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Characterization of *Vibrio cholerae* O1 El Tor Biotype Variant Clinical Isolates from Bangladesh and Haiti, Including a Molecular Genetic Analysis of Virulence Genes▽

Mike S. Son,[1] Christina J. Megli,[1] Gabriela Kovacikova,[1] Firdausi Qadri,[2] and Ronald K. Taylor[1]*

Department of Microbiology and Immunology, Dartmouth Medical School, Hanover, New Hampshire 03755,[1] *and International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Centre for Health and Population Research, Mohakhali, Dhaka, Bangladesh*[2]

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Vibrio cholerae **serogroup O1, the causative agent of the diarrheal disease cholera, is divided into two biotypes: classical and El Tor. Both biotypes produce the major virulence factors toxin-coregulated pilus (TCP) and cholera toxin (CT). Although possessing genotypic and phenotypic differences, El Tor biotype strains displaying classical biotype traits have been reported and subsequently were dubbed El Tor variants. Of particular interest are reports of El Tor variants that produce various levels of CT, including levels typical of classical biotype strains. Here, we report the characterization of 10 clinical isolates from the International Centre for Diarrhoeal Disease Research, Bangladesh, and a representative strain from the 2010 Haiti cholera outbreak. We observed that all 11 strains produced increased CT (2- to 10-fold) compared to that of wild-type El Tor strains under** ***in vitro*** **inducing conditions, but they possessed various TcpA and ToxT expression profiles. Particularly, El Tor variant MQ1795, which produced the highest level of CT and very high levels of TcpA and ToxT, demonstrated hypervirulence compared to the virulence of El Tor wild-type strains in the infant mouse cholera model. Additional genotypic and phenotypic tests were conducted to characterize the variants, including an assessment of biotype-distinguishing characteristics. Notably, the sequencing of** ***ctxB*** **in some El Tor variants revealed two copies of classical** ***ctxB*****, one per chromosome, contrary to previous reports that located** ***ctxAB*** **only on the large chromosome of El Tor biotype strains.**

Vibrio cholerae is a Gram-negative, curved-rod-shaped bacterium that is the causative agent of the watery diarrheal disease cholera. The structure of the cell surface lipopolysaccharide O antigen is used to classify *V. cholerae* into more than 200 serogroups, of which only two, O1 and O139, possess the potential to cause epidemic or pandemic cholera. The O1 serogroup is further divided into two biotypes, classical and El Tor, which evolved from independent lineages (20, 22), and they display genotypic and phenotypic differences.

V. cholerae O1 is distinguished by two of its major virulence factors, cholera toxin (CT) and the toxin-coregulated pilus (TCP). The cholera toxin is encoded by *ctxA* and *ctxB*, which are found on the CTXϕ prophage (49), and is responsible for the manifestation of diarrheal disease with severe water and electrolyte loss. The TCP, encoded by the *tcp* operon in the *Vibrio* pathogenicity island (VPI), is required for *V. cholerae* colonization of the small intestinal epithelium (17, 21, 24, 47). Although these essential virulence factors are regulated primarily by ToxT via the ToxR virulence regulon (in conjunction with AphA and AphB) (26, 27) in both classical and El Tor biotypes, the genes are differentially expressed between the biotypes, particularly under *in vitro* inducing conditions.

Since 1817, the world has experienced seven cholera pandemics, the first six of which were caused by the classical O1 biotype. The current (seventh) pandemic, which began in 1961, is the result of the El Tor O1 biotype, which has essentially displaced the classical biotype globally since 1993 (5, 32, 42). Although the El Tor biotype was first isolated in 1937 and its pandemic potential debuted 24 years later in 1961, both biotypes coexisted until 1992 (32). During this time, the El Tor biotype was responsible for most outbreaks; however, the classical biotype still was responsible for isolated incidents until 1992. These incidents included a large outbreak in west Pakistan in 1968 and the appearance of the classical biotype in Bangladesh in 1979, with a continuous presence until the end of 1992 (5). However, since 2001, a series of reports have been published revealing clinical isolates, from as far back as the early 1990s, that are of El Tor biotype background but possess some classical biotype traits (3, 4, 28, 34, 35, 36, 43). For example, the *ctxA* gene, which is the first gene of the *ctx* phage operon and codes for the A subunit of the cholera toxin, is completely conserved in sequence between the two O1 biotypes. However, *ctxB*, which is the second gene in the operon and codes for the B subunit of the cholera toxin, is identical in sequence between the biotypes except for two bases, a key difference used to distinguish the biotypes. The bases at positions 115 and 203 are cytosines in the classical O1 biotype, whereas in El Tor O1 biotypes they both are thymines. These base transitions, which result in amino acid conversions of $H_{39}Y$ and $T_{68}I$, respectively, are completely conserved within each biotype, allowing them to be used as reliable markers for biotype characterization. These clinical El Tor O1 strains were

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Dartmouth Medical School, Vail Building HB7550, Hanover, NH 03755. Phone: (603) 650-1632. Fax: (603) 650-1318. E-mail: Ronald.K.Taylor@dartmouth.edu.

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found to contain the classical biotype cholera toxin B subunit gene (*ctxB*) and have since been termed El Tor variants (34, 39). These El Tor variants have become a major focus of research, since other El Tor variants also have been identified in Asia and Mozambique (29, 34, 35, 38) and recently have been a focus of attention in Haiti (1, 6, 7, 8, 9). Of particular concern are the recently published results indicating that some of the clinically isolated El Tor variants produce different levels of cholera toxin, including some isolates that produce higher levels than classical biotype strains (16).

Although evidence has been presented demonstrating that the El Tor biotype has globally displaced the classical biotype (19, 42), the emergence of El Tor variants has warranted the use of standard genotypic and phenotypic assays to determine the biotype background of new isolates. Standard phenotypic assays, including polymyxin B resistance, hemolysis assays, phage sensitivities, and the Voges-Proskauer tests, are frequently employed in some combination (4, 11, 45). Genetic markers, such as the *ctxB* gene, have been used to distinguish the cholera toxin between classical and El Tor biotypes (11, 33, 35) to classify isolates as El Tor variants. The sequences of *tcpA*, which codes for the pilin subunits of the TcpA apparatus, and *rstR*, the repressor found in the RS1 element, which codes for the regulatory region for phage replication, also have been used in determining the biotypes of new isolates (4, 11, 39, 45).

El Tor variants were first identified from sporadic strains of *V. cholerae* O1 isolated from Matlab Hospital in Bangladesh (37), and their importance was accentuated with similar El Tor variants identified from a 2004 epidemic in Mozambique (3). Here, we present the characterization of 10 Matlab variant clinical strains (Bgd1, Bgd2, Bgd3, Bgd4, Bgd5, Bgd6, Bgd7, Bgd8, MQ1795, and MQ04) isolated from patients at Matlab Hospital and the recently isolated El Tor variant (BAA-2163), which was identified as the causative agent of the 2010 cholera outbreak in Haiti. We report the results of various genotypic and phenotypic assays used to determine the biotype background for each strain. However, more importantly, we report relative cholera toxin production levels expressed by all variants, the expression levels of the virulence factors TcpA and ToxT, and quantitative data for biofilm production and other phenotypic traits, and we compare these data to those for classical O395, El Tor C6706, and El Tor N16961 wild-type strains. Finally, in agreement with the virulence factor production profiles, we demonstrate the virulence profile of the high producers of cholera toxin (Bgd8 and MQ1795), as well as some of the genetically different representative variant strains (Bgd2, Bgd3, MQ04, and BAA-2163), using the infant mouse cholera model.

