Multilocus sequence typing (MLST) analysis of *Vibrio cholerae* O1 El Tor isolates from Mozambique that harbour the classical CTX prophage

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Vibrio cholerae O1 isolates belonging to the Ogawa serotype, El Tor biotype, harbouring the classical CTX prophage were first isolated in Mozambique in 2004. Multilocus sequence typing (MLST) analysis using nine genetic loci showed that the Mozambique isolates have the same sequence type (ST) as O1 El Tor N16961, a representative of the current seventh cholera pandemic. Analysis of the CTX prophage in the Mozambique isolates indicated that there is one type of *rstR* in these isolates: the classical CTX prophage. It was also found that the *ctxB-rstR-rstA-rstB-phs-cep* fragment was PCR-amplified from these isolates, which indicates the presence of a tandem repeat of the classical CTX prophage in the genome of the Mozambique isolates. The possible origin of these isolates and the presence of the tandem repeat of the classical prophage in them implicate the presence of the classical CTX phage.

INTRODUCTION

Vibrio cholerae is a Gram-negative pathogenic bacterium that causes severe dehydrating diarrhoeal disease (Faruque *et al.*, 1998; Kaper *et al.*, 1995; Mekalanos *et al.*, 1997). The serogroup of *V. cholerae* is determined by the LPS structure. While over 206 different serogroups are currently recognized (Li *et al.*, 2002), only the O1 and O139 serogroups have been linked to epidemic and pandemic cholera in humans. The O1 serogroup is classified into two biotypes, classical and El Tor, which can be distinguished by a number of phenotypic traits (Kaper *et al.*, 1995). The pathogenesis of cholera is related mainly to the production of cholera toxin, encoded by the *ctxAB* genes. The cholera toxin genes *ctxAB*

are located on the genome of a filamentous lysogenic phage designated CTXΦ (Waldor & Mekalanos, 1996). CTXΦ is integrated in the large chromosome of the El Tor biotype, or at two different sites of both chromosomes of the classical biotype (Davis *et al.*, 2000). Various arrays of the CTX prophage and the related genetic element RS1 have been reported on the genomes of the O1 El Tor biotype and the O139 serogroup (Davis *et al.*, 1999; Davis & Waldor, 2000; Heidelberg *et al.*, 2000). The CTX phage and RS1 both contain the genes *rstR*, *rstA* and *rstB*. The phage-encoded protein RstA is required for phage genome replication, and RstB is required for phage genome integration. RstR is a repressor that down-regulates the *rstA* promoter. While there are genes of the phage core region on the CTX prophage genome, RS1 contains *rstC* instead. RstC is an anti-repressor of RstR, and promotes transmission of RS1 and CTXΦ (Davis *et al.*, 2002). While solitary or truncated prophages are generally integrated into both chromosomes of classical biotype strains, classical biotype strains

containing multiple-copy CTX prophages have also been documented (Basu *et al.*, 2000; Davis *et al.*, 2000). The CTX prophages found in El Tor and classical biotypes have a major difference in *rstR*, a repressor of *rstA* that is required for replication of CTXΦ (Kimsey & Waldor, 1998). Recently, genetic hybrids of *V. cholerae* O1 strains possessing both classical and El Tor biotypes were found in Bangladesh (Nair *et al.*, 2002; Nusrin *et al.*, 2004), which may imply that *rstR* is not biotype specific. *V. cholerae* O1 El Tor strain harbouring the classical CTX prophage was isolated in Beira, Mozambique, in 2004 (Ansaruzzaman *et al.*, 2004). We further analysed the genetic relationship of these isolates by using a multilocus sequence typing (MLST) approach. MLST analysis of nine loci showed that the isolates from Mozambique and O1 El Tor strain N16961 (for which the whole genome sequence is known) have the same unique sequence type. We could also amplify the *ctxB-rstR-rstA-rstB-phs-cep* fragment from these isolates, which suggests that there is a tandem repeat of the classical prophage in the genome of the isolates from Mozambique.

