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ToxR Regulates Virulence Gene Expression in Non-O1 Strains of *Vibrio cholerae* That Cause Epidemic Cholera

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***Vibrio cholerae* serogroup O1 has historically been thought to be the exclusive cause of epidemic cholera. O139 is a novel serogroup of *V. cholerae* which emerged on the Indian subcontinent in the last few months of 1992 and is the first non-O1 serogroup of *V. cholerae* to cause epidemic cholera. We have investigated the expression of some of the known virulence factors of classical and El Tor O1 strains of *V. cholerae* in clinical isolates of O139 strains. We show that, in contrast to other non-O1 strains, O139 strains express TcpA, the major subunit of the toxin-coregulated pilus found in O1 strains. As in O1 strains, the expression of cholera toxin and TcpA is coordinately regulated by environmental parameters in O139 strains. Derivatives of O139 strains that contain a *toxR* null mutation were constructed and used to demonstrate that the expression of cholera toxin, TcpA, and the outer membrane protein OmpU in O139 strains, as in O1 strains, is dependent on ToxR. Two kinds of evidence suggest that O139 strains are closely related to El Tor strains of *V. cholerae*. First, both O139 and El Tor strains share a restriction fragment length polymorphism for *tcpA*, which distinguishes El Tor from classical strains of *V. cholerae*. Second, cholera toxin production in O139 strains is greatly enhanced by culture conditions that have been previously shown to promote production of cholera toxin in El Tor strains and not in classical strains of *V. cholerae*. Although O139 is a novel serotype of *V. cholerae*, O139 strains conform to a fundamental theme that has evolved from the study of O1 strains: ToxR mediates coordinate regulation of virulence gene expression.**

Vibrio cholerae serotype O1 is the cause of a severe secretory diarrhea and has historically been the sole cause of cholera epidemics. However, very recently investigators in India and Bangaladesh noted the beginning of a dramatic shift in the epidemiology of cholera. An epidemic of cholera, which emerged in the Tamalnadu state of India in October 1992 and in southern Bangaladesh in January 1993, was caused by a strain of *V. cholerae* with a novel non-O1 serotype (1, 20). This marked the beginning of the first cholera epidemic caused by a non-O1 serotype of *V. cholerae*. Previously, non-O1 strains of *V. cholerae* were known to cause sporadic cases of diarrhea and extraintestinal disease but not epidemics of cholera (18).

This new non-O1 strain of *V. cholerae* has been named *V. cholerae* O139 synomyn Bengal (22). Like O1 strains of *V. cholerae*, O139 strains can cause a massive secretory diarrhea (1, 20). In the 8 months since its emergence, this strain has been responsible for thousands of cholera cases throughout India and in many parts of Bangaladesh and has replaced O1 strains of *V. cholerae* as the predominant cause of cholera on the Indian subcontinent (1, 3, 10, 20–22). Apparently, prior immunity to O1 strains of *V. cholerae* does not protect against O139 strains, since the adult populations in areas where O1 strains of *V. cholerae* are endemic have been susceptible to the current O139 epidemic (3).

Previous cholera epidemics have been caused by O1 strains of either the El Tor or classical biotype of *V. cholerae* (2). Several of the virulence factors produced by these strains of *V. cholerae* have been shown to be coordinately regulated by the transcriptional activator ToxR (4, 17). These ToxR-regulated virulence factors include cholera toxin, an ADP-ribosylating toxin that leads to massive fluid secretion from the small intestine (5), and toxin-coregulated pili (TCP), an essential factor for colonization of the small intestine (7, 23, 24). TCP are composed of a 20.5-kDa subunit called TcpA (24). Cholera toxin production and TcpA expression are coordinately regulated by environmental signals, and this regulation requires ToxR (16). Although previously investigated non-O1 strains have *toxR* sequences (15), most non-O1 strains do not have the genes required for the production of cholera toxin (*ctx* genes) (6, 18), and none have *tcpA* (23) sequences.