TABLE 1. Patient profiles[c]

Strain	Patient sex	Age (yr)	Dehydration status	Acute watery diarrhea status	Serotype
Bgd1	F	17	Severe	Yes	Ogawa
Bgd2	F	7	Severe	Yes	Ogawa
Bgd3	M	19	Severe	Yes	Inaba
Bgd4	F	13	Mild	Yes	Inaba
Bgd5	F	35	Severe	Yes	Inaba
Bgd6	F	5	Severe	Yes	Inaba
Bgd7	F	13	Severe	Yes	Ogawa
Bgd8	M	15	Severe	Yes	Ogawa
MQ1795	M	90	None	Yes	Inaba
MQ04	NA	4	None	Yes	Inaba
BAA-2163[a]	NA	NA[b]	NA	Yes	Ogawa

[a] Haiti 2010 outbreak strain: NCBI strain 2010EL-1786 (accession number AELH00000000).

[b] This isolate was a representative strain of the outbreak in Haiti, where the patient ages ranged from 0 to 84 years old.

[c] F, female; M, male; NA, not available.

MATERIALS AND METHODS

Clinical patient profiles. The clinical profiles of the patients from Matlab Hospital in Bangladesh and representative data for the patients from Haiti are given in Table 1. All patients were hospitalized with clinical manifestations of cholera and were treated accordingly.

Bacterial strains and general growth conditions. All strains used in this study are listed in Table 2. The Matlab clinical isolates were previously identified as El Tor variants as part of a collective pool of clinical isolates (37). The strain BAA-2163, isolated in Haiti, also was identified as an El Tor variant (12). These strains were grown simultaneously under the same conditions with wild-type classical O395, wild-type El Tor strains C6706 and N16961, and classical O395Δ*tcpA* or O395Δ*toxT* (for TcpA or ToxT production, respectively). Polymyxin B resistance was determined by patching strains on Luria-Bertani (LB) medium plates containing polymyxin B (50 IU ml^{-1}) (Sigma-Aldrich, St. Louis, MO), and strain motility was determined by stabbing into 0.3% agar LB plates and incubating at 37°C for 6 h, as previously described (25). Voges-Proskauer medium (MR-VP) (Becton Dickinson, Sparks, MD) was inoculated with each strain as described previously (25). Hemolytic properties were determined by patching the different strains onto blood agar plates (Remel, Lenexa, KS) that then were incubated overnight at 37°C and observed for the degree of hemolysis indicated by clearing on the plate. The ability to use citrate as a sole carbon source was determined by patching the individual strains onto minimal citrate medium plates (48) and incubating them at 37°C overnight. The ability to hydrolyze casein also was determined by patching on milk agar plates followed by incubation at 37°C. Classical biotype-inducing growth conditions were performed in LB (starting pH 6.5) at 30°C with aeration for 15 h. El Tor biotype TCP-inducing conditions were AKI-inducing conditions as previously described (18).

The biofilm assay was performed as previously described (40) on the El Tor variant strains, wild-type classical O395, and wild-type El Tor C6706 and N16961. Briefly, cultures were grown in AKI-inducing medium, without shaking, in 96-well U-bottom plates (Becton Dickinson, Sparks, MD) overnight. The cultures were removed and the plates washed, and then the biofilm formed on the sides of the well was stained with 0.1% crystal violet. The crystal violet then was dissolved with glacial acetic acid, and the amount of crystal violet (Bio-Rad, Hercules, CA) was quantitated at the optical density of 550 nm (OD_{550}).

Cholera toxin production assay. GM_1 ganglioside enzyme-linked immunosorbent CT assays (15) were performed on the culture supernatants of the wild-type classical, wild-type El Tor, and El Tor variant cultures grown under AKI-inducing conditions, and the total ng of CT produced per ml of culture per OD_{600} unit (ng CT ml^{-1} $OD_{600}{}^{-1}$) was determined.

Immunoblot assays for ToxT and TcpA. Whole-cell extracts (WCE) were prepared from all of the cultures grown under AKI-inducing conditions for 3.5 (for ToxT) or 7.5 h (for TcpA) at 37°C. AKI-inducing conditions involve incubation without aeration for the first 3.5 h, followed by aeration for the next 4 h. WCE protein concentrations were determined, and either 10 (for ToxT) or 8 μg (for TcpA) of WCE was run on a 16% Tris-glycine polyacrylamide gel (Invitrogen, Carlsbad, CA), transferred to nitrocellulose, probed with anti-ToxT or anti-TcpA, and visualized using the ECL (enhanced chemiluminescence) detection system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Infant mouse cholera model. The El Tor variants were tested for virulence alongside the two wild-type El Tor strains, C6706 and N16961, using suckling CD-1 mice ($n = 6$) (Charles River Laboratories, Kingston, NY) as previously described (23), with an infectious dose of $\sim 7.5 \times 10^8$ bacteria.

PCR assay. Primers (Integrated DNA Technologies, Coralville, IA) used in this study to amplify *tcpA*, *ctxB*, and the CTX phage/ancestral chromosome (large and small) junction are listed in Table 3. Genomic DNA of all strains was prepared using a DNA purification kit (Qiagen, Valencia, CA). For the amplification and sequencing of *tcpA* in all strains in this study, oligonucleotides *tcpA*-For (primer 32) and *tcp*-Rev (primer 33) were used, and for *ctxB* the

TABLE 2. Strains used in this study[a]

Strain	Relevant description	O1 biotype	Reference or source
E. coli			
S17-1λ*pir*	*recA thi pro hsdR*$^-$ M$^+$ [RP4-2-Tc::Mu::Km^r Tn*7*] (λ *pir*); Tmp^r Str^r	NA	12
V. cholerae			
O395	Wild-type Ogawa; Str^r	Classical	This study; laboratory collection
C6706	Wild-type Inaba; Str^r	El Tor	This study; laboratory collection
N16961	Wild-type Inaba; Str^r	El Tor	This study; laboratory collection
C6706Δ*toxT*	C6706Δ*toxT*; Str^r	El Tor	This study; laboratory collection
O395Δ*toxT*	O395Δ*toxT*; Str^r	Classical	This study; laboratory collection
O395Δ*tcpA*	O395Δ*tcpA*; Str^r	Classical	23
MQ1795	El Tor	El Tor variant	34; Bangladesh
MQ04	El Tor	El Tor variant	34; Bangladesh
Bgd1	El Tor	El Tor variant	This study; Bangladesh
Bgd2	El Tor	El Tor variant	This study; Bangladesh
Bgd3	El Tor	El Tor variant	This study; Bangladesh
Bgd4	El Tor	El Tor variant	This study; Bangladesh
Bgd5	El Tor	El Tor variant	This study; Bangladesh
Bgd6	El Tor	El Tor variant	This study; Bangladesh
Bgd7	El Tor	El Tor variant	This study; Bangladesh
Bgd8	El Tor	El Tor variant	This study; Bangladesh
BAA-2163	El Tor	El Tor variant	This study; Haiti

[a] NA, not applicable; Tmp^r, trimethoprim resistant; Str^r, streptomycin resistant.

oligonucleotides *ctxAB*-Rev (primer 39) and *ctxB*-For (primer 51) were used. For chromosome-specific PCR, the oligonucleotides *ctxAB1*-Rev (primer 56) and *ctxAB2*-Rev (primer 57) were used individually with oligonucleotide *ctxB*-For (primer 51).

Phylogenetic analysis. Phylogenetic analysis was performed on the El Tor variants against classical wild-type O395 and El Tor wild-type N16961 as previously described (2), using the neighbor-joining method and P distance to determine relatedness between the strains. Gene sequences for *groEL* (classical, VC0395_A2237; El Tor, VC2664), *gyrB* (classical, VC0395_A2504; El Tor, VC0015), *pryC* (classical, VC0313; El Tor, VCA0925), and *recA* (classical, VC0395_A0070; El Tor, VC0543) were analyzed for each strain, and sequences were compared using MEGA5 (46). For comparison, a non-O1 environmental *V. cholerae* isolate, 12129, also was incorporated. Additional analyses were conducted comparing *ctxB* and *tcpA* sequences between the variants and both wild-type classical O395 and El Tor N16961.

