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Occurrence in Mexico, 1998–2008, of *Vibrio cholerae* CTX$^+$ El Tor carrying an additional truncated CTX prophage

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The seventh cholera pandemic caused by *Vibrio cholerae* O1 El Tor (ET) has been superseded in Asia and Africa by altered ET possessing the cholera toxin (CTX) gene of classical (CL) biotype. The CL biotype of *V. cholerae* was isolated, along with prototypic and altered ET, during the 1991 cholera epidemic in Mexico and subsequently remained endemic until 1997. Microbiological, molecular, and phylogenetic analyses of clinical and environmental *V. cholerae* isolated in Mexico between 1998 and 2008 revealed important genetic events favoring predominance of ET over CL and altered ET. *V. cholerae* altered ET was predominant after 1991 but not after 2000. *V. cholerae* strains isolated between 2001 and 2003 and a majority isolated in 2004 lacked CTX prophage (Φ) genes encoding CTX subunits A and B and repeat sequence transcriptional regulators of ET and CL biotypes: i.e., CTXΦ$^-$. Most CTXΦ$^-$ *V. cholerae* isolated in Mexico between 2001 and 2003 also lacked toxin coregulated pili *tcpA* whereas some carried either *tcpA*^{ET} or a variant *tcpA* with noticeable sequence dissimilarity from *tcpA*^{CL}. The *tcpA* variants were not detected in 2005 after CTXΦ$^+$ ET became dominant. All clinical and environmental *V. cholerae* O1 strains isolated during 2005–2008 in Mexico were CTXΦ$^+$ ET, carrying an additional truncated CTXΦ instead of RS1 satellite phage. Despite *V. cholerae* CTXΦ$^-$ ET exhibiting heterogeneity in pulsed-field gel electrophoresis patterns, CTXΦ$^+$ ET isolated during 2004–2008 displayed homogeneity and clonal relationship with *V. cholerae* ET N16961 and *V. cholerae* ET isolated in Peru.

The causative agent of cholera, *Vibrio cholerae*, is a genetically versatile bacterial species for which more than 200 serogroups have been identified and for which significant lateral transfer of genes has been demonstrated. Pandemic cholera is generally caused by toxigenic strains of *V. cholerae* serogroups O1 and O139. *V. cholerae* O1 has been divided into two biotypes, classical (CL) and El Tor (ET), differing primarily in phenotypic traits and distinct signature genome sequences (1). Of seven cholera pandemics recorded since 1817, the sixth and presumably the earlier pandemics have been caused by CL biotype (1). *V. cholerae* ET biotype was first recognized in 1905, but not until 1961 was it considered the causative agent of the seventh cholera pandemic, during which the CL biotype was no longer isolated from cholera cases in Asia.

Although cholera has been endemic in Asia for centuries and sporadic cases have been recorded in the Americas, the presence of *V. cholerae* CL biotype as the causative agent of cholera in the Americas has not been clarified. A massive epidemic of cholera occurred in South America during 1991–1992, first reported in Peru in January 1991, after which cholera appeared in other countries of Latin America, notably Mexico, by June 1991. Although the characteristic features of the Latin American strains of *V. cholerae* O1 biotype ET distinguished them from seventh pandemic *V. cholerae* ET isolated in Asia (2), clonal relatedness led some investigators to conclude that the 1991 Latin American cholera epidemic was simply an extension of the seventh pandemic from the Western hemisphere (3).

A significant recent development in cholera epidemiology has been the emergence of toxigenic variant strains of *V. cholerae* ET carrying traits of the CL biotype isolated in Asia and Africa (4, 5). Genetic changes in Latin American strains of *V. cholerae* have been described and the Peruvian *V. cholerae* O1 isolated between 1991 and 2003 has been shown to be similar to the ET of the seventh pandemic prototype, carrying a distinct signature in the VSP-II region that distinguished it from the Asian ET prototype (6). A recent study of *V. cholerae* isolated between 1991 and 1997 from diarrhea patients and surface water sources in Mexico showed both CL and ET biotype strains were present, along with the altered ET involved in epidemic cholera globally (7). In this study, *V. cholerae* isolated between 1998 and 2008 from diarrheal patients and the aquatic environment in Mexico was characterized, using microbiological, molecular, and phylogenetic techniques to elucidate events leading to the outbreaks of cholera in Mexico.

Significance

Vibrio cholerae classical (CL) biotype was isolated, along with biotype El Tor (ET) and altered ET carrying the cholera toxin (CTX) gene of CL biotype, during the 1991 cholera epidemic in Mexico, subsequently becoming endemic until 1997. Microbiological, molecular, and phylogenetic analyses of *V. cholerae* isolated from both clinical and environmental samples during 1998–2008 confirm important genetic events, namely predominance of ET over CL and altered ET in Mexico. Although altered ET is predominantly associated with cholera globally, progression of CTX$^+$ *V. cholerae* ET with truncated CTX prophage to the predominant pathogen causing endemic cholera in Mexico may prove to be yet another key historical point in the global epidemiology of cholera.

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Data deposition: The sequence reported in this paper of the ~1,200-bp amplicon for the cepF/rstRETR primers has been deposited in the GenBank database (accession no. KC952008).