The emergence of the first non-O1 cholera epidemic raises a number of questions. Do O139 strains have the same ToxR-regulated virulence factors as O1 strains? And if so, are these factors coordinately regulated by ToxR as they are in O1 strains? With regard to the origin of O139 strains, ascertaining the presence of particular virulence gene sequences will be helpful in determining whether O139 strains are most related to classical O1, El Tor O1, or non-O1 strains of *V. cholerae*. The investigators who reported the initial O139 outbreak demonstrated that these strains have cholera toxin genes (1, 20). In a recent report, we showed that the chromosomal location of the CTX genetic element and tandem duplications of this element in O139 strains suggested that these strains are related to El Tor strains (27). We also showed that O139 strains contain chromosomal *tcpA* sequences (27).

In this paper, we describe experiments that explore the environmental regulation of the expression of cholera toxin and TcpA in O139 strains of *V. cholerae* isolated from patients in India. We have constructed *toxR* insertion mutations in O139 strains to investigate the importance of ToxR in the expression of cholera toxin and TcpA. As in O1 strains, the expression of these genes was found to be ToxR dependent and coordinately regulated by environmental

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TABLE 1. *V. cholerae* strains used in this study

Strain	City or country of origin	Genotype	Biotype[a]/serogroup	Reference
O395	India	Wild type	Classical/O1	13
LAC-1		O395 Δ*lacZ1*	Classical/O1	This study
TCP2		O395 Δ*tcpA2*	Classical/O1	23
C6709	Lima, Peru	Wild type	El Tor/O1	26
E7946	Bahrain	Wild type	El Tor/O1	13
MO3	Madras, India	Wild type	O139	This study
MO3-55		MO3 *toxR*::pVM55	O139	This study
MO10	Madras, India	Wild type	O139	This study
MO10-55		MO10 *toxR*::pVM55	O139	This study
MO45	Madras, India	Wild type	O139	This study
MO2	Madras, India	Wild type	O139	This study
SG20	Calcutta, India	Wild type	O139	This study
SG34	Calcutta, India	Wild type	O56	This study
VO18	Vellore, India	Wild type	O139	This study
VO7	Vellore, India	Wild type	O37	This study

[a] Biotype is defined only for O1 *V. cholerae* strains.

signals. ToxR-regulated genes were also shown to play an essential role in colonization of the small intestine by O139 strains of *V. cholerae* in the suckling mouse model of cholera.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are described in Table 1. All of the O139 wild-type strains listed in Table 1 are clinical isolates from the current O139 cholera epidemic in India. All of the clinical isolates of O139 strains we tested, except for MO2, were found to exhibit resistance to trimethoprim, sulfamethoxazole, and streptomycin. These antibiotic resistance markers were taken advantage of in some of the analyses described below. The *lacZ* deletion derivative of O395, LAC-1, will be described in further detail in a separate report. Both O395 and LAC-1 are resistant to streptomycin. Bacterial strains were maintained at −70°C in LB broth containing 20% (vol/vol) glycerol. LB (14) and AKI (9) broth were prepared as previously described. The AKI-SW culture conditions that were developed to enhance cholera toxin production by El Tor strains of *V. cholerae* were used as described previously (9). With these culture conditions, bacteria are grown for 4 h in 10 ml of AKI broth in a stationary test tube at 37°C; after this initial period of growth in a low-oxygen environment, the broth culture is transferred to a well-aerated shaken flask for growth overnight at 37°C (9). A modification of the AKI-SW culture conditions was used to promote the autoagglutination of O139 strains. This modification involved the same initial 4-h growth period of bacteria in 10 ml of AKI broth in a stationary test tube (15 cm in height and 1.5 cm in diameter) at 37°C. After this, however, 8 ml of the culture was discarded, and the remaining 2 ml of the culture was then grown for 3 h on a rotator at 60 rpm at 37°C.

Nucleic acid preparation and analysis. *V. cholerae* DNA was purified and Southern blot analysis was carried out as previously described (13), except that the probes were conjugated with horseradish peroxidase and hybridization and development with a chemiluminescent substrate (luminol) was done according to the manufacturer's (Amersham) protocol. The probe for *tcpA* was the plasmid pRTG7H3, which contains the entire *tcpA* sequence (23). The plasmid pVM7, which contains the entire sequence of *toxR*, was used as the probe for *toxR* (16).

