Genetic characteristics of Matlab variants of *Vibrio cholerae* O1 that are hybrids between classical and El Tor biotypes

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The Matlab variants of *Vibrio cholerae* O1, defined as hybrids between the classical and El Tor biotypes, were first isolated from hospitalized patients with acute secretory diarrhoea in Matlab, a rural area of Bangladesh. These variants could not be categorized as classical or El Tor biotypes by phenotypic and genotypic tests, and had representative traits of both the biotypes. A number of virulence-associated genes and/or gene clusters were screened by PCR and DNA sequencing. El Tor-specific gene clusters, *Vibrio* seventh-pandemic islands (VSP)-I and -II and repeat toxin (RTX) were present in the genome of these variants, indicating their El Tor lineage, whereas the nucleotide-sequence-derived CtxB amino acid sequence of these strains grouped them under the classical biotype. Matlab variants possessed all the necessary genes to initiate pandemics. The genetic relatedness of Matlab variants to the *V. cholerae* strains recently isolated in Mozambique is another important observation of this study, which underscores the epidemiological significance of Matlab variants.

Received 20 April 2006

Accepted 18 July 2006

INTRODUCTION

Vibrio cholerae belonging to serogroups O1 and O139 can cause the severe watery diarrhoea known as cholera. To date, more than 200 serogroups of *V. cholerae* have been recognized, based on variable somatic O antigen composition; O1 was the only known epidemic serogroup of *V. cholerae* up to 1991. In 1992, serogroup O139 was recognized as the second epidemic serogroup of *V. cholerae* (Albert *et al.*, 1993; Shimada *et al.*, 1993). There are two well-established biotypes within the *V. cholerae* O1 serogroup, classical and El Tor, which are distinguished from each other by several properties, including haemolysis of sheep red blood cells, agglutination of chicken red blood cells, Voges-Proskauer reaction, and susceptibility to polymyxin B and to biotype-specific phages (Kaper *et al.*, 1995). The first six cholera pandemics are believed to have been caused by the

classical biotype, and in 1961, *V. cholerae* strains of El Tor biotype initiated the seventh cholera pandemic, which is still continuing.

Different studies have shown that the virulence of *V. cholerae* O1/O139 involves a number of genes and/or gene clusters. Two large DNA regions, the *Vibrio* pathogenicity island (VPI)-1 and the CTX prophage, encode the major virulence factors in *V. cholerae* O1/O139 (Faruque *et al.*, 1998). The *tcpA* gene of the VPI-1 encodes the major pilin protein which is involved in colonization of *V. cholerae* in the host intestine and also acts as the receptor for CTXΦ to infect *V. cholerae* strains. The core part of the CTXΦ is composed of the genes *psh*, core-encoded pillin (*cep*), *gIII^{CTX}*, *ace* (accessory cholera enterotoxin), *zot* (zonula occludens) and *ctxAB* (Davis & Waldor, 2003). The key virulence factor of *V. cholerae*, cholera toxin (CT), is encoded by the *ctxAB* genes. *cep* is reported to encode a minor coat protein, the function of which is not yet known. Ace and Zot are two CTXΦ-encoded proteins that play a role in phage assembly (Faruque *et al.*, 1998). The *gIII^{CTX}* gene plays a role in the processes of phage infection and replication (Heilpern & Waldor, 2003). The core part of CTXΦ is usually flanked by a 2.5 kb RS2 region containing

Abbreviations: CCA, chicken red cell agglutination; CT, cholera toxin; MSHA, mannose-sensitive haemagglutinin; RTX, repeat toxin; TLC, toxin-linked cryptic; VPI, *Vibrio* pathogenicity island.