RESULTS

Phenotypic profiles reveal a wide range of properties among the clinical isolates with no unifying trend. (i) Polymyxin B resistance, hemolysis, citrate metabolism, and casein hydrolysis profiles. Standard phenotypic assays were conducted to comprehensively characterize the background of the El Tor variant clinical isolates. All variants were able to grow on LB medium supplemented with polymyxin B just as well as the wild-type El Tor strains C6706 and N16961 (Table 4), whereas the wild-type classical strain exhibited polymyxin B sensitivity. When patched onto blood agar plates to determine hemolytic activity, the variants demonstrated a range of degrees of hemolytic abilities, ranging between the levels for wild-type C6706 and N16961 (Table 4). Wild-type classical O395 did not demonstrate any hemolysis. All of the clinical variants studied also were able to use citrate as a sole carbon source and grew just as well as wild-type El Tor C6706 and N16961 strains, whereas wild-type classical O395 did not grow at all. Akin to wild-type El Tor C6706 and unlike wild-type classical O395 and wild-type El Tor N16961, all of the variants studied here were able to hydrolyze the casein in the milk agar plates, resulting in a clear zone around the inoculation patch.

TABLE 3. Primers used in this study

Primer name	Sequence (5′–3′)	Primer no.
tcpA-For	CCGCACCAGATCCACGTAGGTGGG	32
tcpB-Rev	GTCGGTACATCACCTGCTGTGGGGCAG	33
ctxAB-Rev	CATCATCGAACCACAAAAAAGCTTACTGAGG	39
ctxB-For	GGGAATGCTCCAAGATCATCGATGAGTAATAC	51
ctxAB1-Rev	CGATGCCGCTCTAAAGCTGCTTGCCGCTGAACTC	56
ctxAB2-Rev	CTCAAGCTGCGATCAGCATGGCGTGGTCAGACG	57

(ii) All clinical variants studied demonstrated motility comparable to that of wild-type N16961. The motility of the variants at 37°C also was determined to help characterize their phenotypic characteristics (Table 4). The motility plate assays revealed that all variants studied exhibited motility characteristics similar to those of wild-type El Tor N16961. Motility was measured as an average diameter of growth of three independent trials at 37°C and compared to the diameter of growth of wild-type N16961.

(iii) VP assay results for acetoin production profiles are akin to results for classical O395. The Voges-Proskauer (VP) test was performed to determine whether or not the variants produce acetoin as an end product of glycerol metabolism (Table 4). El Tor biotype strains are known for producing a very bright red color, indicating a positive VP result, whereas classical biotype strains produce no color change, indicating a negative VP result. Surprisingly, all clinical variants studied tested negative or weakly positive (slight pink coloration) for the VP reaction (Fig. 1).

(iv) Biofilm production levels varied among the clinical variants studied. The El Tor variant strains were tested for the ability to produce biofilm, and their production levels were compared to the levels of biofilm produced by both of the wild-type El Tor strains as well as the wild-type classical O395

TABLE 4. Summary of phenotypic traits of the El Tor variants compared to wild-type classical biotype strain O395 and wild-type El Tor biotype strains C6706 and N16961[a]

Phenotype	O395	C6706	N16961	Bgd1	Bgd2	Bgd3	Bgd4	Bgd5	Bgd6	Bgd7	Bgd8	MQ1795	MQ04	BAA-2163
Polymyxin B resistance	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemolysis	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer	−	+++	+++	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	−	+/−	+/−
Citrate metabolism	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility at 37°C	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Casein hydrolysis	−	+	−	+	+	+	+	+	+	+	+	+	+	+
Biofilm production	++	+++	+	++	+	+	+	+	+	+	+	+	+++	+

[a] +, ++, and +++, degree of positivity; −, negative; +/−, weakly positive.

strain (Table 4). Both initial visual observations of biofilm production and quantitated amounts of dissolved crystal violet (at OD_{550}) indicated that all clinical variants studied here, except Bgd1 and MQ04, produced levels of biofilm comparable to that of wild-type El Tor N16961, which is much less than that produced by either wild-type El Tor C6706 or wild-type classical O395.

The El Tor variants produced more cholera toxin than wild-type El Tor under AKI-inducing conditions. Cell-free culture supernatant of each strain was used to determine the amount of cholera toxin produced under both classical and AKI-inducing conditions. As expected, under classical inducing conditions, only the classical wild-type O395 produced detectable levels of cholera toxin (~7,000 ng CT ml^{-1} $OD_{600}{}^{-1}$; data not shown). However, under AKI-inducing conditions (Fig. 2), the El Tor variants produced larger amounts of cholera toxin than all three wild-type strains, classical O395, El Tor C6706, and El Tor N16961 (~2,420, ~470, and ~1,800 ng CT ml^{-1} $OD_{600}{}^{-1}$, respectively). Bgd8 (~10,900 ng CT ml^{-1} $OD_{600}{}^{-1}$) and MQ1795 (~12,500 ng CT ml^{-1} $OD_{600}{}^{-1}$) produced the largest amounts of toxin, with average production of ~6- and ~7-fold larger amounts than El Tor N16961. The other nine variants also produced more cholera toxin than N16961, but in a smaller range of >2- to 5-fold.

***In vitro* ToxT and TcpA production profiles differ across the El Tor variants.** ToxT (32 kDa) is an AraC family transcriptional regulator which activates the expression of the *tcp* operon in *V. cholerae* under virulence gene-inducing conditions. TcpA (20.5 kDa) is the major pilin protein of the TCP apparatus and is an essential virulence factor in *V. cholerae*. TcpA is encoded by the first gene in the *tcp* operon, *tcpA*, which is activated by ToxT. After growing the El Tor variant strains and the appropriate wild-type strains (classical O395 and El Tor C6706 and N16961) under AKI-inducing conditions, the levels of TcpA and ToxT proteins produced were observed. As seen in Fig. 3A, variants MQ1795 and Bgd8 produced higher levels of TcpA than the wild-type El Tor N16961. The other El Tor variants were observed to produce TcpA levels that were less than that of N16961 and more comparable to that of C6706. Interestingly, classical O395 produced TcpA at levels similar to those of wild-type El Tor N16961 under AKI-inducing conditions.

However, a different pattern of protein production was observed in levels of ToxT across the strains (Fig. 3B). We observed that El Tor variants Bgd8, MQ1795, and MQ04 produced noticeably more ToxT than both wild-type El Tor C6706 and N16961, with levels comparable to that of classical O395. The other eight variants produced levels of ToxT comparable to that wild-type El Tor C6706, all of which were greater than that of N16961. Wild-type classical O395 produced more ToxT than wild-type El Tor C6706 and N16961 strains.

Variants Bgd8 and MQ1795 are hypervirulent in the infant mouse cholera model compared to wild-type El Tor N16961 and C6706. The virulence of the El Tor variant strains was determined relative to the virulence of the wild-type El Tor strains C6706 and N16961 by infecting the infant mice (infectious dose of $\sim 7.5 \times 10^8$ in this study) and observing the length of time for half the infected infant mouse population ($n = 6$) to succumb to the cholera infection (LT_{50}) (Fig. 4). Of the 11 El Tor variant strains available, only certain representative strains were used in the infant mouse cholera model based on the different genotypes, particularly the number of predicted copies of the *ctxAB* operon, the mutations found in *tcpA*, and the heptad copy number (Table 5). The strains used in this study include MQ1795, which produced the most CT and carries two copies of the *ctxAB* operon (Fig. 5C), all three *tcpA*

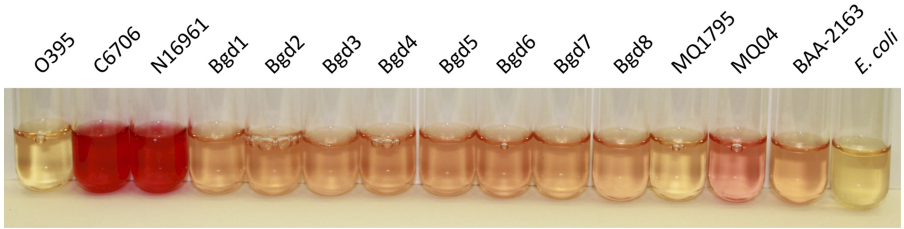

FIG. 1. Voges-Proskauer (VP) results for acetoin production by the El Tor variants. *V. cholerae* classical biotype strain O395 is negative for the VP test. El Tor biotype strains C6706 and N16961 are bright pink, which is characteristic of a positive VP test result. The El Tor variants in this study all show various degrees of no pink to slight pink color, which is interpreted as a negative or weakly positive VP result, respectively. For comparison, VP-negative *E. coli* S17-1λ*pir* also is shown.