METHODS

Bacterial strains and growth conditions. Forty *V. cholerae* isolates collected at the Cholera Treatment Centre, Beira, Mozambique, were analysed at the International Vaccine Institute (IVI) in Korea. For the comparison of sequences, five isolates each of *V. cholerae* O1 El Tor biotype (strain numbers 2201969, AR-17384, AR-11698, AR-17379 and AR-11585), classical biotype (strain numbers C-19385, F-2427, H-18, X-19850 and Y8661) and O139 (strain numbers AR196157, AR-18096, 2206945, 2206252 and AR-9954) were obtained from the culture collection of the enteric microbiology laboratory of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh.

Serotyping and biotype analysis. The *V. cholerae* isolates from Mozambique were confirmed serologically by the slide agglutination test using specific antiserum to *V. cholerae* polyvalent O1 and serotype-specific antisera to Inaba and Ogawa serotype strains. For biotype analysis, we used chicken erythrocyte agglutination, haemolysis of sheep erythrocytes, the Voges–Proskauer reaction, sensitivity to polymyxin B, and Mukerjee classical phage IV and Mukerjee El Tor phage 5 tests (Basu & Mukerjee, 1968).

MLST and sequence analysis. Bacterial genomic DNA was prepared from a single colony grown overnight on nutrient agar by using the PrepMan Ultrakit (Applied Biosystems). All oligonucleotides used for the MLST analysis, PCR amplification and sequencing were purchased from Genotech (Taejon, Korea). Nine loci (*dnaE*, *lap*, *rstA*, *gmd*, *recA*, *pgm*, *gyrB*, *cat* and *chi*) were used for MLST analysis. Each locus was amplified by using PCR and primers as described previously (Garg *et al.*, 2003). In the report of Garg *et al.* (2003), *rstA* was denoted as *rstR*, despite the fact that the amplified fragment was *rstA*. Here, we wish to clarify that *rstA* was used for MLST, and *rstR* was used to differentiate between the classical and El Tor biotype prophages.

The primer sets were as follows, and their locations on the CTX prophage genome are shown in Fig. 3: primer set 1 for El Tor biotype-specific *rstR-rstA* fragment amplification (forward, 5′ TGTCTTATC-AGCATACTTTC 3′; reverse, 5′ GAGTGAATCGTCGTG 3′), primer set 2 for *rstB-rstC* (or *cep*)-*rstR-rstA* fragment amplification (forward, 5′ ACGTTTGACAATGAGCCAG 3′; reverse, 5′ GAGTGAATC-GTCGTG 3′), primer set 3 for *ctxB-rstR-rstA-rstB-psh-cep* fragment

amplification (forward, 5′ TATGCACATGGAACACCTCAAA 3′; reverse, 5′ AACCCCGAGTGAAAGCGTG 3′), primer set 4 for classical-biotype-specific *rstR-rstA* fragment amplification (forward, 5′ AGCCTCCATCAAAATGAATA 3′; reverse, 5′ GAGTGAATCGT-CGTG 3′) and *rstC* primer set (forward, 5′ GGATGTTTAC-GATAGCCTAGAA 3′; reverse, 5′ TTGAGTTGCGGATTTAGGC 3′).

The amplified products were confirmed on agarose gel and purified with the UltraClean PCR Clean up DNA purification kit (MO BIO Laboratories). The purified PCR products were sequenced in both directions by using a Big Dye cycle sequencing kit (ABI) according to the manufacturer's instructions. Sequencing was performed on an ABI 3770 automatic sequencer. For alignment and phylogenetic analyses, we used several software programs: MultAlin [available at http://prodes.toulouse.inra.fr/multalin/multalin.html (Corpet, 1988)], PHYDIT (available at http://plaza.snu.ac.kr/~jchun/phydit) and NTSYspc version 2.11h (Exeter software).

RESULTS AND DISCUSSION

Subtyping of Mozambique isolates

All 40 Mozambican isolates were identified as *V. cholerae* O1 Ogawa serotype, as described previously (Ansaruzzaman *et al.*, 2004). All isolates agglutinated with chicken cell erythrocytes, were positive for El Tor-type haemolysin by the modified tube agglutination method, yielded a positive Voges–Proskauer reaction, and were resistant to polymyxin B. They were sensitive to Mukerjee El Tor phage 5 but resistant to the classical phage IV (Table 1). The isolates were therefore classified as El Tor biotype.