The GenBank/EMBL/DDBJ accession numbers for the *ctxB* gene sequences of *Vibrio cholerae* O1 and O139 are DQ523199–DQ523219.

three ORFs, namely *rstA*, *rstB* and *rstR*, which function in the replication, regulation and integration processes, respectively. In El Tor and O139 strains of toxigenic *V. cholerae* only, CTX Φ is often flanked by an element termed the RS1 element (Waldor *et al.*, 1997; Davis *et al.*, 2000). The RS1 and RS2 regions are similar, except for the *rstC* gene, which is present in the RS1 element alone (Waldor *et al.*, 1997). Mannose-sensitive haemagglutinin (MSHA) is another virulence factor, and encodes a type IV pilus and consists of six ORFs (Jonson *et al.*, 1991; Faruque *et al.*, 1998). Repeat toxin (RTX) is also reported to be virulence-associated, and encodes a protein that has cytotoxic activity (Lin *et al.*, 1999). This cluster comprises four ORFs, *rtxABCD*, of which the *rtxC* gene is seen in the El Tor biotype only. Dziejman *et al.* (2002) have recently reported two other clusters of genes, namely *Vibrio* seventh pandemic island (VSP)-I and -II, which are unique to the *V. cholerae* seventh pandemic El Tor strains. The other genes that may be linked to the pathogenicity of *V. cholerae* include *hlyA*, which encodes a haemolysin that is cytolytic for a variety of erythrocytes and mammalian cells in culture and is rapidly lethal for mice (Kaper *et al.*, 1995); toxin-linked cryptic (TLC), a 4.7 kb DNA fragment whose functional relationship to CTX Φ is not yet understood (Rubin *et al.*, 1998); *pilE*, which encodes a putative fimbrial assembly protein (Heidelberg *et al.*, 2000); and *intI4*, which encodes a previously unknown integrase that recognizes a family of *V. cholerae* repeated sequences (VCRs) associated with a 'gene-VCR' organization, similar to that of the well-characterized antibiotic-resistance integrons (Mazel *et al.*, 1998; Recchia & Hall, 1997).

In our previous studies, we described *V. cholerae* O1 strains that possess both the classical and El Tor attributes and designated these strains as Matlab types I, II and III, as they were first detected in Matlab, a rural area in Bangladesh (Nair *et al.*, 2002). Different phenotypic traits (haemolysis of sheep erythrocytes, agglutination of chicken erythrocytes, sensitivity to polymyxin B and to specific phages, and reaction to the Voges–Proskauer test) and genotypic traits (*ctxA*, *acfB*, *tcpA* and *rstR*) examined in the previous study failed to classify them into classical or El Tor biotypes (Nair *et al.*, 2002). Further characterization of the Matlab type strains by PFGE revealed that type I strains had a classical biotype lineage, whereas types II and III were more closely related to the El Tor biotype (Safa *et al.*, 2005). In this study, we employed extensive PCR-based screening and a DNA-sequence-based approach to examine the different virulence genes and/or gene clusters of Matlab variants and other similar strains, and compared the results with those obtained with reference strains of the classical and El Tor biotypes.

METHODS

Bacterial strains. From the 38 strains of *V. cholerae* O1 and O139 Matlab variants available in our collection, 17 were included in this study. Of these, five *V. cholerae* O1 strains were isolated

between 1992 and 1994, and were previously designated Matlab type I (MJ1236, MJ1485), II (MG116226) and III (MG116025, MG116926). The remaining 12 strains (10 *V. cholerae* O1 and two *V. cholerae* O139) were isolated between 1994 and 1997, and have been characterized previously (Nusrin *et al.*, 2004). Three representative strains from previously characterized strains of *V. cholerae* O1 El Tor biotype, isolated in 2004 from Beira, Mozambique, were also included in this study (Ansaruzzaman *et al.*, 2004). All strains were grown in Luria–Bertani (LB) broth and stored as frozen stocks in LB broth with 25%, v/v, glycerol. *V. cholerae* strain N16961 of El Tor biotype and strain 569B of classical biotype were used as reference strains.

Biotyping. Tests for polymyxin B susceptibility, chicken red cell agglutination (CCA) and phage sensitivity were performed using standard procedures (WHO, 1987).