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MICROBIOLOGY

Results

Source and Distribution of Cholera Cases and *V. cholerae* in Mexico. *V. cholerae* was isolated from hospitalized diarrheal patients and from surface water sources (Table 1) collected from nearly every province of Mexico, including those bordering the United States in the north and Guatemala in the south (Fig. 1). Of 91 *V. cholerae* isolates included in this study, all produced characteristic colonies on taurocholate tellurite gelatin agar, reacted to polyvalent O1 antisera, were agglutinated by either monovalent Inaba or Ogawa antisera, and amplified primers for the O-biosynthetic gene *wbe* (O1), confirming all to be serogroup O1. Among these 91 *V. cholerae* O1, 48 were Inaba and 43 Ogawa (Table 1). The *ctxA* gene was present in 58 strains, confirming CTX$^+$ *V. cholerae* O1 (Table 1) and 33 were confirmed CTX$^-$.

Phenotypic and Related Genotypic Characteristics. Phenotypic and related genetic characteristics of *V. cholerae* O1 strains are presented in Table 1. All *V. cholerae* O1 strains were resistant to CL biotype-specific phage IV, all but 11 were responsive to El Tor (ET)-specific phage V, and all showed ET-specific phenotypic traits, including the ability to agglutinate chicken blood cells (CCA) and resistance to polymyxin B (poly-B). The *V. cholerae* strains amplified primers for repeat in toxin *rtxC*, an ET-specific marker gene, and ET-specific hemolysin $hlyA^{ET}$ (Table 1). All but 13 amplified primers for *tcpA*, 74 amplified primers for $tcpA^{ET}$, and 4 for $tcpA^{CL}$ (Table 1). Of 58 CTX$^+$ strains, 16 amplified primers for $tcpA^{ET}$, $ctxB^{CL}$, $rstR^{ET}$, and $rstR^{CL}$, indicating *V. cholerae* altered (variant) ET, as reported previously (7). All altered ET and two ET (prototype) strains amplified primers specific for phage encoded antirepressor *rstC* of the RS1 satellite phage flanking ET CTXΦ. Forty-two CTX$^+$ strains amplified primers for $rstR^{ET}$ and $ctxB^{ET}$, confirming *V. cholerae* ET of seventh pandemic prototype, of which 40 failed to amplify *rstC* of the RS1 element. Thirty-three *V. cholerae* ET strains lacked *ctxA*, *ctxB*, and *rstR*, suggesting major genetic truncation, of which 13 lacked *tcpA*, whereas the remaining strains carried either ET-specific *tcpA* ($tcpA^{ET}$) or CL-specific *tcpA* ($tcpA^{CL}$) in their ET biotype background.

Year-wise analysis showed that most of the clinical *V. cholerae* strains isolated between 1998 and 2004 were Ogawa (Table 1), with Inaba dominant among the environmental isolates. Inaba was the only serotype isolated in Mexico between 2005 and 2008. The three *V. cholerae* strains isolated in 1998 and four in 1999

Table 1. Phenotypic and genotypic characteristics of *V. cholerae* O1 strains (n = 91) isolated from clinical and environmental samples collected in Mexico (1998–2008)