Sequence type (ST) of *V. cholerae* isolates from Mozambique

Though a number of MLST studies have been conducted on *V. cholerae*, most have focused on O139 strains, and only a few O1 strains have been analysed with a limited number of loci for MLST analysis (Garg *et al.*, 2003; Kotetishvili *et al.*, 2003). We applied the MLST analysis method of Garg *et al.* (2003) to O1 El Tor and classical biotypes. Among 40 Mozambique *V. cholerae* O1 isolates transported to IVI, 23 were fully sequenced at nine MLST loci, and all 23 had identical DNA sequences at the loci. The remaining 17 isolates were sequenced at six to eight MLST loci, and each sequenced locus was identical to that of the other isolates. We therefore concluded that the Mozambican isolates belonged to one ST. When the sequence of each allele in our study was identical to previously reported allele sequences, the allele type number was denoted with the same number. The allele types and ST results are summarized in Table 2. We found one new allele type in *rstA*, *recA* and *chi* loci in all five classical biotype strains, and one new *rstA* allele type in the O139 strains. We also compared the DNA sequence of nine loci of the Mozambique isolates with those of the El Tor N16961 strain, for which the whole genome sequence is available (Heidelberg *et al.*, 2000). As shown in Table 2, the ST of the Mozambique isolates was identical to that of *V. cholerae* O1 El Tor N16961. The allele profile of O1 El Tor N16961 and Mozambique isolates is 1, 1, 1, 2, 1, 1, 1, 1, 1 (in the order *dnaE*, *lap*, *rstA*, *gmd*, *recA*, *pgm*, *gyrB*, *cat* and *chi*).

Table 1. Biotype characterization of *V. cholerae* O1 isolated from Beira, Mozambique

R, Resistant; S, sensitive.

Test	Mozambique strain ($n = 40$)	Classical reference strain	El Tor reference strain
Chicken erythrocyte agglutination	+	−	+
Voges–Proskauer reaction	+	−	+
Sensitivity to:			
Polymyxin B (50 i.u.)	R	S	R
Mukerjee classical phage IV	R	S	R
Mukerjee El Tor phage 5	S	R	S

This allele profile does not match any of the O139 strains previously reported (Table 2; Garg *et al.*, 2003). As this MLST method can be applied to O1 El Tor and classical biotypes, we propose further MLST analyses of O1 strains.

Based on allele profiles of *V. cholerae*, allele profile similarities were calculated and divided into four groups. The highest similarity value was found between *V. cholerae* Mozambique and *V. cholerae* O1 El Tor. After determining the *V. cholerae* allele profiles, we constructed a dendrogram using unweighted pair grouping with mathematical averaging (UPGMA) (Fig. 1). The Mozambique isolates were closely related to *V. cholerae* O1 El Tor compared to strains O139 and O1 classical biotype. When all sequences of the nine loci were used to calculate sequence similarity, the *V. cholerae* isolates from Mozambique and *V. cholerae* O1 El Tor strains were the most similar. On the basis of the allele profile, the dendrogram and the sequence similarities, we concluded that *V. cholerae* isolates from Mozambique are closely related to *V. cholerae* O1 El Tor.

CTX prophage of Mozambique *V. cholerae* isolates

A 1447 nt DNA fragment, encompassing the first 288 (of 336) nt of classical *rstR*, ig-2, and the first 1032 nt of *rstA*, was amplified only from classical strains and Mozambique

isolates using primer set 4. The DNA sequence of Mozambique isolates showed 100 % homology with that of the classical biotype *rstR* and ig-2. With primer set 1, a 1460 nt fragment was amplified from all five O1 El Tor strains and all five O139 strains; however, there was no amplification of this DNA fragment in classical strains or in the Mozambique isolates. From these results, we concluded that only the classical *rstR* was present in the CTX prophage of *V. cholerae* isolates from Mozambique.