Genomic DNA isolation and purification. Genomic DNA was extracted and purified from the strains following the method of Sambrook *et al.* (1989), with some modifications. In brief, cells were harvested by centrifugation from overnight-grown cultures in LB and treated with TES (10 mM Tris, pH 8.0, 10 mM EDTA, 100 mM NaCl) and 10% SDS at 65 °C for 10–15 min. After proteinase K treatment at 50 °C for 18 h, DNA was extracted with phenol/chloroform–isoamyl alcohol (25:24:1) and purified by ethanol precipitation, and dried before dissolving in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). RNase treatment was performed at 37 °C for 1–2 h, and final purification was done by ethanol precipitation. The purified DNA was dissolved in TE buffer and stored at –20 °C, and used for PCR analysis. The purity of the DNA was assayed with a spectrophotometer (GeneQuant) by using automatic calculation of the ratio of A_{260} to A_{280} .

PCR analysis. PCR was used to assay for 11 virulence-associated genes and/or gene clusters in the genome of the 20 *V. cholerae* isolates using 31 sets of PCR primers and conditions described previously (Chow *et al.*, 2001; O'Shea *et al.*, 2004; Rivera *et al.*, 2001). Further, using PRIMER3 software (available at http://www.genome.wi.mit.edu/genome_software/other/primer3.html), six sets of primers were designed (Table 2) to amplify regions that are uniquely present in all El Tor strains but absent in classical strains (Dziejman *et al.*, 2002). PCR was performed in a 25 μ l reaction mixture as follows: an initial denaturation step at 96 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, 1 min of primer extension at 72 °C and 7 min of final extension at 72 °C for one cycle. Amplicons were separated by agarose gel electrophoresis (1%) in 0.5 $\times$ Tris/borate/EDTA buffer. Products were stained with ethidium bromide, destained with distilled water, and visualized under UV light and photographed on a gel-documentation system.

PCR amplification and nucleotide sequencing of *ctxB*. PCR amplification of the *ctxB* gene was performed in a 25 μ l reaction mixture in an automated Peltier thermal cycler (PTC-200, MJ Research). PCR primers and conditions were as previously described (Mitra *et al.*, 2000). PCR products were purified with a Microcon centrifugal filter device (Millipore) according to the manufacturer's instructions, and a cycle sequencing reaction was performed in a 20 μ l reaction mixture containing 40 ng purified PCR product, 4 μ l ready reaction mixture and 3.2 pmol primer. After 25 cycles of amplification, unincorporated nucleotides were removed from the reaction mixture by ethanol precipitation. Dried products were then resuspended in template suppression reagent (TSR) and sequenced based on the dideoxynucleotide chain-termination method with an ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer Applied Biosystems) on an ABI PRISM 310 automated sequencer.

DNA and protein sequence analysis. The chromatogram sequencing files were inspected using Chromas 2.23 (Technelysium). Nucleotide sequences of the test isolates were compared with the corresponding sequences of the N16961 El Tor reference strain (NC_002505), the 569B classical reference strain (U25679), the O139 reference strain (X76391), and the B33 (AY648939) and B65 (AY648940) strains, retrieved from GenBank using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997). Multiple sequence alignments were developed using CLUSTALX 1.81.13, and DNA sequences were translated using GeneDoc version 2.6.002 alignment editor.