				Phenotypic properties				Genetic screening by PCR									
Year of isolation	No. of isolates	Serotype	Source	Chick cell aggl	Poly B (50U)	Phage IV CL	Phage V ET	*ompW*	*wbeO1*	*ctxA*	*CtxB* type*	*tcpA*	*rtxC*	*hlyA*	*rstC*	*rstR* type	Deduced biotype
1998	3	Ogawa	Hum.	+	R	R	S	+	+	+	C	E	E	E	+	E,C	Alt-ET
1999	4	Ogawa	Hum.	+	R	R	S	+	+	+	C	E	E	E	+	E,C	Alt-ET
	5	Ogawa	Hum.	+	R	R	S	+	+	–	–	E	E	E	–	–	ET
	1	Ogawa	Hum.	+	R	R	R	+	+	–	–	E	E	E	–	–	ET
	1	Inaba	Hum.	+	R	R	S	+	+	–	–	E	E	E	–	–	ET
2000	2	Ogawa	Hum.	+	R	R	S	+	+	+	E	E	E	E	+	E	ET
	8	Ogawa	Hum.	+	R	R	S	+	+	+	C	E	E	E	+	E,C	Alt-ET
	1	Ogawa	Hum.	+	R	R	R	+	+	+	C	E	E	E	+	E,C	Alt-ET
	1	Ogawa	Hum.	+	R	R	S	+	+	–	–	E	E	E	–	–	ET
	4	Inaba	Env.	+	R	R	S	+	+	–	–	–	E	E	–	–	ET
2001	1	Ogawa	Hum.	+	R	R	S	+	+	–	–	E	E	E	–	–	ET
	2	Ogawa	Env.	+	R	R	S	+	+	–	–	E	E	E	–	–	ET
	2	Ogawa	Env.	+	R	R	S	+	+	–	–	C	E	E	–	–	TCP-Var
	3	Ogawa	Env.	+	R	R	R	+	+	–	–	–	E	E	–	–	ET
	1	Inaba	Env.	+	R	R	R	+	+	–	–	–	E	E	–	–	ET
2002	1	Ogawa	Hum.	+	R	R	S	+	+	–	–	–	E	E	–	–	ET
	1	Ogawa	Hum.	+	R	R	S	+	+	–	–	E	E	E	–	–	ET
	1	Inaba	Env.	+	R	R	S	+	+	–	–	E	E	E	–	–	ET
2003	1	Ogawa	Hum.	+	R	R	S	+	+	–	–	–	E	E	–	–	ET
	1	Ogawa	Env.	+	R	R	S	+	+	–	–	–	E	E	–	–	ET
	1	Inaba	Env.	+	R	R	S	+	+	–	–	–	E	E	–	–	ET
	1	Ogawa	Env.	+	R	R	S	+	+	–	–	C	E	E	–	–	TCP-Var
2004	1	Ogawa	Hum.	+	R	R	S	+	+	–	–	C	E	E	–	–	TCP-Var
	1	Ogawa	Hum.	+	R	R	R	+	+	–	–	–	E	E	–	–	ET
	3	Ogawa	Env.	+	R	R	S	+	+	–	–	E	E	E	–	–	ET
	4	Inaba	Env.	+	R	R	S	+	+	+	E	E	E	E	–	E	ET
2005	10	Inaba	Env.	+	R	R	S	+	+	+	E	E	E	E	–	E	ET
2006	11	Inaba	Env.	+	R	R	S	+	+	+	E	E	E	E	–	E	ET
2007	1	Inaba	Hum.	+	R	R	S	+	+	+	E	E	E	E	–	E	ET
	3	Inaba	Hum.	+	R	R	R	+	+	+	E	E	E	E	–	E	ET
	2	Inaba	Env.	+	R	R	S	+	+	+	E	E	E	E	–	E	ET
2008	8	Inaba	Env.	+	R	R	S	+	+	+	E	E	E	E	–	E	ET
	1	Inaba	Env.	+	R	R	R	+	+	+	E	E	E	E	–	E	ET
N16961	ET	Inaba	Hum.	+	R	R	S	+	+	+	E	E	E	E	+	E	El Tor
O395	CL	Ogawa	Hum.	–	S	S	R	+	+	+	C	C	–	C	–	C	Classical

Alt, altered; C, classical; Chick cell aggl, chicken cell agglutination; CL, classical biotype; E, El Tor; Env., environment; ET, El Tor biotype; Hum., human; Poly B, polymixin B; R, resistant; S, sensitive; TCP-Var, TCP-variant; +, positive; –, negative.
*Determined by mismatch amplification mutation assay (MAMA) PCR (38).

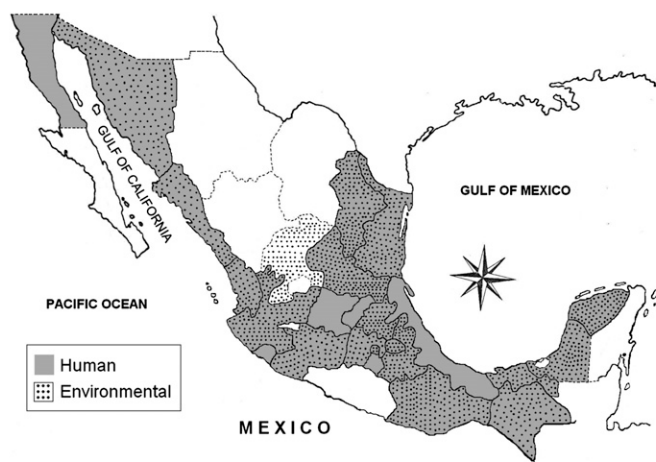

Fig. 1. Geographic map of Mexico showing provinces and locations near the Gulf of Mexico where samples from human and environmental sources were collected and tested for *V. cholerae* O1 as part of a national surveillance between 1991 and 2008.

were CTXΦ$^+$ altered ET whereas the remaining seven isolated in 1999 were CTXΦ$^-$ ET. *V. cholerae* CTXΦ$^+$ altered ET was isolated in 2000 in relatively larger proportions, together with *V. cholerae* ET strains with and without the CTXΦ, but, thereafter, CTXΦ$^+$ *V. cholerae* was not consistently isolated from clinical cases (Table 1). *V. cholerae* O1 biotype ET isolated between 2001 and 2003 lacked CTXΦ. However, most remarkable was the isolation in 2004 of CTXΦ$^+$ *V. cholerae* ET, and, thereafter, between 2005 and 2008, all Mexican isolates were the CTXΦ$^+$ *V. cholerae* ET seventh pandemic prototype (Table 1).