The DNA sequence of the 618 nt *rstA* internal fragment for MLST analysis (from nt 415 to 1032, out of 1083 nt of the full-length *rstA*) of Mozambique isolates was identical to that of the most common allele type of O139 and O1 El Tor N16961 (allele type 1). However, the rest of the *rstA* of the Mozambique isolates was different from *rstA* of El Tor N16961. When we compared the full-length sequences of *rstA* of Mozambique isolates to those of other *V. cholerae* strains, we found an overall sequence similarity of over 99 %. We identified 10 polymorphic sites (Fig. 2). The first four variable sites (27, 162, 183 and 258) of Mozambique isolates were identical to the those of the classical biotype, and the last six variable sites that belong to the internal fragment used for the MLST analysis were the same as those of the El Tor biotype. The positions of all polymorphic-site nucleotides within their codons were third positions, and all differences were synonymous changes when translated. Although the *rstA* of the Mozambique isolates seems to be a

Table 2. Allele profiles of *V. cholerae* O1 classical, O1 El Tor and O139 strains, and Mozambique isolates of this study

Numbers are previously reported allele profile numbers (Garg *et al.*, 2003). N, New allele type identified in this study.

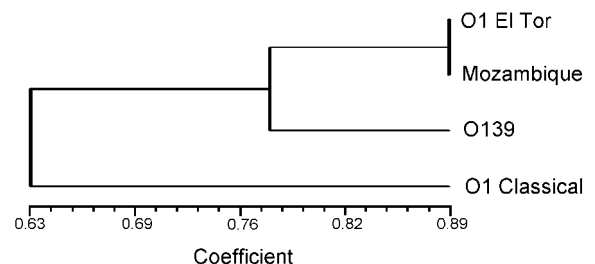| Strain or isolate	Locus								
	dnaE	*lap*	*rstA*	*gmd*	*recA*	*pgm*	*gyrB*	*cat*	*chi*
O1 El Tor	1	1	2	2	1	1	1	1	1
O1 classical	1	1	12 (N)	2	3 (N)	1	1	1	6 (N)
O139	1	1	13 (N)	1	1	1	1	1	1
Mozambique	1	1	1	2	1	1	1	1	1
O1 El Tor N16961	1	1	1	2	1	1	1	1	1

Fig. 1. Dendrogram of *V. cholerae* strains based on allele profiles created by using unweighted pair grouping with mathematical averaging (UPGMA). *V. cholerae* isolates from Mozambique were closely related to *V. cholerae* O1 El Tor strains.

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                       1 1 2 3 5 5 5 6 7
                       2 6 8 5 4 1 4 7 0 7
                       7 2 3 8 5 6 0 9 9 4
      O1 classical    T T A C T A G C C T
        Mozambique    T T A C G G A T T C
          O1 El Tor   C C C G G G A T T C
         O139 9803    C T A T G G G C T C
         O139 AS207   T T A G G G A C T T
         O139 86015   C T C G G A G C T C
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Fig. 2. Variable sites of the *rstA* gene among O1 classical and O1 El Tor (strain N16961) biotypes, O139 strains, and Mozambique isolates. The vertical numbers indicate the positions of variable sites, numbered from the first nucleotide of *rstA*. The first four variable sequences (bases 27, 162, 183 and 258) of *rstA* of the Mozambique isolates are identical to those of O1 classical biotype, but six variable sites (from base 345 to base 774) are identical to those of O1 El Tor N16961. However, variations similar to those of the Mozambique isolates were also present in O139 isolates. The accession nos of O139 strains at the NCBI database are: O139 9803, AF302794; O139 AS207, AF110029 (Davis *et al.*, 1999); O139 86015, AF220606.

hybrid of classical biotype and El Tor biotype, the generation mechanisms of this mosaic structure would be quite complicated. Therefore, we propose the presence of a classical CTX phage with the same variable sites in *rstA* as those of the Mozambique isolates.