RESULTS

All strains tested were resistant to polymyxin B and positive for CCA. Phage typing of seven of the 10 *V. cholerae* O1 strains tested showed resistance to phage IV and susceptibility to phage 5. The remaining strains were resistant to both phages (Table 1). We examined for the presence of the three important virulence-associated genes, namely *tcpA*, *toxT* and *acfB*, of the VPI-1 cluster. Fifteen strains, including three previously characterized strains from Mozambique, showed the presence of *tcpA* of El Tor type, and the remaining five Matlab reference strains (MT-I, MT-II and MT-III) showed *tcpA* of the classical type (Table 1). The remaining two genes were positive for all strains. All strains, including the reference strains, were positive for *rstA*, *gIII^{CTX}* and *zot* located in the CTX prophage region. Seven strains, including the El Tor N16961 reference strain, were positive for the *rstC* gene, indicating the presence of the RS1 element, while the remaining strains, including the classical reference strain 569B, were negative for *rstC* (Table 1). PCR analysis of all strains tested showed the presence of the MSHA gene cluster. All test strains, including El Tor N16961, showed a positive amplification for the *rtxC* gene of the RTX gene cluster, whereas only classical 569B reference strain was negative for the same gene. Another gene in this cluster, *rtxA*, was present in all the test strains, including classical and El Tor reference strains. Genes in the VSP-I and -II gene clusters were present in all the strains, including the El Tor reference strain, but were absent from the classical reference strain. Four pairs of primers were used to examine four individual loci, namely *hlyA*, *pilE*, *tlc* and *int14*. Of these, *pilE* and *int14* were present in all strains tested, including the classical and El Tor reference strains, *hlyA* was present in all except two strains, whereas *tlc* was absent in all strains that were negative for *rstC* (Table 1). PCR analysis of all the six loci that are unique to El Tor strains but absent in classical strains showed the expected size of PCR product in all Matlab type reference strains (MT-I, MT-II and MT-III), including the El Tor reference strain (Fig. 1), but none in the classical reference strain. The deduced amino acid sequence of the CtxB subunit of the single representative strain of Matlab type II and one of two Matlab type III representative strains showed 100% homology to the N16961 El Tor reference strain of genotype 3, which has tyrosine at position 39, phenylalanine at position 46 and isoleucine at position 68 (Table 1). In contrast, the amino acid sequence of the CT-B subunit of the remaining 12 strains, including two

O139 strains, was identical to that of the 569B classical reference strain of genotype 1 (histidine at position 39, phenylalanine at position 46 and threonine at position 68) (Table 1).

DISCUSSION

Molecular studies have shown that the two biotypes of *V. cholerae* O1 originated from separate lineages (Kaper *et al.*, 1982; Karalois *et al.*, 1995). Over time, some of the biotype-specific phenotypic traits have shown variable reactions, as seen in the 1960 pandemic El Tor isolates of *V. cholerae*, which lyse sheep or goat erythrocytes, whereas classical strains are non-haemolytic. The haemolytic trait is now of limited value in differentiating biotypes, since El Tor strains isolated in later years are mostly non-haemolytic (Barrett & Blake, 1981). However, in 1994, El Tor strains from the Gulf Coast of the USA were reported to be strongly haemolytic (Kaper *et al.*, 1995). In our previous study, biotyping of *V. cholerae* O1 strains isolated in Matlab showed variable reactions and therefore we were unable to biotype these strains. We therefore resorted here to genetic screening of different virulence markers in order to understand better the genetic organization of Matlab variants.

The VPI-1 is a large DNA region that is composed of two gene clusters, namely toxin-coregulated pilus (TCP) and accessory colonization factor (ACF) (Herrington *et al.*, 1988; Kovach *et al.*, 1996). The *tcpA* gene of the TCP cluster has alleles specific for classical and El Tor biotypes of O1. In this study, the *tcpA* gene analysis showed variable results, and either a single allele or both the alleles were found among the strains tested (Table 1). Another important gene of this cluster is *toxT*, which encodes a second positive regulatory protein that directly activates a number of virulence genes (DiRita *et al.*, 1996). This gene was present in all the strains tested and indicated the presence of an active virulence-gene regulatory system. Immediately adjacent to and downstream of the TCP cluster the ACF gene cluster is located. This cluster comprises four ORFs (ABCD), and the presence of *acfB*, which encodes a potential colonization factor, in all the strains is significant from the viewpoint of virulence potential. The genes of the MSHA cluster are expressed by strains of the El Tor biotype, but are only rarely expressed by strains of the classical biotype (Jonson *et al.*, 1991). However, all strains in this study were positive for the MSHA gene cluster, including the classical reference strain. The *hlyA* gene is reported to be present in all classical, El Tor and non-O1 strains of *V. cholerae* (Brown & Manning, 1985). In this study, two strains were negative by PCR for this gene, and a further examination can indicate whether the gene is deleted or is present in a mutated form in these two strains. The presence of all these genes, including *pilE* and *int14*, further indicated the pathogenic potential of these strains.