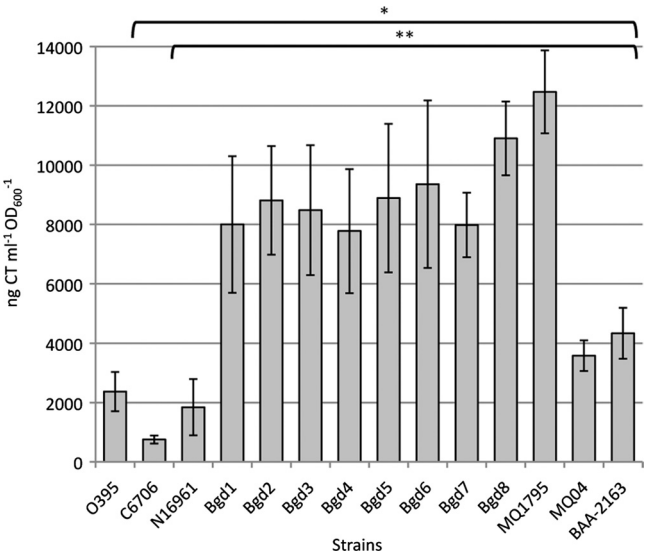

FIG. 2. Enzyme-linked immunosorbent assay (ELISA) for total cholera toxin production by El Tor variant strains. Total cholera toxin (CT) produced by wild-type classical O395, wild-type El Tor C6706, wild-type El Tor N16961, and the El Tor variants Bgd1, Bgd2, Bgd3, Bgd4, Bgd5, Bgd6, Bgd7, Bgd8, MQ1795, MQ04, and BAA-2163 under AKI-inducing conditions was determined by a colorimetric ELISA. The CT assay was conducted in triplicate, and the mean CT produced (ng CT ml^{-1} $OD_{600}{}^{-1}$) is shown with standard deviations for each strain. A two-tailed standard *t* test yielded *P* values of <0.05 when the El Tor variants were compared to wild-type El Tor C6706 (*) and wild-type El Tor N16961 (**).

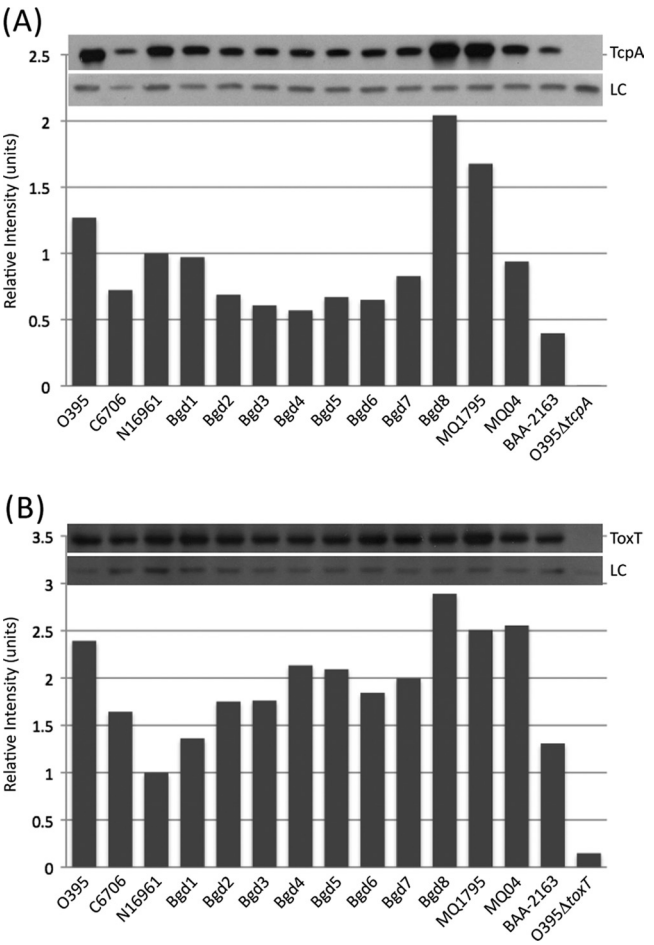

FIG. 3. Immunoblot assays and densitometry for *V. cholerae* virulence factors TcpA and ToxT. Whole-cell extracts (WCE) were prepared, and 8 and 10 μg of total protein for TcpA and ToxT, respectively, was loaded onto a 16% Tris-glycine polyacrylamide gel. The resulting band intensities were quantitated and normalized against wild-type El Tor N16961. Anti-TcpA (A) or anti-ToxT (B) antibody was used to probe for TcpA and ToxT, respectively, in wild-type classical O395, wild-type El Tor C6706, wild-type El Tor N16961, and El Tor variants Bgd1, Bgd2, Bgd3, Bgd4, Bgd5, Bgd6, Bgd7, Bgd8, MQ1795, MQ04, and BAA-2163. O395Δ*tcpA* and O395Δ*toxT* are shown as controls. Nonspecific bands serving as loading controls (LC) are shown below each respective immunoblot.

mutations, and four heptad repeats (TTTTGAT), which serve as binding sites for the activators ToxR (30, 31, 41) and ToxT (10, 50); MQ04, which contains two potential copies of *ctxAB* (Fig. 5C) but produced elevated levels of ToxT and wild-type N16961 levels of TcpA (Fig. 3), contains all three *tcpA* mutations and four heptad repeats; Bgd2 contains two potential copies of *ctxAB* (Fig. 5C), does not have all three *tcpA* mutations (no $A_{266}G$), but does have three heptad repeats; Bgd3 contains only one copy of *ctxAB* (akin to wild-type El Tor N16961 [Fig. 5B]) but all three *tcpA* mutations as well as three heptad repeats; Bgd8 produced the second highest levels of CT (Fig. 2), the highest levels of TcpA and ToxT (Fig. 3), carries two copies of *ctxAB* (Fig. 5C), has only the $C_{128}T$ mutation in *tcpA*, and has three heptad repeats; and BAA-2163, which was the causative agent of the cholera outbreak in Haiti in 2010, carries only one copy of the *ctxAB* operon (akin to wild-type El Tor N16961 [Fig. 5B]), has all three *tcpA* mutations but also carries an additional $C_{58}A$ mutation in *ctxB* and five heptad repeats, neither of which have ever been observed in any previous strains.

As shown in Fig. 4A and B, El Tor variants Bgd8 and MQ1795 demonstrated an increase in virulence at the inoculation dose tested compared to that of both wild-type El Tor N16961 and C6706. The LT_{50} for El Tor variant MQ1795 was 22 h, 6 h sooner than that for wild-type N16961, whereas the LT_{50} for variant Bgd8 was 25 h, 3 h sooner than that for N16961. El Tor variants BAA-2163 (Fig. 4A), Bgd2, and Bgd3 (Fig. 4B) exhibited LT_{50}s of 27, 26, and 29 h, respectively, which is comparable to that of wild-type N16961. Wild-type C6706 demonstrated an LT_{50} of 43 h, much later than that of N16961, whereas variant MQ04 did not kill any mice during the course of the 48-h experiment (Fig. 4A).