Chronologically, the majority of CTXΦ$^-$ *V. cholerae* ET isolated in 1999 and 2000 possessed ET biotype *tcpA* (Table 1), and all ET strains isolated during 2001 and thereafter were CTXΦ$^-$. The majority of CTXΦ$^-$ *V. cholerae* isolated in Mexico between 2001 and 2003 lacked *tcpA* whereas some CTXΦ$^-$ *V. cholerae* carried the *tcpA* allele of either ET or CL type in an ET host; the latter were designated TCP variant. Only *V. cholerae* CTXΦ$^-$ ET was isolated from environmental sources between 2001 and 2003. *V. cholerae* CTXΦ$^-$ ET was no longer isolated in Mexico after 2004, indicating clonal switching. During 2005–2008, CTXΦ$^+$ *V. cholerae* O1 ET Inaba was the dominant serotype (Table 1). The Mexican CTXΦ$^+$ *V. cholerae* O1 ET isolated during 2005–2008 exhibited phenotypic and molecular traits of the seventh pandemic prototype.

Nucleotide Sequencing of Targeted Genes. Data on the *ctxB* (460 bp) sequence of CTXΦ$^+$ strains from each year class (1998–2008) showed that altered ET strains isolated before 2004 presented amino acid sequences identical to CL biotype CT (*ctxB* genotype 1), with histidine and threonine at positions 39 and 68, respectively. However, *ctxB* sequences of ET strains isolated in 2004 and thereafter matched ET biotype CT (*ctxB* genotype 3): i.e., the cholera toxin type of seventh cholera pandemic strains.

Genome Organization of CTXΦ and Flanking Region. The Mexican CTXΦ$^+$ ET strains isolated between 2004 and 2008 failed to amplify *rstC* of the RS1 element. To follow up on this finding, representative ET biotype-specific *ctxB*^{ET} and *rstR*^{ET} were tested for genetic arrangements in CTXΦ and its flanking region by primer walking. Results showed that, unlike ET reference strain N16961, none of the *rstC*-negative strains amplified the ∼1.6 kb and 2.1 kb targeted RS1 sequences primed by *rstA*-F/*rstC*-R and *rstR*^{ET}-F/*rstC*-R, respectively (Table S1), confirming the absence of RS1 in the genome of the Mexican *V. cholerae* ET strains.

Thus, the Mexican *V. cholerae* ET lacked RS1, a characteristic of the CL biotype observed in ET variant strains from Bangladesh and Mozambique (8). A PCR assay was performed using primers CIIF and CIIR targeting *dif2* (9) to determine the presence of CTXΦ in the small chromosome (Chr II). As shown in Table S1, all strains possessed an ∼747-bp amplicon, suggesting the absence of CTXΦ in Chr II. Because the ET strains were CTXΦ$^+$, primer sets ctxAF/RTX5R and TLC3F/rstAR were used to locate the exact position of CTXΦ. As shown in Table S1, amplification of ∼1.4-kb and 1.8-kb DNA fragments, respectively, confirmed CTXΦ to be present in the large chromosome (Chr I). The presence or absence of CTXΦ tandem repeat was determined using the primer pair ctxBF/cepR. This amplicon was not found using template DNA prepared from the Mexican strains (Table S1), indicating that a tandem repeat of CTXΦ was not present in either chromosome.

Primer sets cepF/rstR^{ET}R, cepF/rstAR, and cepF/rstBR were used to detect truncated CTXΦ at the upstream region of CTXΦ for strains lacking RS1. All yielded amplicons, ∼1.2 kb, 2.2 kb, and 2.7 kb for cepF/rstR^{ET}R, cepF/rstAR, and cepF/rstBR primers, respectively, as did CL reference strain O395, confirming the presence of a truncated CTXΦ at the upstream region of CTX. DNA sequence analysis of the ∼1.2-kb amplicon, using cepF/rstR^{ET}R primers, confirmed the presence of a truncated *orfU* subunit (453 bp) within the truncated CTXΦ. Thus, the results showed that *V. cholerae* O1 ET strains isolated in Mexico between 2004 and 2008 had a truncated CTXΦ, in addition to CTX prophage, in the large chromosome, with neither an RS1 element nor a tandem array of CTX prophage. A schematic genetic map displaying the deduced chromosomal localization of CTXΦ and its flanking genetic region is provided in Fig. 2.

Sequencing and Phylogenetic Analysis of *tcpA*. The results of DNA sequencing of the *tcpA*^{ET} allele of the Mexican ET strains possessing only ET type *tcpA* showed that the sequences were identical to reference ET N16961(accession no. AF536868.1). The nucleotide sequences of *tcpA*^{CL} of four *V. cholerae* ET strains (TCP variant ET, Mex-2174, Mex-3358, Mex-3065, and Mex-2058) were identical, with 81% sequence homology with reference CL and 74% with ET biotype *tcpA* alleles. The nucleotide sequence of the *tcpA*^{CL} allele of the Mexican *V. cholerae* ET strains matched exactly the *tcpA* sequence in GenBank for nontoxigenic *V. cholerae* O1 ZJ59 (accession no. EU622531) and LN93094 (accession no. AF512422) reported from China. The deduced amino acid sequences revealed substitutions at positions 74 [valine to isoleucine (V→I)], 99 [proline to serine (P→S)], 145 [alanine to glycine (A→G)], and 189 [threonine to alanine (T→A)], suggesting genetic differences from CL, ET, and other types of *tcpA* alleles described previously (10).