Biochemical analyses. *V. cholerae* strains were grown either in LB broth overnight at 30°C on a roller incubator (for maximal production of cholera toxin by classical *V. cholerae* strains) or in AKI broth at 37°C for 4 h in a stationary test tube and then overnight in a flask with shaking as described previously (9) (for maximal production of cholera toxin by El Tor and O139 strains of *V. cholerae*). Supernatants from these cultures were assayed for cholera toxin with a GM1 ganglioside-dependent enzyme-linked immunosorbent assay (ELISA) (8). The amount of cholera toxin produced is expressed as micrograms of toxin per optical density unit at 600 nm. Preparations of total cell protein from the same cultures were electrophoresed in 11% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) (11). After electrophoresis, either gels were stained with Coomassie brilliant blue or proteins were transferred by electrophoresis to nitrocellulose sheets (25). Immunoblotting of the nitrocellulose with a rabbit anti-TcpA antiserum was performed as described previously (19) except that in the second step, goat anti-rabbit antibody was conjugated to horseradish peroxidase (Bio-Rad). The horseradish peroxidase was then detected with a chemiluminescent substrate (luminol; Amersham).

Isolation of *toxR* insertion mutations in O139 *V. cholerae* strains. Plasmid pVM55 is a suicide plasmid that carries the R6K origin of replication and therefore requires the product of the *pir* gene to replicate (16). It also carries a 630-bp internal fragment of *toxR* and can be mobilized from the *Escherichia coli* SM10 λ*pir* into *V. cholerae*, where it cannot replicate since there is no *pir* gene in *V. cholerae* (16). Selection for ampicillin resistance, which this plasmid also confers, requires integration of the plasmid by homologous recombination via the *toxR* sequences. This results in an insertion mutation in *toxR* that provides a null phenotype. The mating of SM10 λ *pir* pVM55 with MO3 and MO10 was carried out overnight on LB agar plates with a donor-to-recipient ratio of approximately 1:10. After this, the cells were scraped off the LB agar plates and grown for 2 h in LB broth containing trimethoprim (160 μg/ml) and sulfamethoxazole (800 μg/ml) to counterselect the donor and ampicillin (50 μg/ml) to select against unmated recipients and then were plated on LB agar plates containing the same antibiotic concentrations for isolation of transconjugates. DNA from the transconjugates was analyzed by Southern hybridization

to confirm the correct integration of pVM55 into the *toxR* locus essentially as described previously (16).

Mouse colonization assay. A competition assay between a *lacZ* deletion mutant of O395, LAC-1, and different test strains (which were all *lacZ*$^+$) was done essentially as described previously (24). The LAC-1 strain has been shown to colonize the suckling mouse small intestine as well as O395 does (unpublished observations). It serves as a standard to facilitate comparison of the intestinal colonization properties of different test strains. Three- to five-day-old suckling mice (Charles River) were inoculated orally with *V. cholerae* strain mixtures and sacrificed 20 h later. There were six animals per group. An in vitro competition was done concomitantly with the same cell mixtures used to inoculate the suckling mice. In the in vitro competition, the cells were grown at 37°C in LB broth for 6 h (a time point when the cultures were near saturation). Viable cell counts were obtained by plating dilutions of the intestinal homogenates or of the broth cultures on LB agar plates containing streptomycin (10 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (40 μg/ml). The ratio of the numbers of the two strains was determined by scoring the ratio of blue (*lacZ*$^+$) colonies to white (Δ*lacZ*) colonies on X-Gal plates. When intestinal homogenates of uninfected suckling mice were plated on the same selection plates, no colonies were recovered.

RESULTS

Demonstration of *tcpA* restriction fragment length polymorphism. We have previously shown by Southern analysis that O139 strains have *tcpA* sequences (27). There is a *Pst*I restriction fragment length polymorphism in *tcpA* that can be used to differentiate classical from El Tor strains of *V. cholerae* O1 (23). As shown in Fig. 1, all of the O139 strains tested had chromosomal sequences that hybridized to the *tcpA* probe on 2.8- and 2.1-kilobase-pair (kb) *Pst*I restriction fragments. The sizes of these fragments were identical to those seen in the two El Tor strains E7946 and C6709 (Fig. 1, top). This is in contrast to the 2.6- and 1.4-kb fragments which were seen in the classical strain O395 (Fig. 1). The two non-O1 strains SG34 (O56) and VO7 (O37), like non-O1 strains previously studied (23), did not have sequences which hybridized to the *tcpA* probe.