Genetic analysis of variants reveals El Tor *tcpA*, with previously unreported mutations, classical *ctxB* genes, and duplication of the *ctxAB* operon on the small chromosome. Primers listed in Table 3 were used to amplify and sequence the *tcpA* and *ctxB* genes to determine whether these genes are of the classical or El Tor biotype. PCR using primers for amplifying *tcpA* yielded a product of 1,420 bp, while the *ctxAB* primers yielded a 580-bp product. The sequencing of *tcpA* in the variants showed that the variants possessed El Tor versions of *tcpA*, but it also revealed some point mutations compared to the sequence of N16961. A cytosine-to-thymine point mutation 128 bp upstream of the ATG start site of *tcpA* was common to

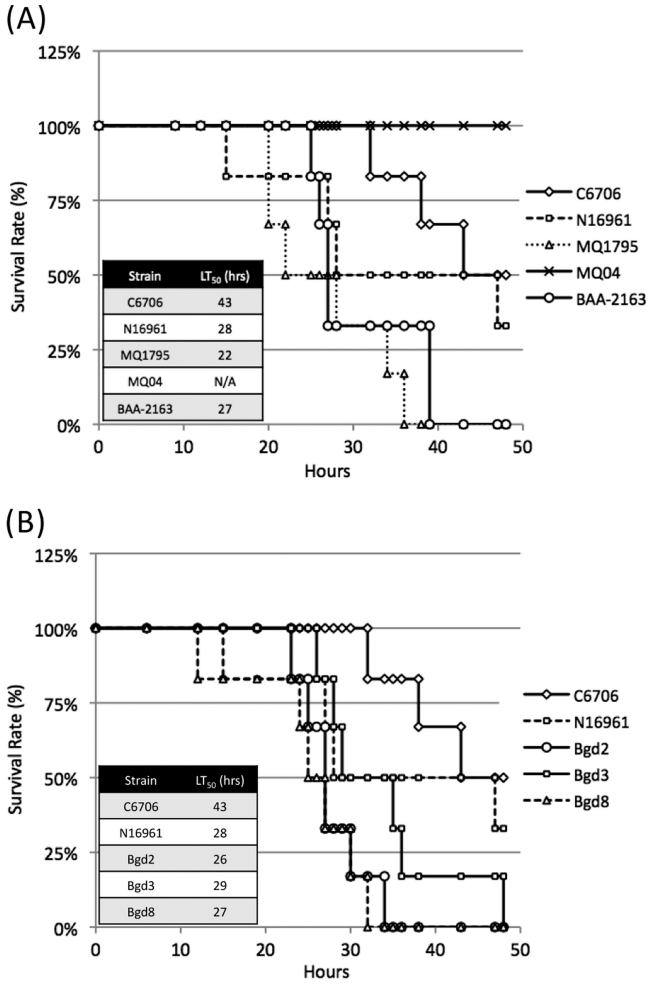

FIG. 4. Determining the relative virulence of the El Tor variant strains using the infant mouse cholera model. The El Tor variant strains were individually inoculated (infectious dose of $\sim 7.5 \times 10^8$) into infant mice ($n = 6$) to determine the length of time required before half the population succumbs to infection (LT_{50}) (inset). Wild-type El Tor C6706 and N16961 also were separately inoculated as controls and to determine the relative virulence (hypo-, equi-, or hypervirulent) of the El Tor variant strains MQ1795, MQ04, and BAA-2163 (A) and Bgd2, Bgd3, and Bgd8 (B).

the variants. An additional $A_{266}G$ point mutation, resulting in a residue change of $N_{89}S$, also was found in variants Bgd3, Bgd4, Bgd5, Bgd6, MQ1795, MQ04, and BAA-2163. A third mutation of an additional adenine base insertion 130 bp upstream of the ATG start site also was identified in all of the variants tested except Bgd8. Although these mutations in *tcpA* and the upstream region were the basis for representative strain selection for the LT_{50} assay (Fig. 4), no correlation could be made between these mutations and the amounts of TcpA produced.

When the *ctxB* sequence was analyzed, all of the variants tested were found to carry the classical version of *ctxB*, which contains the residues H39 and T68, as opposed to Y39 and I68, respectively, which were found in El Tor N16961 (Table 5). Interestingly, the strain isolated from Haiti, BAA-2163, in addition to being of the classical biotype, also was found to have an additional mutation of a cytosine to an adenine at position 58, resulting in an $H_{20}N$ residue substitution, which is not common to either classical O395 or El Tor N16961 (Table 5).

The downstream region of the CTXϕ also was characterized in the variants to determine if the chromosomal location(s) of the phage is similar to that observed in wild-type El Tor (a single copy on the large chromosome) or to wild-type classical (two copies, one on each chromosome) (Fig. 5). Using downstream primers that were specific for either the large (*ctxAB2*-Rev) or small (*ctxAB1*-Rev) chromosome and a common upstream primer (*ctxB*-For), it was determined that unlike the wild-type N16961, variants Bgd2, Bgd7, Bgd8, MQ1795, and MQ04 had the CTXϕ inserted on both chromosomes (Fig. 5C), akin to classical O395, which has not been previously reported for any El Tor variant strains. Although duplicated CTXϕ have been previously reported in some El Tor variants, the reported duplications were tandem duplications of the CTXϕ on the large chromosome (14). In light of this report, the tandem duplication of the CTXϕ also was considered in the El Tor variants of this study; however, analysis through PCR revealed that this is not the case, and CTXϕ duplication is not found in tandem but individually on each chromosome.

Phylogenetic analysis groups the clinical El Tor variants and the Haiti strain in the same clade as wild-type El Tor N16961. The sequences of four genes (*recA*, *pyrC*, *gyrB*, and *groEL*; classical accession numbers VC0395_A0070, VC0395_0313, VC0395_A2504, and VC0395_A2237, respectively, and El Tor accession numbers VC0543, VCA0925, VC0015, and VC2664, respectively) were analyzed in the clinical El Tor variant strains and were compared to wild-type classical O395 and wild-type El Tor N16961 sequences to distinguish evolutionary lineages. The alignment of the sequences and phylogenetic analysis revealed that the El Tor variants belonged to the same phylogenetic clade as wild-type El Tor N16961 (Fig. 6A), lending further support that the variants are of an El Tor background. For comparison, the same genes in the environmental *V. cholerae* strain 12129 also were analyzed and incorporated into the phylogenetic analysis (Fig. 6A). As expected, the environmental non-O1 *V. cholerae* strain 12129, isolated from the Gulf Coast, was completely separate from both classical and El Tor strains.

Phylogenetic analyses also were conducted that compared *ctxB* (Fig. 6B) and *tcpA* (containing the upstream promoter region) (Fig. 6C and D) of the variants to genes of the two wild-type *V. cholerae* strains, N16961 and O395. As illustrated in Fig. 6B, the *ctxB* gene clustered the variants with classical O395, although the Haitian strain BAA-2163 grouped alone within the same clade (Fig. 6B). In contrast, when analyzing *tcpA* and the upstream promoter region, the variants grouped with El Tor N16961 (Fig. 6C). Further dichotomy within this clade was resolved in Fig. 6D, as variants Bgd1, Bgd2, Bgd7, and Bgd8 grouped with wild-type N16961 and Bgd3, Bgd4, Bgd5, Bgd6, MQ1795, MQ04, and BAA-2163 clustered together.