A phylogenetic tree was constructed using *tcpA* sequences of the two Mexican strains (Mex-2058 and Mex-3065) together with *tcpA* gene sequences from GenBank (Fig. S1). The Mexican and Chinese strains with analogous *tcpA* sequences formed a separate cluster to the 10 clusters for *tcpA* alleles reported to date (11). Overall, results of the analysis clearly indicate that *tcpA* of Mex-2058 and Mex-3065 have a lineage distinct from the seventh pandemic ET.

PFGE and Cluster Analysis. The results of genomic fingerprinting determined by pulsed-field gel electrophoresis (PFGE) showed Mexican *V. cholerae* ET strains carrying *tcpA*^{ET}, and ET biotype-specific CTX-prophage marker genes yielded an overall banding pattern characteristic of seventh pandemic ET N16961 (Fig. 3). *V. cholerae* CTXΦ$^-$ ET and TCP variants differed significantly from the signature PFGE banding patterns of the CTXΦ$^+$ ET isolated in Mexico between 1998 and 2008.

Cluster analysis, performed using PFGE patterns of *Not*I-digested genomic DNA, separated genetically heterogeneous CTXΦ$^-$ ET and TCP variants from pre- and post-2004 (1991 and 2008) *V. cholerae* ET, the latter comprising a cluster with Peruvian *V. cholerae* ET and *V. cholerae* N16961. The Mexican CTXΦ$^+$ *V. cholerae* ET strains (1991–2008) formed a tight cluster including ET N16961 and closely linked to *V. cholerae* ET strains from Peru.

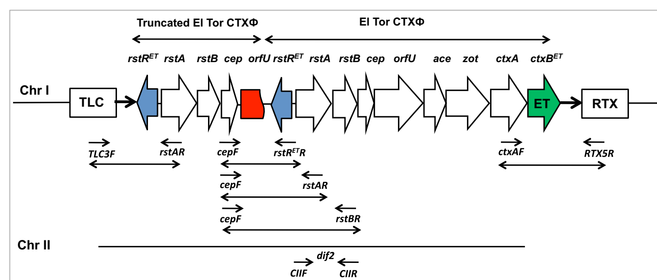

Fig. 2. Genetic mapping of CTXΦ lacking RS1 in *V. cholerae* O1 biotype El Tor strains isolated in Mexico during 2004–2008. The deduced genetic organization shows integration of an additional truncated CTXΦ in the upstream region of the CTXΦ in the large chromosome (Chr I) of *V. cholerae* whereas the *dif2* site in the small chromosome did not harbor CTXΦ. Arrows indicate transcription directions of each of the genes in the CTXΦ and flanking regions. The map is without a scale bar. $rstR^{ET}$ (blue arrow), El Tor type *rtsR*; $ctxB^{ET}$ (green arrow), El Tor type *ctxB*; *orfU* (red box), truncated *orfU* subunit; TLC, toxin-linked cryptic; RTX, repeat in toxin. Locations of primers are also shown.

Discussion

Reports of the emergence and pandemic spread of an altered *V. cholerae* El Tor (ET) suggest that the pattern of global cholera is changing, a phenomenon that appears to be occurring in Mexico, where the *V. cholerae* CL biotype of pre-1991 cholera has been isolated, as well as altered *V. cholerae* ET, the latter dominant among *V. cholerae* CL and ET biotype progenitors associated with cholera outbreaks between 1991 and 1997 (7). To date, the CL biotype may no longer be associated with human disease in Mexico; and altered ET, previously considered the major cause of endemic cholera (7), was no longer isolated after the emergence in 2000 and predominance of the $CTXΦ^-$ population, including a unique TCP variant of *V. cholerae* ET. Characterization of *V. cholerae* population in subsequent years (2004–2008) suggests another major clonal shift, with the emergence and predominance of a CTX^+ *V. cholerae* O1 ET lacking the RS1 element, with a truncated CTXΦ in the upstream region of the CTXΦ in the large chromosome.

Although the *V. cholerae* ET strains varied in PCR primer amplification for *ctxB*, *tcpA*, and *rstR* biotype-specific alleles (12, 13), overall serobiotyping results confirmed that the strains were a *V. cholerae* O1 ET seventh pandemic prototype. The genetic transitions in Asia from *V. cholerae* biotypes CL to ET (14) and ET prototype to altered ET are considered chronological events occurring in the 1980s and in 2001, respectively (4, 15, 16). In Mexico, both *V. cholerae* CL and ET biotype progenitors were isolated during cholera outbreaks, together with altered ET, dominant between 1991 and 1997 (7). The consistent association of *V. cholerae* CL, ET, and altered ET with local cholera outbreaks in the Americas between 1991 and 1997 and their occurrence in the natural aquatic environment, together with non-O1 phenotypically and genetically O1 CL, suggest that the emergence of altered *V. cholerae* ET has occurred locally in Mexico (7, 17) and is not clonal expansion from endemic regions of Asia (14), as has been proposed for Africa (5). According to our data, *V. cholerae* altered ET was not isolated in Mexico after 2000 nor was the CL biotype reported in Mexico until 1997 (7). However, *V. cholerae* altered ET continues to be routinely isolated from clinical cholera cases in Asia and Africa (4, 5, 15, 18), and both are reported to be capable of causing a more severe disease (19) and of spreading globally (20). The clonal shift observed in this study may well have implications for global cholera, considering that altered ET strains, such as those isolated from cholera cases in Asia and Africa, are dominant pandemic pathogens (5, 18, 20, 21).