CT genes were previously identified in all strains examined here (Ansaruzzaman *et al.*, 2004; Nair *et al.*, 2002; Nusrin

Table 1. Phenotypic and genotypic traits of *V. cholerae* O1 and O139 strains

Abbreviations: R, resistant; S, sensitive; C, classical; E, El Tor; Cal, Calcutta type; Env, environmental type; +, positive; –, negative.

Strains	Geographic origin	Year of isolation	Sero-group	Phenotype				Sequence type	Genotype*							
				Polymyxin B	CCA IV	Phage 5	CtxB subunit sequence	VSP-I (VCO175 VCO180)	Hly (VCO489)	VSP-II (VCO490 VCO517)	VPI-1 (<i>tcpA</i>)	RTX (<i>rtxC</i>)	RS1 (<i>rstC</i>)	CTX (<i>rstR</i> †)	TLC (<i>tlc</i>)	
MJ1236 (MT-I)‡	Bangladesh	1994	O1	R	—	R	R	39 (H), 68 (T)	+	+	+	C	+	—	C	—
MJ1485 (MT-I)‡	Bangladesh	1994	O1	R	—	R	R	39 (H), 68 (T)	+	+	+	C	+	—	C	—
MG116226 (MT-II)‡	Bangladesh	1991	O1	S	—	R	S	39 (Y), 68 (I)	+	+	+	C	+	+	E	+
MG116025 (MT-III)‡	Bangladesh	1991	O1	R	—	R	S	39 (Y), 68 (I)	+	+	+	C	+	+	E	+
MG116926 (MT-III)‡	Bangladesh	1991	O1	R	—	R	S	39 (H), 68 (T)	+	—	+	C	+	+	C, E	+
ML3090	Bangladesh	1996	O1	R	+	R	S	39 (H), 68 (T)	+	—	+	E	+	—	C	—
MJ206	Bangladesh	1994	O1	R	+	R	R	39 (H), 68 (T)	+	+	+	E	+	—	C	—
MJ4298	Bangladesh	1994	O1	R	+	R	S	39 (H), 68 (T)	+	+	+	E	+	—	C	—
MJ5002	Bangladesh	1994	O1	R	+	R	S	39 (H), 68 (T)	+	+	+	E	+	—	C	—
MJ5340	Bangladesh	1994	O1	R	+	R	S	39 (H), 68 (T)	+	+	+	E	+	—	C	—
MJ2894	Bangladesh	1994	O1	R	+	R	R	39 (H), 68 (T)	+	+	+	E	+	—	C	—
MJ591	Bangladesh	1994	O1	R	+	R	R	39 (H), 68 (T)	+	+	+	E	+	—	C	—
MJ382	Bangladesh	1994	O1	R	+	R	S	39 (H), 68 (T)	+	+	+	E	+	—	C	—
MJ1347	Bangladesh	1994	O1	R	+	R	S	39 (H), 68 (T)	+	+	+	E	+	+	C, E	+
MP2071	Bangladesh	1997	O1	R	+	R	S	39 (H), 68 (T)	+	+	+	E	+	+	E, Env	+
MP1950	Bangladesh	2000	O139	R	+	S	S	39 (H), 68 (T)	+	+	+	E	+	+	C, E, Cal	+
MP2044	Bangladesh	2000	O139	R	+	S	S	39 (H), 68 (T)	+	+	+	E	+	+	C, Cal	+
B33‡	Mozambique	2004	O1	R	+	R	S	39 (H), 68 (T)	+	+	+	E	+	—	C	—
B64‡	Mozambique	2004	O1	R	+	R	S	39 (H), 68 (T)	+	+	+	E	+	—	C	—
B180‡	Mozambique	2004	O1	R	+	R	S	39 (H), 68 (T)	+	+	+	E	+	—	C	—
Classical 569B	India	1948	O1	S	—	S	R	39 (H), 68 (T)	—	+	—	C	—	—	C	+
El Tor N16961	Bangladesh	1971	O1	R	+	R	S	39 (Y), 68 (I)	+	+	+	E	+	+	E	+

