 26), the El Tor *tcpA* deletion mutant was deficient in colonization to a level of less than 10^{-5} in some mice. In contrast, the El Tor *mshA* deletion mutant showed no significant defect in its ability to colonize compared with the parental strain. This same trend was repeated in the MO3 and CVD112 O139 backgrounds. Incorporation of Δ*lacZ3* into the chromosome was shown to have a negligible effect on colonization, as determined by the competition assay between C6706str2 and the Δ*lacZ3* derivative KSK262, which yielded competitive indices in four mice ranging from 0.25 to 1.5 with a mean of 0.8. These results demonstrate that elaboration of TCP is required for efficient intestinal colonization by both El Tor and O139 strains, whereas the production of MSHA does not appear to significantly contribute to this process.

Since the Δ*tcpA10* mutants demonstrated a marked defect in colonization ability, it was important to rule out the possibility that a polar effect on *toxT* expression was a major factor contributing to this phenotype. Mutations that have a polar effect on *toxT* expression, such as *tcpA*::Tn*phoA* insertions, result in a 10-fold or greater decrease in expression of cholera toxin (2). To examine toxin production, the strains in this study were grown under AKI-SW conditions (15, 37) to induce toxin gene expression, and the concentration of toxin present in the spent culture supernatants was determined by using a ganglioside binding assay (10, 13). The toxin concentrations in the culture supernatant were 670 ng per unit of optical density at 600 nm (OD_{600}) for the El Tor Δ*lacZ3* strain KSK262 and 380 ng/OD_{600} unit for the Δ*tcpA10* mutant strain KHT52. In the case of O139, the toxin concentrations for the MO3 strain pair were 680 ng/OD_{600} unit for Δ*lacZ3* strain KHT75 and 750 ng/OD_{600} unit for Δ*tcpA10* mutant strain KHT74, and those for the CVD112 strain pair were 88 ng/OD_{600} unit for Δ*lacZ3* strain KHT76 and 114 ng/OD_{600} unit for Δ*tcpA10* mutant strain KHT47. In a second independent assay, the absolute levels of toxin varied somewhat from those in the first assay, but the ratios between the parental and *tcpA* mutant derivatives remained constant. Although the in-frame Δ*tcpA10* mutation resulted in a twofold decrease in cholera toxin levels in the El Tor background, perhaps due to reduced *toxT* expression, the effect would not be expected to be large enough to account for the 4- to 5-log colonization defect of the *tcpA* mutants. As no decrease in toxin level in the O139 background was observed, it seems likely that the colonization defect in these strains is also due to their lack of TCP production.

In summary, the potential contributions of TCP and MSHA in the colonization of *V. cholerae* O1 El Tor biotype and O139 Bengal strains has previously been either unclear or not known. Since these strains are the causative agents of the current pandemic, it is critical to determine the contributions of various potential colonization factors in these strains. The experiments described here using in-frame deletions in *tcpA* that were experimentally determined to have a minimal polar effect on downstream gene expression have provided unequivocal evidence for the role of TCP in colonization of both El Tor and O139 strains. The infant mouse cholera model used in this study has been a reliable indicator of the colonization requirements for humans (12). Thus, despite contradictory reports as to the surface expression of TCP in various strain backgrounds, TCP must be expressed as functional structures in vivo during infection with El Tor and O139 strains as previously determined for classical strains. In contrast to the role for TCP, no deleterious effect on colonization was observed when the *mshA* pilin subunit gene was deleted in either the El Tor or the O139 background. Thus, we are unable to establish a role for the cell-associated MSHA in colonizing the infant mouse. Classical-biotype strains do not express MSHA (7), and it is not clear what contributions this pilus may provide for the strains that do express it. One possibility is that it permits the bacterium to colonize other niches encountered in the environment and in this way might contribute to the persistence of El Tor and O139 strains in regions where they have largely displaced the classical biotype.

The extension of the colonizing role of TCP to the strains

included in this study, coupled with the results of previous experiments demonstrating the protective efficacy of antibodies directed against biotype-specific TCP, strongly suggests that TCP should be a component in cholera vaccine formulations. These would include live, attenuated vaccine strains, in which TCP has the added role of being required for efficient colonization; TCP could also be used as an antigen in killed whole-cell or component formulations. The construction of strains that constitutively express TCP and other ToxR-regulated surface components may prove valuable in such formulations (5).

Thus far TCP remains the major factor for *V. cholerae* colonization. However, the process of colonization is multifactorial, with contributions from a variety of different cell surface and secreted components (22). The precise molecular mechanisms by which TCP contributes to this process are still being elucidated, and a specific host receptor for TCP interaction has yet to be identified. It is likely that successful colonization of the intestine requires more than just a lectin-receptor interaction such as is classically provided by colonization pili or fimbriae. TCP contributes many properties to the bacterium, including those of autoagglutination and serum resistance (3), which may be as critical for colonization as direct bacterial attachment to host cell receptors. Continued genetic analysis of the multiple putative colonization factors of *V. cholerae* will provide a means to determine the contributions each makes to this process.

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