Given that the ST of the Mozambique isolates is identical to that of El Tor strain N16961 and that the CTX prophage of the Mozambique isolates is the classical type, it is likely that the Mozambique strain originated from the El Tor biotype. One possible (and the simplest) mechanism for the generation of this strain is the infection of the El Tor biotype strain by the classical CTX phage that has the Mozambique *rstA*. This classical CTXΦ either integrated into an O1 El Tor biotype strain that had lost the existing El Tor prophage or replaced the existing El Tor prophage. Since the classical CTX phage particle, along with the classical biotype strains of *V. cholerae*, is believed to have been extinct from 1994 onwards (Nair *et al.*, 2002; Safa *et al.*, 2005), the acquisition of the classical CTX phage genome by the El Tor biotype remains an open question. Epidemic cholera in Mozambique, caused by *V. cholerae* O1, Ogawa, biotype El Tor strains, was first reported in 1997 (Folgosa *et al.*, 2001). Those strains contained two copies of CTX prophage, and were resistant to sulfonamides and trimethoprim, characteristics similar to those of the Mozambique isolates. The relationship between the isolates from the 1997 and 2004 epidemics can be established with the methods presented here. Further studies will be needed to understand the origin of the Mozambique strains, since no classical biotype strains have reached the African continent during the past 30 years of the seventh cholera pandemic. The investigation of the pathogenicity of this hybrid strain and the intensity of symptoms in patients should also be investigated, because the O1 El Tor and classical biotype strains differ in these aspects.

CTX prophage array in the genome of Mozambique *V. cholerae* isolates

We examined the CTX prophage array of the Mozambique isolates with respect to two aspects: 1) the presence of the RS1 element or the truncated prophage, and 2) the presence of the tandem repeat of the CTX prophage. To see the presence of the RS1 element or 3′ truncated CTX′ on the 5′ region of the prophage, we used primer set 2, designed to amplify the fragment between *rstB* and *rstA*, as shown in Fig. 3. The same primer set produced a different size DNA fragment from the classical biotype and El Tor biotype strains. A 1962 nt fragment was amplified from the classical biotype strains C-19385, F-2427, H-18, X-19850 and Y-8661, which implies that there are truncated CTX prophages in these strains, as shown in Fig. 3. From all five El Tor strains and from three O139 strains (AR-196157, AR-18096 and AR-9954), a 2543 nt fragment was amplified, showing the presence of the RS1-CTX prophage array. No DNA fragment was amplified from the Mozambique isolates with the same primer set, indicating that no RS1 element is present upstream of the CTX prophage, or the CTX′ truncated prophage. We confirmed the absence of the RS1 element in the Mozambique isolates with an *rstC* primer set. No DNA amplification was obtained from Mozambique isolates, but a 173 nt fragment was amplified from all O1 El Tor and O139 strains (data not shown).

We used primer set 3 to evaluate the presence of the tandem repeat of the CTX prophage on the genome of the Mozambique isolates. The PCR product encompassing *ctxB*-ig1-*rstR*-ig2-*rstB*-*psh*-*cep* can be amplified only from a tandem repeat of the CTX prophage. As expected, there was no amplification from classical biotype strains (data not shown). This DNA fragment was PCR-amplified from only one O1 El Tor strain (2201969) and two O139 strains (AR196157 and 2206945). The DNA sequence of the fragment from the O139 isolates showed that these strains had a similar genetic structure to that of strain O139 AS207, which contains one El Tor prophage followed by a tandem repeat of a new-type prophage, CTX Calcutta, as shown in Fig. 3 (Davis *et al.*, 1999). A DNA fragment of similar size was amplified from most Mozambique isolates, and we analysed the DNA sequence of this fragment (GenBank accession no. DQ012295). Mozambique isolates contain an ig-1 that is homologous to that of the CTX Calcutta prophage (18 nt different out of 730 nt), followed by a classical biotype *rstR* instead of the Calcutta type *rstR*. The potential genetic structure and the array of CTX prophages of the Mozambique isolate are shown in Fig. 3. Since the infectious CTX virion particles (CTXΦ) can be produced from the tandem repeat prophage array (Davis *et al.*, 2000), we expect that this tandem-repeat classical prophage array can yield classical CTX phage particles.