*Other genes and/or gene clusters, namely MSHA, VPI-1, RTX, *rstA*, *gIII*^{CTX}, *zot*, *ctxAB* and *int14* were also tested in this study and were positive for all strains.†*rstR* results have been previously published in Ansaruzzaman *et al.* (2004); Nair *et al.* (2002); Nusrin *et al.* (2004).‡Polymyxin B, CCA, phage IV and phage 5 results for these strains have been previously published in Ansaruzzaman *et al.* (2004); Nair *et al.* (2002); Nusrin *et al.* (2004).

Table 2. Oligonucleotide primer sequences used in PCR assays for six genetic loci present only in El Tor strains of *V. cholerae*

Annotation no.	Primer sequence	No. of bases	Product size (bp)	Function (Dziejman <i>et al.</i> , 2002)
VC1449F	CAGAAATCCAGCAGCAACAA	21	266	Hypothetical protein
VC1449R	TGGGGAAATTTATGATGGAGA	20		
VCA0316F	TCATGCTGATAGCCTGCATT	20	335	Putative acetyl transferase
VCA0316R	TATGCCTATCCCCAATCTCG	20		
VCA0417F	CCAAGCTCTCGGAATCCATA	20	249	Putative acetyl transferase
VCA0417R	CTATCCCCAATCTCGCTCAG	20		
VCA0728F	CCGGTTCGTCAAATCTTCAT	20	758	Hypothetical protein
VCA0728R	TAAAGCGCTCTCAGGTGGTT	20		
VCA0729F	CCAGCATCCACAGAAACCTT	20	462	Hypothetical protein
VCA0729R	TGTGTCTGTCCATTGCCACT	20		
VCA0730F	CACCTTCCAGCTCTTGTTCC	20	486	Hypothetical protein
VCA0730R	ATACCTACGCCTTGACACCAC	20		

et al., 2004). However, PCR identification of other neighbouring genes (*rstA*, *gIII*^{CTX} and *zot*) indicated the presence of an intact core region of the CTX prophage genome. *rstR* is a regulatory gene and present in the RS2 part of the CTX prophage. This gene has four variants: *rstR*^{classical}, *rstR*^{El Tor}, *rstR*^{Calcutta} and *rstR*^{environmental}, and our previous studies (Ansaruzzaman *et al.*, 2004; Nair *et al.*, 2002; Nusrin *et al.*, 2004) have shown that either a single *rstR* variant or an assortment of *rstR* variants is present in all the strains tested. Kimsey *et al.* (1998) first reported the presence of different types of *rstR* in a single *V. cholerae* strain, but the implication of this assortment is not yet known. Faruque *et al.* (2002) have recently shown that RS1 is a self-transducing phage, and that the *rstC* gene acts as an antirepressor in the phage replication process (Davis & Waldor, 2003). This gene is unique to the strains of the El Tor biotype and is absent in classical strains. Thus, we checked all the strains for the *rstC*

gene and found that 10 strains, including Mozambique strains, lacked the *rstC* gene (Table 1). In other words, the absence of the *rstC* gene classified these strains as classical type, which is contrary to the earlier findings with the *tcpA* gene, but matches the *rstR* result obtained in our previous studies (Ansaruzzaman *et al.*, 2004; Nusrin *et al.*, 2004). Notably, all the *rstC*-negative strains were also negative for the TLC (Table 1), and require further examination to understand the relationship.