DISCUSSION

All of the clinical El Tor variant strains in this study display differential cholera toxin and TcpA and ToxT expression profiles. The emergence of *V. cholerae* El Tor variants has not

TABLE 5. Summary of genotypic traits of wild-type classical biotype strain O395, wild-type El Tor biotype strains C6706 and N16961, and the El Tor variants[a]

Characteristic	Presence of CTXϕ	CTXϕ location		*ctxB* type	Presence of $C_{58}A$ results in $H_{20}N$ substitutions in *ctxB*	*tcpA* type	Presence of *tcpA* variant with:			No. of heptads
		Large chrom	Small chrom				$A_{266}G$[b] results in $N_{89}S$ substitution	A[c] insertion	C to T[b,d] substitution	
O395	Yes	Yes	Yes	Class	No	Class	NA	NA	NA	7
C6706	Yes	Yes	No	El Tor	No	El Tor	No	No	No	4
N16961	Yes	Yes	No	El Tor	No	El Tor	NA	NA	NA	4
Bgd1	Yes	Yes	No	Class	No	El Tor*	No	Yes	Yes	3
Bgd2	Yes	Yes	Yes	Class	No	El Tor*	No	Yes	Yes	3
Bgd3	Yes	Yes	No	Class	No	El Tor*	Yes	Yes	Yes	3
Bgd4	Yes	Yes	No	Class	No	El Tor*	Yes	Yes	Yes	4
Bgd5	Yes	Yes	No	Class	No	El Tor*	Yes	Yes	Yes	4
Bgd6	Yes	Yes	No	Class	No	El Tor*	Yes	Yes	Yes	3
Bgd7	Yes	Yes	Yes	Class	No	El Tor*	No	Yes	Yes	3
Bgd8	Yes	Yes	Yes	Class	No	El Tor*	No	No	Yes	3
MQ1795	Yes	Yes	Yes	Class	No	El Tor*	Yes	Yes	Yes	4
MQ04	Yes	Yes	Yes	Class	No	El Tor*	Yes	Yes	Yes	4
BAA-2163	Yes	Yes	No	Class	Yes	El Tor*	Yes	Yes	Yes	5

[a] Chrom, chromosome; Class, classical biotype strain; El Tor, El Tor biotype strain; El Tor*, El Tor biotype variant strains (see the text); NA, not applicable.
[b] The numbering refers to the sequence of wild-type El Tor N16961.
[c] Insertion is 130 bp upstream of the translational start site.
[d] Substitution is 128 bp upstream of the translational start site.

been limited to Bangladesh and Mozambique, it also has been isolated and identified in parts of Asia (38, 45) and now in Haiti (1, 6, 7, 8), with cases originating from Haiti being reported in different parts of the United States (9) and even Canada. With the differences in the locations of where these diverse variants are being isolated come the inherent differences in genotypic and phenotypic profiles, as previously described elsewhere (28, 29, 33, 39, 44, 45). An alarming concern has been the growing number of unpublished reports and one recently published report (16) revealing various amounts of cholera toxin being produced by the different El Tor variants isolated. Our initial clinical patient profiles (Table 1) revealed that the patients all suffered from severe watery diarrhea, a hallmark manifestation of cholera. With the previous knowledge of El Tor variants and anecdotal reports of various cholera toxin production levels at the time this investigation was initiated, we proceeded to characterize the clinical El Tor variants isolated from various patients in Bangladesh and the representative strain from Haiti.

A recent report had determined that 19 different El Tor variants isolated between 1996 and 2007 produced CT levels equivalent to that of wild-type classical strains (16), which was on the level of approximately 20-fold higher than that of wild-type El Tor strains. Similarly, the El Tor variants in this study all demonstrated an increase in the amount of CT produced, although the level of increased production varied among the strains. Variants Bgd8 and MQ1795 produced 6- and 7-fold larger amounts of CT, respectively, than both wild-type classical and El Tor strains (Fig. 2).

Our initial hypothesis was that the differing amounts of CT produced by the variants (Fig. 2) would be directly linked to the master virulence factor regulator, ToxT, and directly correlated to the differing amounts of other virulence factors produced, primarily TcpA. We first examined the levels of TcpA and ToxT produced by the 11 clinical El Tor variant isolates, and we observed that under AKI-inducing conditions (virulence gene expression-inducing conditions for El Tor biotypes), variants Bgd8 and MQ1795 produced much higher levels of both TcpA and ToxT (Fig. 3). However, the TcpA and ToxT levels were not duplicated in the remaining variants, all of which produced lower levels of TcpA and various higher levels of ToxT than wild-type El Tor N16961. Since ToxT is the master regulator (activator) for virulence gene expression, it would make intuitive sense that higher levels of ToxT would in turn activate higher levels of *tcp* gene expression, resulting in increased amounts of TcpA being produced. We initially hypothesized that the higher level of TcpA observed in MQ1795 was the result of a mutation in the promoter region of *tcpA* resulting in a *tcpA* promoter with a higher affinity for ToxT. However, sequence analysis of the upstream region of *tcpA* identified two point mutations in the ToxT binding region, neither of which were unique to MQ1795. The different combinations of the three point mutations varied across the 11 variant strains and do not point to a common mutation that could be attributed to any specific strain to account for the observed phenotype of various ToxT and TcpA production levels (Table 5).

Another explanation may reside with mutations in *toxT* that may be attributable to the increased levels of ToxT production in MQ1795; however, sequence analysis of *toxT* in all variants studied revealed no alterations from the sequence of wild-type N16961. Without further analysis of the entire genome through such techniques as deep sequencing, it is very difficult to even hypothesize what could account for the differences observed in ToxT and TcpA expression levels across the variants, especially since none of the mutations are unique to any one strain.

An alternative explanation for the increased CT production observed resides with the copy number of the *ctxAB* operon. This El Tor variant carries two copies of the *ctxAB* operon, one on each of the large and small chromosomes, akin to classical