The CTX prophage integration site on the large chromosome is well defined (Heidelberg *et al.*, 2000), and we could detect the integration site on the large chromosome of O1 El
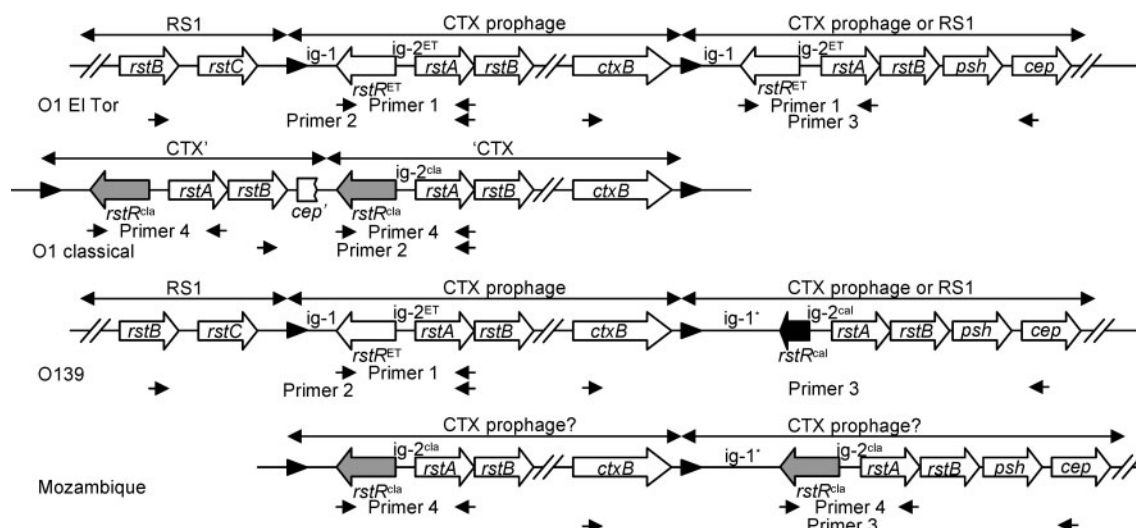
Fig. 3. Comparison of genetic structures and arrays of CTX prophage and RS1 of *V. cholerae*. Block arrows indicate the direction of transcription of each gene (not drawn to scale). Black triangles on the genome indicate repeat sequences flanking the integrated phage DNA. The CTX prophage core region is abbreviated, except for the *ctxB* gene, in this diagram. *V. cholerae* O1 El Tor and O139 have various arrays of CTX prophage. This diagram presents one example of each strain to show the PCR primer locations. *V. cholerae* O1 classical biotype strains contain either a solitary prophage or truncated fused prophages (CTX′, 3′ truncated; ′CTX, 5′ truncated prophage), as shown here and described elsewhere (Davis *et al.*, 2000). *rstR*ᴱᵀ, *rstR* in the CTX prophage of O1 El Tor biotype; *rstR*ᶜˡᵃ, *rstR* in the CTX prophage of O1 classical biotype; *rstR*ᶜᵃˡ, *rstR* in the CTX phage in O139 Calcutta type AS207 strain; ig-1*, Calcutta and Mozambique ig-1 that are homologous but different from El Tor type ig-1. The same abbreviations are used to indicate different types of ig-2.

Tor, classical and O139 strains by using PCR primer pairs that straddled both ends of the prophage. However, we could not define the integration site of the CTX prophage of the Mozambique strains with the same primers (data not shown). Identification of the CTX prophage location and full sequencing of the tandem repeat prophage are planned.

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REFERENCES

Ansaruzzaman, M., Bhuiyan, N. A., Nair, G. B., Sack, D. A., Lucas, M., Deen, J. L., Ampuero, J., Chaignat, C.-L. & The Mozambique Cholera Vaccine Demonstration Project Coordination Group (2004). Cholera in Mozambique, variant of *Vibrio cholerae*. *Emerg Infect Dis* **10**, 2057–2059.

Basu, S. & Mukerjee, S. (1968). Bacteriophage typing of Vibrio eltor. *Experientia* **24**, 299–300.

Basu, A., Mukhopadhyay, A. K., Garg, P., Chakraborty, S., Ramamurthy, T., Yamasaki, S., Takeda, Y. & Nair, G. B. (2000). Diversity in the arrangement of the CTX prophages in classical strains of *Vibrio cholerae* O1. *FEMS Microbiol Lett* **182**, 35–40.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16**, 10881–10890.