Recent studies have reported that *rtxC*, which encodes the activator protein, is absent from strains of classical biotype and present in the El Tor biotype only (Dziejman *et al.*, 2002; Lin *et al.*, 1999; O'Shea *et al.*, 2004). The presence of the *rtxC* gene grouped all the strains as El Tor biotype. The VSP-I gene cluster encompasses a 16 kb region from VC0175 to VC0185, and most of the genes encode hypothetical or

Fig. 1. Gel image showing amplified genes indicated by the annotation numbers given in Table 2. Lane M, molecular size marker (100 bp ladder); lanes 1–5, 8–11, 14–18, 21–25, 28–32 and 34–38 represent Matlab type strains MJ1236 (MT-I), MJ1485 (MT-I), MG116226 (MT-II), MG116025 (MT-III) and MG116926 (MT-III), respectively; lanes 6–7, 12–13, 19–20, 26–27, 33–34 and 39–40 represent *V. cholerae* El Tor N16961 and the classical 569B reference strain.

conserved proteins with no known function. On the other hand, the VSP-II region is a ~27 kb region that encompasses VC0490 to VC0516 (O'Shea *et al.*, 2004). As these two clusters are unique to the El Tor strains of the seventh pandemic, finally we checked for the presence of VSP-I and -II in the genome of these strains. All strains examined were positive for VSP-I and -II, pointing to the seventh-pandemic El Tor ancestry of these strains. The presence of VSP-I and -II also indicated that these strains have the potential to initiate pandemic spread, as these two regions are thought to be associated with the pandemicity of the El Tor biotype. Dziejman *et al.* (2002) also identified six genetic loci that are unique to strains of the El Tor biotype but absent from the classical biotype (Fig. 1). All these loci encode either hypothetical proteins or a putative acetyl transferase. All the six loci were present in all strains tested, except the classical reference strain, which further underscores the El Tor lineage of these strains.

On the basis of DNA and protein sequencing, heterogeneity within the B subunit was first reported in the early 1990s (Brickman *et al.*, 1990; Kaper *et al.*, 1995), and since then *ctxB* typing has been used as a tool for differentiating *V. cholerae* strains. To date, three *ctxB* genotypes of *V. cholerae* O1 strains of both biotypes and serotypes have been identified globally (Mekalanos *et al.*, 1993; Olsvik *et al.*, 1993). Based on the amino acid residue substitution at the three positions 39, 46 and 68, all classical and El Tor strains from the Gulf Coast of the USA are categorized as *ctxB* of genotype 1, El Tor strains associated with the Australian environmental reservoir are genotype 2, and El Tor strains of the seventh pandemic and the recent Latin American epidemic are classified as *ctxB* genotype 3. In this study, all except two strains examined possessed the classical type of *ctxB* DNA sequence or genotype 1, which is in contrast to the results obtained by PCR from different virulence-associated genetic regions. Notably, the PFGE results from our previous study (Safa *et al.*, 2005) that clustered reference strains (MT-I, MT-II and MT-III) of Matlab variants with the El Tor biotype also disagree with the *ctxB* typing result.

The overall analysis showed that in all these Matlab variants most of the genetic traits tested are El Tor-like, indicating that these have an El Tor genome backbone. In contrast, the classical biotype attributes in these strains are mainly confined to the CTX prophage region, which is typically the classical CTX prophage. Lee *et al.* (2006) have recently shown that the Mozambique strains of El Tor biotype carry a tandem array of the classical prophages. Furthermore, in 1961, with the beginning of the seventh pandemic, the classical strains were gradually replaced by the El Tor biotype, and in Bangladesh the replacement started from 1968 and ended by 1973. However, the classical biotype reappeared in 1982 (Samadi *et al.*, 1983) in Bangladesh and caused disease until 1993. Interestingly, the Matlab variants were isolated during a period when the classical biotype was disappearing from Bangladesh for the second time. The hybrid make-up of these strains thus appears to be built on

the El Tor backbone, in which classical biotype attributes might have been acquired through horizontal gene transfer from classical strains that were on the verge of extinction. The similarity of PCR results and identical DNA sequence of the *ctxB* gene of these Matlab variants to those of the strains isolated during a recent outbreak in Mozambique are also key observations of this study which underscore the epidemiological significance of these variants.

ACKNOWLEDGEMENTS

The Japan Health Science Foundation funded this study. ICDDR,B acknowledges with gratitude the commitment of the Japan Health Science Foundation to the Center's research efforts.

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