Environmental regulation of virulence gene expression. Initial studies showed that O139 strains of *V. cholerae* made very little cholera toxin when grown under conditions optimal for cholera toxin production by classical strains (i.e., in LB broth at 30°C). Since our Southern analyses of *tcpA* (Fig. 1) and *ctx* (27) had revealed that O139 strains were very similar to El Tor O1 strains, we investigated the use of AKI-SW culture conditions, which have been shown to optimize cholera toxin production by El Tor strains and not by classical strains (9).

When grown at 37°C in AKI broth with a switch from a stationary test tube to a shaken flask (AKI-SW culture conditions), the two O139 strains MO10 and MO3 each showed an approximately 100-fold increase in cholera toxin production compared with the amounts of cholera toxin these strains produced when they were grown in LB broth at 30°C (Fig. 2, bottom). This relative increase in cholera toxin production was similar to that measured for the El Tor strain C6709. In contrast, the classical strains O395 and TCP2 made considerably less cholera toxin when grown in AKI-SW culture conditions than when grown in LB broth at 30°C (Fig. 2). The non-O1 strain VO7 lacks the CTX genetic element (27), and therefore no cholera toxin production was measured after this strain was grown in either culture condition.

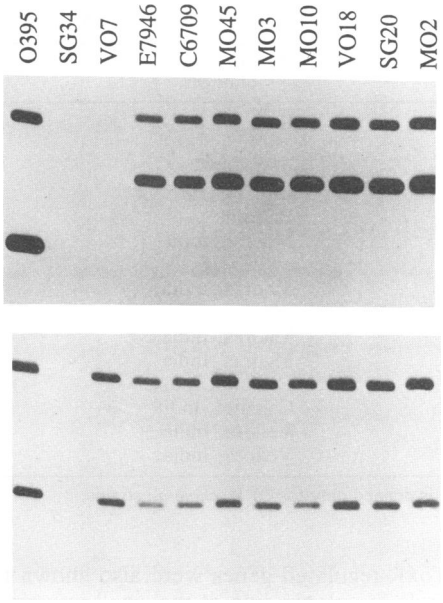

FIG. 1. Southern blot analysis of *tcpA* and *toxR* loci in classical and El Tor O1 and non-O1 strains of *V. cholerae*. The classical O1 strain O395, the El Tor O1 strains E7946 and C6709, the O139 strains MO45, MO3, MO10, VO18, SG20, and MO2, and the non-O1 strains SG34 and VO7 were used in this analysis. Chromosomal DNAs from the indicated strains were digested with *Pst*I, electrophoresed, transferred to nitrocellulose, and probed with a *tcpA* (top) or *toxR* (bottom) probe.

The same culture conditions which enhanced cholera toxin production by the El Tor and O139 strains also increased dramatically the production of another ToxR-regulated gene product, TcpA, in the O139 *V. cholerae* strains. Western blot (immunoblot) analyses showed that MO3 and MO10 as well as C6709 made the 20.5-kDa protein TcpA only when they were grown in AKI-SW culture conditions and not in LB broth at 30°C (Fig. 2). With O395, however, TcpA expression, like cholera toxin production, was enhanced in LB broth compared with AKI broth. TCP2 carries a deletion of *tcpA* and serves as a negative control for TcpA expression in the Western blot analyses. In Fig. 2, TcpA is apparent on the Coomassie blue-stained SDS-PAGE gels only when strain O395 was grown in LB medium at 30°C. TcpA was also apparent on Coomassie blue-stained SDS-PAGE gels of whole-cell lysates of cultures of O139 strains after they were grown with the modified AKI-SW culture conditions described in Materials and Methods (data not shown).

Not all ToxR-regulated gene products respond in the same way to changes in culture conditions. Production of OmpT and OmpU, which are ToxR-regulated major outer membrane proteins of *V. cholerae*, like production of cholera toxin and TcpA, is regulated by the osmolarity and amino acid content of the culture medium (16). However, unlike production of cholera toxin and TcpA, OmpT and OmpU production is not regulated by the pH or temperature of the culture medium (16). When Miller and Mekalanos (16) studied the effects of osmolarity on the expression of ToxR-regulated gene products, they found that OmpT production