Davis, B. M. & Waldor, M. K. (2000). CTXΦ contains a hybrid genome derived from tandemly integrated elements. *Proc Natl Acad Sci U S A* **97**, 8572–8577.

Davis, B. M., Kimsey, H. H., Chang, W. & Waldor, M. K. (1999). The *Vibrio cholerae* O139 Calcutta bacteriophage CTXΦ is infectious and encodes a novel repressor. *J Bacteriol* **181**, 6779–6787.

Davis, B. M., Moyer, K. E., Boyd, E. F. & Waldor, M. K. (2000). CTX prophages in classical biotype *Vibrio cholerae*: functional phage genes but dysfunctional phage genomes. *J Bacteriol* **182**, 6992–6998.

Davis, B. M., Kimsey, H. H., Kane, A. V. & Waldor, M. K. (2002). A satellite phage-encoded antirepressor induces repressor aggregation and cholera toxin gene transfer. *EMBO J* **21**, 4240–4249.

Faruque, S. M., Albert, M. J. & Mekalanos, J. J. (1998). Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol Rev* **62**, 1301–1314.

Folgosa, E., Mastrandrea, S., Cappuccinelli, P., Uzzau, S., Rappelli, P., Brian, M. J. & Colombo, M. M. (2001). Molecular identification of pathogenicity genes and ERIC types in *Vibrio cholerae* O1 epidemic strains from Mozambique. *Epidemiol Infect* **127**, 17–25.

Garg, P., Aydanian, A., Smith, D. J., Glenn, M. J., Nair, G. B. & Stine, O. C. (2003). Molecular epidemiology of O139 *Vibrio cholerae*: mutation, lateral gene transfer, and founder flush. *Emerg Infect Dis* **9**, 810–814.

Heidelberg, J. F., Eisen, J. A., Nelson, W. C. & 29 other authors (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**, 477–483.

Kaper, J. B., Morris, J. G., Jr & Levine, M. M. (1995). Cholera. *Clin Microbiol Rev* **8**, 48–86.

Kimsey, H. H. & Waldor, M. K. (1998). CTXΦ immunity: application in the development of cholera vaccines. *Proc Natl Acad Sci U S A* **95**, 7035–7039.

Kotetishvili, M., Stine, O. C., Chen, Y., Kreger, A., Sulakvelidze, A., Sozhamannan, S. & Morris, J. G., Jr (2003). Multilocus sequence typing has better discriminatory ability for typing *Vibrio cholerae* than does pulsed-field gel electrophoresis and provides a measure of phylogenetic relatedness. *J Clin Microbiol* **41**, 2191–2196.

Li, M., Shimada, T., Morris, J. G., Jr, Sulakvelidze, A. & Sozhamannan, S. (2002). Evidence for the emergence of non-O1 and non-O139 *Vibrio cholerae* strains with pathogenic potential by exchange of O-antigen biosynthesis regions. *Infect Immun* **70**, 2441–2453.

Mekalanos, J. J., Rubin, E. J. & Waldor, M. K. (1997). Cholera: molecular basis for emergence and pathogenesis. *FEMS Immunol Med Microbiol* **18**, 241–248.

Nair, G. B., Faruque, S. M., Bhuiyan, N. A., Kamruzzaman, M., Siddique, A. K. & Sack, D. A. (2002). New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *J Clin Microbiol* **40**, 3296–3299.

Nusrin, S., Khan, G. Y., Bhuiyan, N. A. & 9 other authors (2004). Diverse CTX phages among toxigenic *Vibrio cholerae* O1 and O139 strains isolated between 1994 and 2002 in an area where cholera is endemic in Bangladesh. *J Clin Microbiol* **42**, 5854–5856.

Safa, A., Bhuiyan, N. A., Alam, M., Sack, D. A. & Nair, G. B. (2005). Genomic relatedness of the new Matlab variants of *Vibrio cholerae* O1 to the classical and El Tor biotypes as determined by pulsed-field gel electrophoresis. *J Clin Microbiol* **43**, 1401–1404.

Waldor, M. K. & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**, 1910–1914.