The toxin coregulated pili (TCP), a receptor for lysogenic CTXΦ (22), is encoded by a 40-kb gene cluster, *Vibrio* pathogenicity island (VPI) (23). Like CTXΦ, TCP is biotype-specific, with two distinct alleles of *tcpA* encoding the major protein "pilin" (10, 11). In Mexico, the TCP variant of *V. cholerae* O1 strains carries a *tcpA* sequence that is different from *tcpA* types reported to date, including CL and ET (10, 11), although an analog of the Mexican type *tcpA* deposited in GenBank is from a nontoxigenic O1, reportedly isolated in 2006 from China. *V. cholerae* O1 strains from Mexico carrying the variant *tcpA* are nontoxigenic. Thus, nontoxigenic O1 strains with this *tcpA* can occur in geographically different ecosystems, namely Mexico and China.

As in the case of lysogenic CTXΦ that propagate by infecting susceptible nontoxigenic strains of both *V. cholerae* and *Vibrio mimicus* (22, 23), converting the host to $CTXΦ^+$, excision can also occur, transforming the host to $CTXΦ^-$ (24). In the present study, *V. cholerae* O1 strains isolated in Mexico during 2000–2004 were predominantly $CTXΦ^-$, TCP^-. Although VPI is known to be horizontally transferred in *V. cholerae* (25), it is not generally considered a virion. In any case, $CTXΦ^-$, TCP^- *V. cholerae* in Mexico can be considered to have originated from a toxigenic progenitor via excision of both CTXΦ and VPI.

Bacterial clonal switching in *V. cholerae* can have a profound epidemiological influence (14, 19). Factors playing a role in clonal selection can be biotic, abiotic, or both, in natural aquatic ecosystems where *V. cholerae* is an autochthonous presence (24, 26, 27). In the study reported here, a unique clonal shift was observed whereby toxigenic *V. cholerae* O1 in Mexico was replaced, starting in 2001, with $CTXΦ^-$, TCP^- *V. cholerae* and superseded by toxigenic *V. cholerae* in 2005. It can be hypothesized that nontoxigenic *V. cholerae* in the aquatic environment will outnumber pathogenic strains when fecal–oral transmission is brought under control by application of stringent public health measures, as was done following the 1991–1997 epidemics in Mexico (7, 17, 28). Also important to note is that many bacterial pathogens including *V. cholerae* become nonculturable after release from human host into the aquatic environment, becoming noncompetitive with environmental *V. cholerae*, a phenomenon that may have occurred in Mexico between 2001 and 2004 (26, 27). An alternative hypothesis is that the predominance of a toxigenic or nontoxigenic clone may occur if phages provide selective advantage to a *V. cholerae* subpopulation (27, 29).

Nontoxigenic *V. cholerae* O1 is associated with a mild but rather broad spectrum of human disease. A clinical link for nontoxigenic *V. cholerae* O1 ET was first reported by the US Centers for Disease Control and Prevention (CDC) between 1977 and 1991 (30). During the past three decades, there have been reports of isolation of nontoxigenic *V. cholerae* O1 from clinical, sewage, oyster, and surface-water samples collected in several countries, including Bangladesh, Guam, Brazil, Peru, Japan, England, the United States, and Mexico (7, 30). CT^- *V. cholerae* O1 strains occur sporadically as the causative agent of cholera outbreaks, but not at as high a frequency as in Mexico between 2000 and 2004. It is important to note that nontoxigenic *V. cholerae* O1 was associated with a large cluster of cases of cholera in India (31). In addition to *V. cholerae* O1 CT^- strains, currently available data suggest that a significant proportion of non-O1 strains possessing CT or non–O1-specific heat-stable enterotoxin (NAG-ST) are associated with cases of diarrhea (32).

In toxigenic *V. cholerae* O1 ET and O139 Bengal strains, the CTXΦ genome often is flanked by satellite phage RS1 carrying genes *rstA, rstB, rstC,* and *rstR* that determine integration of plasmids transporting portions of CTXΦ into the bacterial genome (23). The RS2 genetic element also is a satellite phage but differs from RS1 in lacking *rst*C (22). *V. cholerae* O1 CL biotype CTX prophages lack RS elements and exist either as a solitary prophage or as arrays of two truncated, fused prophages (8). *V. cholerae* O1 ET and O139 Bengal strains carrying RS1 generally yield infectious CTXΦ, but CL biotype strains do not. *V. cholerae* altered ET carrying the *ctxB* allele of the CL biotype recently was reported to have been isolated in Bangladesh (Matlab variant) and Mozambique (Mozambique variant), lacking RS1 (8, 18). Pathogenic *V. cholerae* ET strains isolated in Mexico between 2004 and 2008 lack RS1, but, unlike the Matlab and Mozambique