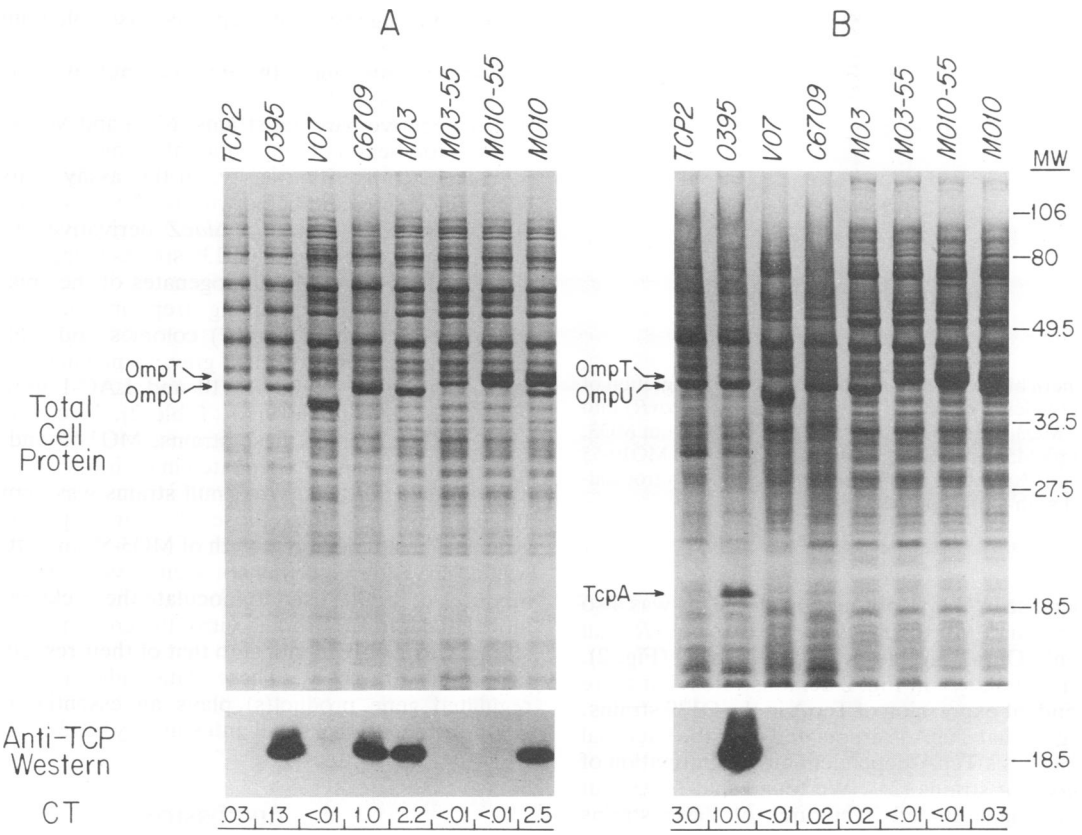

FIG. 2. Effect of culture conditions on the expression of ToxR-regulated gene products in classical and El Tor O1 and O139 strains of *V. cholerae*. The classical O1 strains TCP2 and O395, the non-O1 strain VO7, the El Tor O1 strain C6709, and the O139 strains MO3 and MO10 and their *toxR* null mutant derivatives MO3-55 and MO10-55 were used in these analyses. The ToxR-regulated gene products TcpA and cholera toxin were measured by immunoblot and ELISA, respectively. *V. cholerae* strains were grown either in the AKI-SW culture conditions at 37°C (A) or in LB broth at 30°C (B). Whole-cell lysates from each culture were prepared in sample buffer and analyzed by SDS-PAGE. Then, either the gels were stained with Coomassie blue (top) or the proteins were transferred to nitrocellulose and probed with an anti-TcpA antiserum (bottom). The amount of cholera toxin produced in each culture is shown below its corresponding lane and is expressed as micrograms per optical density unit at 600 nm. Molecular weights (MW) are in the thousands.

changed inversely to changes in the production of OmpU, cholera toxin, and TcpA. None of the O1 or O139 strains shown in Fig. 2 showed a detectable shift in the ratio of OmpT to OmpU production between the two culture conditions used here. Thus, after O395, C6709, and MO3 were grown in either AKI medium at 37°C or LB medium at 30°C, only OmpU is apparent. MO10 made both OmpT and OmpU under both culture conditions (Fig. 2). Since temperature and pH are the most salient differences between these two culture conditions, the lack of a shift from OmpU to OmpT production when the bacteria were grown in either AKI-SW culture conditions or LB broth was not unexpected. The SDS-PAGE analyses of total cellular proteins from MO3 and MO10 also showed that the proteins produced by these O139 strains were very similar to the proteins produced by the classical and El Tor O1 strains tested (Fig. 2, top). Consistent with its lack of relatedness to O139 and O1 strains, the non-O1 strain VO7 did exhibit a pattern of cellular proteins different from those of the other strains shown (Fig. 2).