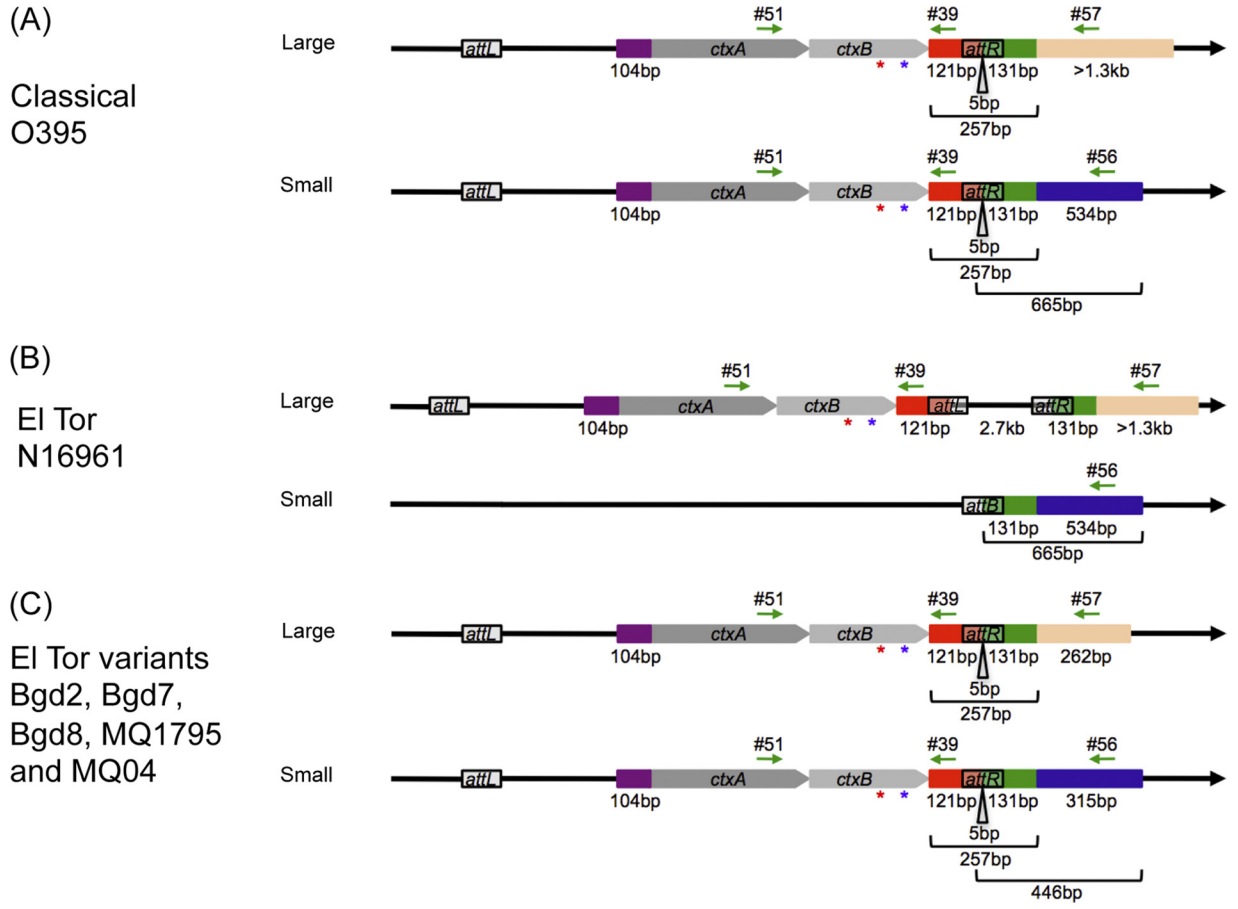

FIG. 5. Comparative schematic illustrating the hypothesized arrangement of the CTXϕ integration in some of the El Tor variants. The orientation of the *ctxAB* operon in the large and small chromosomes of wild-type classical biotype O395 (A), wild-type El Tor biotype N16961 (B), and some of the El Tor biotype variants (MQ1795, MQ04, Bgd2, Bgd7 and Bgd8) (C) are shown. The *ctxB* gene was amplified in the El Tor variants by PCR using classical large and small chromosome-specific primers (primers 57 and 56, respectively). PCR and sequencing revealed that *cxtB* was present on both the large and small chromosomes of the variants, and both copies were that of the classical *ctxB* sequence (red asterisk, H39 in classical and El Tor variants and Y39 in El Tor N16961; blue asterisk, T68 in classical and El Tor variants and I68 in El Tor N16961). Relative *attL* and *attR* sequences (*attB* in El Tor N16961 small chromosome) are shown as shaded boxes. Identical sequences across the strains are color-coded and indicated with size (in bp). Positions of primers used for PCR and sequencing (primers 39, 51, 56, and 57) all are indicated with directional green arrows.

biotype strains. Unlike the wild-type El Tor biotype N16961, which only has a single copy of the operon on the large chromosome, this duplication of the *ctxAB* operon may account for the observed increase in the CT produced even though ToxT levels do not vary, much like the difference observed between low- and high-copy expression plasmids.

To further investigate this possibility, sequence analysis was performed on all of the El Tor variants in this study through chromosome-specific PCR. El Tor variant strains Bgd2, Bgd7, Bgd8, MQ1795, and MQ04 have a duplicated *ctxAB* operon, one copy residing on each chromosome. PCR with a forward *ctxB* primer (primer 51; Table 3) and either a small or large chromosome-specific primer (primer 56 or 57, respectively; Table 3) generated chromosome-specific products, much like wild-type classical O395. Neither wild-type El Tor C6706 nor N16961 produced a PCR product when using the small chromosome-specific primer (data not shown). However, as increased levels of CT also were observed in the variants that have only a single copy of the *ctxAB* operon (i.e., Bgd1, Bgd3, Bgd4, Bgd5, Bgd6, and BAA-2163), the duplication of the *ctxAB* operon cannot be the only explanation. Furthermore, as there is no consistency between the differences in phenotypes (Table 4) and the genotypes (Table 5) of these variants, the observations made most likely are due to the result of alternative mechanisms. It is plausible that this mechanism could be identified by looking at the genome sequence of the variants and identifying mutations in the DNA sequences, particularly of regulatory proteins or promoter regions. These data suggest a higher level of complexity in the regulatory cascade than originally predicted.

El Tor variants Bgd8 and MQ1795 are hypervirulent in the infant mouse cholera model. The inoculation of infant mice has been used previously to determine *V. cholerae* strain virulence (23) by interpolating the length of time required for half the inoculated population to succumb to the infection (LT_{50}). Using the infant mouse cholera model, the representative clinical El Tor variants were individually inoculated (~7.5×10^8

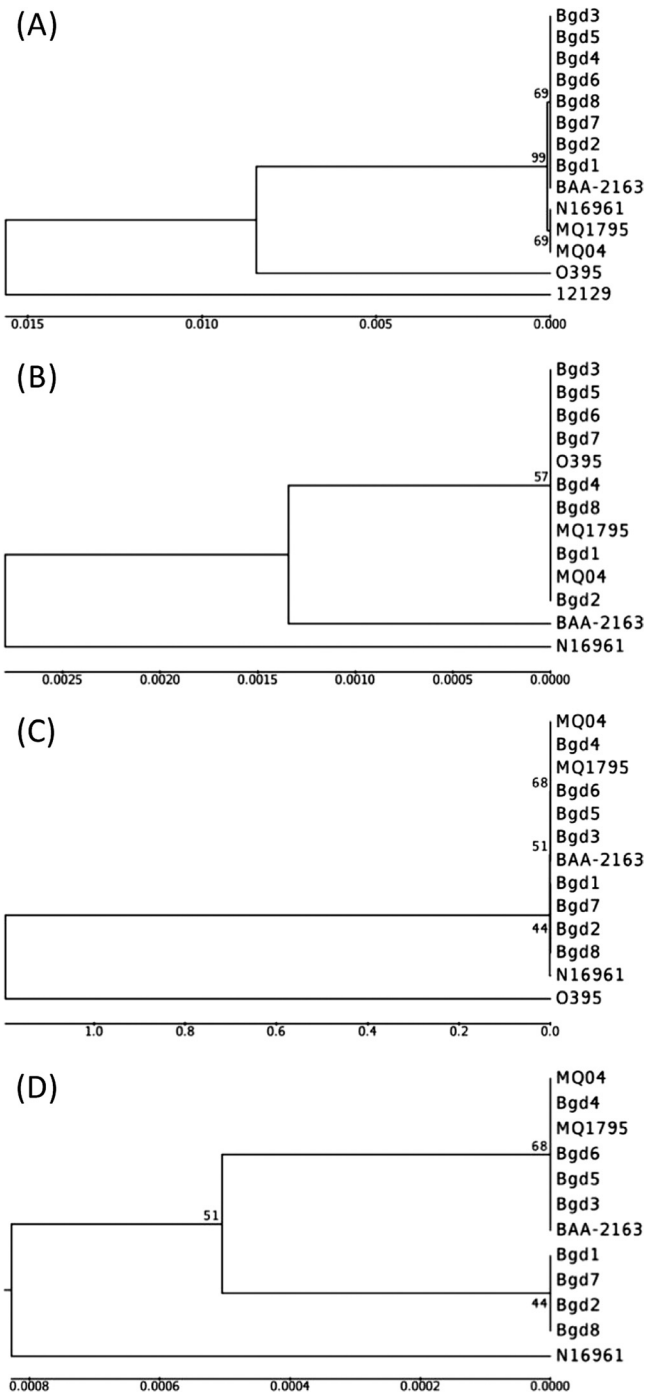

FIG. 6. Phylogenetic analysis of the El Tor variants. (A) The four genes *recA*, *pyrC*, *gyrB*, and *groEL* were used to determine the phylogenetic relatedness of the El Tor variants MQ1795, MQ04, Bgd1, Bgd2, Bgd3, Bgd4, Bgd5, Bgd6, Bgd7, Bgd8, and BAA-2163 to wild-type El Tor biotype strain N16961 and classical biotype strain O395. The environmental strain, 12129, also was included in panel A for comparative purposes. Phylogenetic relatedness of the variants was compared to both O1 classical and El Tor wild-type strains with respect to *ctxB* (B) and *tcpA* (C and D) with the promoter region. (D) The clades generated in the *tcpA* branch showing the divergence of the variants and wild-type N16961 were further resolved. The bootstrap number at the node indicates the confidence for the clade grouping as determined by MEGA5 software. The scale bar represents the branch distance between the different strains.

bacteria) alongside both wild-type El Tor strains, and the mice were observed for a period of 48 h.