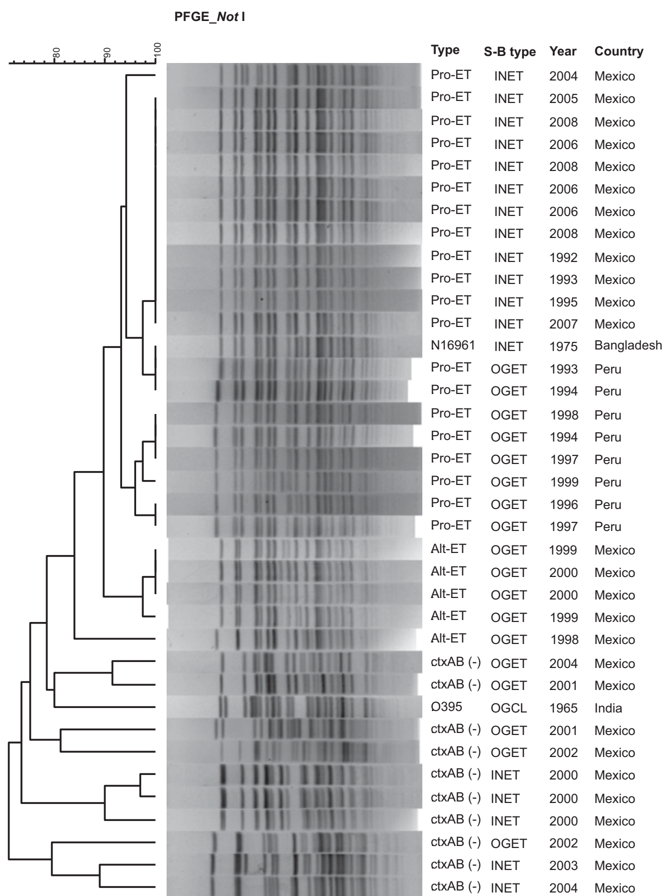

Fig. 3. Dendrogram of PFGE patterns of *V. cholerae* O1 prototype ET and transitional CTX^{-} ET possessing TCP of ET or CL biotype. S-B type, sero-biotype. The tight clustering of Mexican *V. cholerae* O1 prototype ET strains indicates clonality whereas CTX^{-} ET and transitional TCP variants of ET demonstrate heterogeneous patterns, indicating divergence. Peruvian prototype ET strains clustered differently from Mexican prototype *V. cholerae* ET (not identical because differentiating regional signatures were observed in the patterns). Alt-ET, altered ET; INET, Inaba ET; OGET, Ogawa ET; Pro-ET, prototype ET.

variants (8, 18), the *ctxB* allele of these strains is an ET biotype. In addition, ET biotype strains isolated in Mexico during 2004–2008 exhibited a novel genetic array, with a truncated CTX prophage instead of RS1 element in the upstream region of the ET-specific CTXΦ located in the large chromosome (Chr I) of *V. cholerae*. It has been proposed that gene capture via plasmids or phages contributes to rapid adaptation and evolution in *V. cholerae* (33), a probable situation in Mexico, with clonal CTX^{+} ET displacing all other preexisting subtypes (7). Although the epidemiological significance of the newly emerged *V. cholerae* CTX^{+} ET in Mexico is yet to be understood, the truncated CTXΦ upstream could surrogate RS elements in facilitating replication of the lysogenic CTXΦ genome, producing infectious phage particles.

An aquatic reservoir of *V. cholerae* in the Americas has previously been documented, including association of *V. cholerae* with plankton in coastal waters of Peru and Mexico (26, 28, 34). Although the *V. cholerae* ET isolates causing epidemic cholera in Latin America in 1991 were initially considered to be homogeneous (3), divergence was demonstrated soon thereafter (2, 35, 36). Divergence was clearly evident among *V. cholerae* ET in Peru (6) and Mexico, and *V. cholerae* associated with the 1991 epidemic and subsequent endemic cholera in Mexico comprises a diverse population of serogroup O1 strains, including biotypes CL and ET, together with altered variant ET (7). *V. cholerae* O1 endemic cholera along the Gulf of Mexico coast after 1997 recently has been confirmed, with changes in ribosomal patterns separating strains into two distinct chronological groups, those isolated before and after 1997. Results presented here are in agreement with those of Lizarraga-Partida et al. (28), who described a new CTX prophage^{-} *V. cholerae* lineage emerging shortly after 1997 in Mexico and continuing to displace preexisting *V. cholerae* O1 CL and ET strains, including altered ET strains (7). The CTX^{+} *V. cholerae* ET with a truncated CTX prophage isolated during endemic cholera in Mexico may prove to be a historical point in the global epidemiology of cholera.

Materials and Methods

Bacterial Strains. *Vibrio cholerae* O1 strains included in the present study (n = 91) together with source, location, and year of isolation (Table 1), were provided by the Department of Public Health, Faculty of Medicine, National Autonomous University of Mexico (UNAM) and Centro de Investigación Científica y de Educación Superior de Ensenada. The strains were isolated from cholera patients (n = 36) and from surface-water samples (n = 55) as part of a nationwide cholera surveillance program conducted between 1998 and 2008. These isolates were identified as *V. cholerae* using a combination of biochemical and molecular procedures, as described previously (24).