Construction of *toxR* insertion mutations. In classical and El Tor O1 strains of *V. cholerae*, expression of cholera toxin and TcpA is regulated by ToxR (16, 17). To test whether the expression of these virulence factors was also ToxR dependent in O139 strains, *toxR* null derivatives of O139 strains were constructed. Initial Southern blot analysis showed that a *toxR* probe hybridized to the same-sized *Pst*I restriction fragments in O139 strains as in the classical and El Tor O1 strains tested (Fig. 1, bottom). This suggested that the *toxR* locus in O1 and O139 strains is similar. One of the two non-O1 strains tested, SG34, did not have sequences that hybridized to the *toxR* probe (Fig. 1). SG34 is unusual in this respect, since the non-O1 strains previously tested all have had *toxR*-homologous sequences on Southern blots (15).

Derivatives of two O139 strains, MO3 and MO10, containing insertion mutations in *toxR* were constructed by using pVM55, a nonreplicating plasmid which carries an internal fragment of *toxR* (16). The Southern blot analysis presented in Fig. 3 shows that the two *toxR* insertion mutation strains MO3-55 and MO10-55 have the restriction fragments expected after the insertion of pVM55 (16).

***toxR* regulation of virulence gene expression.** We then tested the *toxR* insertion mutation strains MO3-55 and MO10-55 for the production of the ToxR-regulated gene products cholera toxin, TcpA, OmpU, and OmpT. MO3-55 and MO10-55 made no detectable cholera toxin or TcpA regardless of the culture conditions (Fig. 2). In *toxR* null insertion mutant derivatives of classical O1 strains of *V. cholerae*, there is a characteristic decrease in OmpU pro-

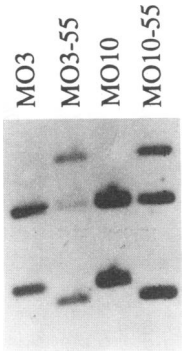

FIG. 3. Southern blot analysis of the chromosomal integration of plasmid pVM55 (which contains an internal fragment of *toxR*) into the O139 strains MO3 and MO10. Chromosomal DNAs from MO3, MO10, and their pVM55 integrant derivatives MO3-55 and MO10-55 were digested with *Pst*I, electrophoresed, transferred to nitrocellulose, and probed with a *toxR* probe.

duction and increase in OmpT production (16). This was also seen in MO3-55 and MO10-55. Both of these *toxR* null strains made only OmpT and no detectable OmpU (Fig. 2).

The Western blot shown in Fig. 2 demonstrates that there is a ToxR-dependent expression of TcpA in the O139 strains. Other data suggest that TcpA is associated with the bacterial cell surface. There is a TcpA-dependent autoagglutination of classical *V. cholerae* strains (24). We found that MO3, but not MO3-55, autoagglutinated if cultures of these strains were grown by a minor modification of the AKI-SW method (described in Materials and Methods) and then allowed to stand at room temperature for about 1 h. Also, MO3 but not MO3-55 was agglutinated in a slide agglutination assay with an anti-TcpA antiserum after the bacteria were grown in the modified AKI-SW culture conditions. Both the autoagglutination and antibody-mediated agglutination of MO3 suggest that TcpA is in fact expressed on the cell surface. Preliminary electron microscopic studies of modified AKI-SW cultures of strains MO3 and MO3-55 have shown the presence of masses of pili in MO3 cultures that were not observed in MO3-55 cultures (data not shown). Together, these data suggest that TcpA is assembled into a pilus structure when expressed optimally by MO3.