With the characteristics of MQ1795 demonstrated in the CT assay and Western blot analysis, our prediction of MQ1795 being more virulent than both wild-type El Tor strains was validated. The LT_{50} of MQ1795 was determined to be 22 h postinoculation, which was 6 h faster than that of wild-type N16961. This result was not surprising, as a strain that produces greater levels of the master virulence gene regulator, ToxT, and therefore more TcpA and cholera toxin, would be more virulent. Increased levels of TcpA would allow the bacterium to better establish itself in the intestinal tract, while increased levels of CT would give rise to a substantially increased lethal effect. These results, when considered with the virulence factor expression profile described above and this variant's lack of detectable biofilm production (Table 4), strongly suggests that this particular El Tor variant has been well adapted to be the most fit in the host rather than in the environment, where the ability to produce a good biofilm would be more favorable.

Variant Bgd8, which produced the second highest levels of CT but the highest levels of both TcpA and ToxT, also proved to be more virulent in the infant mouse cholera model than wild-type El Tor strains N16961 and C6706. Akin to MQ1795, a similar increase in virulence in Bgd8 can be attributed to the increased levels of the master regulator ToxT and its effects on the virulence genes downstream.

Variants Bgd2, Bgd3, and BAA-2163, although genetically different from each other in terms of both *ctxAB* operon number and *tcpA* sequence (Table 5), did not exhibit any noticeable differences in virulence compared to that of wild-type N16961, despite producing greater levels of CT (Fig. 2). It appears that the copy number of the *ctxAB* operon alone does not give rise to increased levels of CT. Bgd2 contains two copies of the *ctxAB* operon, whereas Bgd3 and BAA-2163 only carry one copy (Table 5). However, Bgd2 and Bgd3 produced relatively equal levels of CT (Fig. 2), and BAA-2163 produced half that of either Bgd2 or Bgd3. All three of these variants differ in their *ctxB* sequence and *tcpA* mutations yet produce no significant differences in CT, TcpA, or ToxT production levels, and they are relatively as virulent as wild-type N16961.

Variant MQ04 produced wild-type levels of TcpA but produced elevated levels of ToxT and CT, suggesting that this strain has the potential to be hypervirulent, much like variant MQ1795. However, in the infant mouse cholera model this variant proved to be avirulent at an infectious dose of $\sim 7.5 \times 10^8$ bacteria, as none of the inoculated mice died within the 48-h incubation period. In lieu of the levels of ToxT, TcpA, and CT produced, it is unusual that this variant did not kill any of the hosts. However, further analysis revealed that this strain is unable to effectively colonize the intestines of the infant mouse cholera model (the competitive index was decreased by 2 log [data not shown]), although wild-type levels of TcpA are produced. Interestingly, variant MQ04 produced the most biofilm out of the three variants studied. This observation, in conjunction with the lack of virulence, suggests a different evolutionary path for this variant compared to that for variant MQ1795. Contrary to the hypervirulent MQ1795, which did not produce detectable levels of biofilm, variant MQ04 displays phenotypic traits that suggest that this variant is more fit for survival in an

environment where the ability to produce a biofilm is more advantageous rather than inside a host.

The results of our genotypic and phenotypic analyses of the 11 El Tor variants and those of the wild-type El Tor C6706 and N16961 are summarized in Tables 4 and 5. The phenotypic assays confirmed that all variants in this study are of the El Tor biotype background. Of interest were the results of the Voges-Proskauer (VP) test that is used to distinguish between classical and El Tor biotypes (36). Classical biotype strain O395 is VP negative, as it does not produce the intense bright pink coloration observed in the El Tor biotype strains C6706 and N16961 (Fig. 1). Although all variants in this study are of the El Tor background, the results of the VP test were not as clear-cut as those observed in the wild-type classical and El Tor strains. The VP test on the clinical isolates resulted in negative (no coloration in MQ1795) or weakly positive (slightly pink coloration in the remaining 10 isolates) results, while the El Tor wild-type strains were positive (Fig. 1). It is obvious that the metabolic capabilities of these variants have been altered over the course of their independent evolutions, and it is not unreasonable to believe that environmental pressures have inadvertently or purposefully favored mutations in these strains. Further investigation into the metabolic characteristics of these strains would identify these and any other metabolic differences.

Genetic analysis of the *tcpA* gene also is routinely conducted to verify the biotype background of *V. cholerae* isolates (4, 11, 34, 45) and was used in this study. The *tcpA* gene in all clinical variants in this study was sequenced and confirmed to be of the El Tor biotype, with three mutations (Table 5). Among these mutations was a base change that was conserved across all El Tor variants of a cytosine to a thymine 128 bp upstream of the *tcpA* ATG start site. A missense mutation of an $A_{266}G$ in the coding region, resulting in an amino acid change from asparagine to serine ($N_{89}S$), was found in Bgd3, Bgd4, Bgd5, Bgd6, MQ1795, MQ04, and BAA-2163. The third mutation was an insertion of an extra adenine in the stretch of adenines approximately 130 bp upstream of the ATG start site of *tcpA*. This mutation was found in all of the variants except Bgd8. As previously mentioned, the consequences of these mutations or combination of mutations have not yet been elucidated and are under investigation. However, the mutations do not seem to have a direct influence over *tcpA* expression or virulence, as no direct correlation could be made and no unifying theme could be identified.

To determine the phylogenetic similarity between the El Tor variants and the wild-type classical and El Tor biotype strains, sequence analyses of several genes (*recA*, *pyrC*, *gyrB*, and *groEL*) were used. Although all previous assays strongly suggested the variants are of El Tor lineage, a comparison of these genes lends more evidence that these variants are of the El Tor background. The strong evolutionary lineage to the El Tor wild-type N16961 strain, as evidenced by inclusion in the same phylogenetic clade, further illustrates the observations of classical strain displacement by El Tor strains. These sequence and phylogenetic analyses also have revealed that the El Tor variants can be further subdivided based on *tcpA* and its upstream promoter region, which raises interesting questions regarding the evolution of the variants within a smaller geographic location. The inclusion of BAA-2163 as an El Tor variant is not surprising, as its origin has been one of the central foci to emerge from the Haitian outbreak. However, the sequence of the *ctxB* locus in BAA-2163 has identified a unique mutation never observed in any other El Tor variants, $C_{58}A$ (Table 5). This single mutation could call into question not only the true origin of this variant but also the evolutionary timeline that this particular strain has followed. Another intriguing observation was the identification of an unusual number of heptad repeats (TTTTGAT) found in the promoter region of the *ctxAB* operon. Where classical O395 has seven heptad repeats and El Tor has four, the 10 Bangladesh El Tor variants have either three or four. However, the Haitian strain BAA-2163 was found to have five heptad repeats in the promoter region of the *ctxAB* operon, which has never been reported in any other strain. This particular feature, in conjunction with the unique mutation found within the *ctxB* locus, suggests an alternative origin or lineage (Fig. 6B).

The emergence and prevalence of *V. cholerae* El Tor variants has been reported in several geographic regions (1, 6, 12, 13, 33, 38, 45), and their independent evolution has led to various combinations of classical traits possessed by the different variant strains identified. Even in the 11 clinical El Tor variants isolated from the same hospital and from Haiti presented here, there exists significant variation among them, with regard not only to virulence gene expression but also to other phenotypic properties, such as alteration in basic metabolic pathways, biofilm production, and differing hemolytic activities. These differences, especially the potential of duplicated or deleted sequences, such as that of the CTXϕ genome, warrants further investigation. Alternative approaches, such as deep sequencing and *de novo* genome synthesis, may reveal more information that can help elucidate the differences observed and differences yet to be observed.

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