Serogroup Analysis. *V. cholerae* strains were screened serologically by slide agglutination, using polyvalent antisera specific for *V. cholerae* O1 or O139, followed by monoclonal antibody specific for each serogroup (1). Serogroups were reconfirmed using polyvalent O1 and monovalent Inaba and Ogawa antisera (7).

Biotype Analysis. *V. cholerae* O1 strains were screened for chicken erythrocyte agglutination, sensitivity to polymyxin B, and Mukerjee classical (CL)-specific phage IV, and Mukerjee El Tor (ET)-specific phage V (1).

Storage. All *V. cholerae* strains were subcultured on gelatin agar (GA), and a single representative colony from each GA plate was inoculated into T1N1 broth (1% Trypticase and 1% NaCl), incubated at 37 °C for 4–6 h, and stored at −80 °C with 15% (vol/vol) glycerol for future use.

Complementation of Serogrouping and Biotyping Results by PCR Assays. Genomic DNA was extracted from each of the *V. cholerae* strains following previously described methods (7). *V. cholerae* strains identified primarily by phenotypic characteristics were reconfirmed by PCR for species-specific *ompW* (7). Serogroup-specific O biosynthetic *wbe*O1, *wbf*O139, and cholera toxin subunit A encoding *ctxA* were also examined by multiplex PCR (37). Furthermore, PCR targeting biotype-specific genetic markers, including *tcpA* (CL or ET), *hlyA* (CL or ET), and *rstR* (CL or ET) (13), were performed to complement phenotypic test results. Mismatch amplification mutation assay (MAMA)-PCR was performed to determine biotype-specific *ctxB*, as described previously (38).

DNA Sequencing of *ctxB* and *tcpA*. Nucleotide sequencing of *ctxB* and *tcpA* of randomly selected representative strains was carried out using an ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer Applied Biosystems) on an ABI PRISM 310 automated sequencer, as described previously (4). Nucleotide sequences were compared with the corresponding sequences of *V. cholerae* ET N16961 (GenBank accession no. NC_002505.1), and *V. cholerae* CL O395 (GenBank accession no. CP001235.1), retrieved from GenBank by Basic Local Alignment Search Tool (BLAST).

Genetic Analysis of CTXΦ and Flanking Region. Mexican *V. cholerae* ET strains possessing $ctxB^{ET}$ and $rstR^{ET}$ and unable to amplify *rstC* of the RS1 element were further analyzed using primers *rstAF*/*rstCR* targeting the RS1 element (39). *V. cholerae* N16961, possessing RS1 downstream of CTXΦ, and *V. cholerae* O395, lacking RS1, were used as controls, considering that N16961 has CTXΦ only in the large chromosome, whereas O395 possesses CTXΦ in both the large and small chromosomes. The genetic structure of CTXΦ and its flanking region in representative El Tor strains harboring $ctxB^{ET}$ and $rstR^{ET}$ was analyzed using similar primers and primer walking and sequencing as described previously (39, 40). Primers cepF/rstR^{ET}R, cepF/rstAR, and cepF/rstB were used to detect truncated CTXΦ in strains lacking RS1 element and tandem repeat of CTX prophage. The ∼1,200-bp amplicon for the cepF/rstR^{ET}R primers was sequenced for confirmation of truncated CTXΦ and subsequently was deposited in GenBank under accession no. KC952008.

Phylogenetic Analysis of *tcpA*. To determine the genetic relatedness of *tcpA* in the Mexican *V. cholerae* O1 strains, *tcpA* of representative ET, CL, and TCP variants was sequenced, using primers and PCR conditions as described previously (11). Multiple sequence alignment of trimmed *tcpA* (600 bp, encoding mature protein) and phylogenetic analysis of *tcpA* alleles were done using the ClustalW multiple alignment program and the Molecular Evolutionary Genetic Analysis program, version 5.05 (MEGA5), respectively. *TcpA* sequences of two Mexican strains (Mex-2058 and Mex-3065) were used for construction of a phylogenetic tree with 33 *tcpA* sequences, of which 31 were of serogroup O1 and non-O1/non-O139 from a previous study (11), and two of nontoxigenic O1 strains from China (ZJ59 and LN93097, accession nos. EU622531 and AF512425, respectively, in GenBank). A neighbor-joining tree with 1,000 bootstrap was generated to determine the phylogenetic relationships on the basis of *tcpA* sequences.

Pulsed-Field Gel Electrophoresis. Agarose-embedded genomic DNA was prepared from each of the *V. cholerae* strains. Genomic DNA was digested by *Not*I restriction enzyme (GIBCO-BRL). *Salmonella braenderup* DNA digested by *Xba*I served as molecular size markers. PFGE was carried out using a contour-clamped homogeneous electrical field (CHEF-DRII) apparatus (Bio-Rad), following procedures described elsewhere (6, 41). Gel fingerprint patterns were analyzed using the Bionumeric Software Package (Applied Maths). After background subtraction and gel normalization, fingerprint patterns were subjected to typing based on banding similarity and dissimilarity, using Dice similarity coefficient and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering, as recommended by the manufacturer. Results are graphically represented as a dendrogram.

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