In vivo attenuation of the *toxR* null mutants. The *toxR* null mutant strains MO3-55 and MO10-55 were compared with their respective parental strains, MO3 and MO10, in regard to their abilities to colonize the infant mouse small intestine by using a competition assay. In this assay 3- to 5-day-old CD-1 mice were coinfected with two strains of *V. cholerae*. One strain (LAC-1) was a Δ*lacZ* derivative of O395; the other strain was one of the O139 strains being studied. After 20 h of in vivo growth, homogenates of the small intestine were plated on agar containing streptomycin and X-Gal, and the numbers of blue (*lacZ*$^+$) colonies and white (Δ*lacZ*) colonies were counted. MO3 grew somewhat better in vivo than did LAC-1, while MO10 and LAC-1 grew approximately equivalently in vivo (Table 2). In comparison, the *toxR* null mutants of these strains, MO3-55 and MO10-55, were both profoundly attenuated in their in vivo growth. The in vivo yields of these *toxR* null strains was approximately 10,000 times less than those of their respective parental strains. The attenuated growth of MO3-55 and MO10-55 that was observed in vivo was not seen in vitro. When the same mixtures of strains used to inoculate the suckling mice were inoculated into LB broth in vitro, the growth of the *toxR* null strains was nearly identical to that of their respective parental strains (Table 2). These data indicate that a ToxR-regulated gene product(s) plays an essential role in the colonization of the small intestine by O139 strains of *V. cholerae*.

DISCUSSION

O139 is the first non-O1 serotype of *V. cholerae* to cause an epidemic of cholera. Since the emergence of O139 strains in the last few months of 1992, they have spread throughout the Indian subcontinent and have caused thousands of cases of cholera (1, 3, 10, 20–22). The emergence of this novel non-O1 serotype, O139, raises immediate questions about the origin of this strain and the pathogenesis of cholera caused by O139 strains. The data presented in this report begin to address these questions.

We have presented two kinds of evidence to suggest that O139 *V. cholerae* is derived from an El Tor strain of O1 *V.*

TABLE 2. Intestinal colonization assay of O139 strains and their *toxR* null derivatives

Competing strains	Inoculum (10^5)	Inoculum ratio[a]	In vivo competition		In vitro competition	
			CFU recovered[b]	Recovery ratio[c]	CFU recovered	Recovery ratio
MO3	1.5	1.0	1.4×10^7	11.6	5.0×10^9	12.5
LAC-1	1.5		1.2×10^6		4.0×10^8	
MO3-55	1.5	1.0	1.7×10^3	0.00036	4.5×10^9	11.2
LAC-1	1.5		4.7×10^6		4.0×10^8	
MO10	0.7	0.47	5.7×10^6	1.2	3.2×10^9	10.7
LAC-1	1.5		4.6×10^6		3.0×10^8	
MO10-55	0.9	0.6	1.2×10^2	0.000054	3.4×10^9	8.5
LAC-1	1.5		2.2×10^6		4.0×10^8	

[a] Ratio of the number of CFU of the test strain (*lacZ*$^+$) to the number of CFU of LAC-1 cells (Δ*lacZ*) in the inoculum mixtures.

[b] Mean number of CFU from each group. There were six mice per group. The numbers of CFU of MO3 and MO10 were significantly different from those of their respective *toxR* null mutant derivatives MO3-55 and MO10-55 ($P < 0.02$ for both by the Wilcoxon rank sum test).

[c] Ratio of the number of CFU of the test strain to the number of CFU of LAC-1 cells in the intestinal homogenates from the in vivo competitions or in the LB broth from the in vitro competitions.

cholerae. First, the Southern blot analysis presented here showed that *tcpA* is present on the same-sized *Pst*I restriction fragments in O139 strains as in El Tor strains and that these fragments differ in size from those seen in classical *V. cholerae* strains. Our previous Southern analyses also showed that the chromosomal organization of another virulence locus, the CTX genetic element, in O139 strains also resembled that of El Tor and not classical *V. cholerae* (27). Second, the in vitro environmental conditions that enhance cholera toxin and TcpA production by O139 strains are different from those that optimize cholera toxin production by classical strains and are in fact the conditions that have been previously shown to enhance cholera toxin production by El Tor strains. Thus, we saw an approximately 100-fold increase in cholera toxin production when O139 strains were grown at 37°C in AKI-SW culture conditions compared with when they were grown in LB broth at 30°C.

Theoretically, the O139 strain might have been derived from a non-O1 strain which acquired virulence genes like *tcpA* and *ctx* through genetic recombination. However, the similarity between O139 and El Tor O1 strains in the chromosomal organizations of the *ctx* and *tcpA* loci and in the environmental stimuli which lead to the expression of these genes suggests that O139 strains are derived from an El Tor O1 strain which has acquired a variant O antigen. Since antibodies to the O antigen of lipopolysaccharide constitute a significant portion of the human vibriocidal immune response to *V. cholerae* (12), we can speculate that in areas where O1 strains are endemic (as on the Indian subcontinent) there is selective pressure for alterations in O antigen structures. There is a precedent for serotype variation in the current epidemic of cholera in Latin America. There, the Ogawa serotype strains have become more prevalent than the Inaba serotype strains as the epidemic has persisted (26).

The data presented here are the first demonstration of TcpA in a non-O1 strain of *V. cholerae*. Southern analysis showed the presence of chromosomal sequences in O139 strains that hybridized to a *tcpA* probe. Western blotting confirmed the production of TcpA. The construction of *toxR* null mutant O139 strains allowed the demonstration that TcpA production in O139 strains, as in O1 strains, is ToxR dependent. The autoagglutination and anti-TcpA antibody-mediated agglutination of MO3 strongly suggest that the TcpA is associated with the bacterial cell surface. Immunogold electron microscopic studies will be useful to confirm the production of TCP by O139 strains.

The finding that the first non-O1 strain of *V. cholerae* to be associated with epidemic cholera (O139) expresses TcpA lends further support to the idea that TCP are an essential virulence factor. Previous studies clearly demonstrated that *tcpA* null mutant strains of classical *V. cholerae* are severely attenuated in vivo in both mice (24) and humans (7). The *toxR* null mutant strains MO3-55 and MO10-55 do not make TcpA and are also severely attenuated in the suckling mouse model of cholera. To date, among the known ToxR-activated genes, only null mutations of *tcpA* have resulted in the degree of in vivo attenuation that was seen for MO3-55 and MO10-55. Therefore, the attenuation seen in these *toxR* null mutant strains is probably attributable to the reduced expression of TCP seen in these strains. However, since the *toxR* null mutation is pleiotropic, we cannot rule out the possibility that some other ToxR-regulated gene product besides TCP is responsible for the observed colonization defect.

Our studies have shown that although O139 is a novel strain of *V. cholerae*, the regulation of its virulence factors conforms to the themes that have previously been described for O1 strains of *V. cholerae*. There are environmental signals that lead to the coordinate expression of several of the ToxR-regulated genes in O1 strains. This was also the case for O139 strains of *V. cholerae*. Thus, in the AKI-SW culture conditions, production of both cholera toxin and TcpA by O139 strains increased significantly compared with when these strains were grown in LB broth. In O1 strains, ToxR coordinately regulates the expression of several virulence factors. This was also the case for O139 strains. The *toxR* null mutant O139 strains MO3-55 and MO10-55 demonstrated that as in O1 strains of *V. cholerae*, the production of cholera toxin, TcpA, and OmpU are all ToxR dependent. Also, in vivo experiments showed that colonization of the infant mouse small intestine by O139 strains was ToxR dependent.

Although O139 strains of *V. cholerae* share with O1 strains ToxR-regulated virulence factors such as TcpA and cholera toxin, there are important differences between O1 and O139 strains which remain to elucidated. For example, the structure of and the genes coding for the O139 O antigen need to be investigated. Also, the factors which have led to the displacement of the endemic O1 El Tor strains of *V. cholerae* on the Indian subcontinent by O139 strains are not yet known. The rapid spread of O139 strains throughout the Indian subcontinent suggests that O139 strains will give rise to the next pandemic of cholera. Regardless of the differences that remain to be discovered between O1 and O139 strains of *V. cholerae*, the similar chromosomal organizations of the CTX genetic element in O1 and O139 strains have allowed us to construct O139 strains with deletions of this element (27). These O139 deletion mutants will be tested as potential live vaccines to protect against cholera caused by O139 strains of *V. cholerae*.

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ADDENDUM IN PROOF

Recently, Hall and colleagues reported the similarity of *tcpA* in O139 strains and in El Tor O1 strains, as we have shown in this report. This group used PCR with *tcpA* primers to detect an amplified fragment in an O139 strain that was identical in size to the El Tor O1 *tcpA* amplified product (R. C. Hell et al., Lancet **342:**430, 1993).

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