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Classification of Hybrid and Altered *Vibrio cholerae* Strains by CTX Prophage and RS1 Element Structure

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Analysis of the CTX prophage and RS1 element in hybrid and altered *Vibrio cholera* O1 strains showed two classifiable groups. Group I strains contain a tandem repeat of classical CTX prophage on the small chromosome. Strains in this group either contain no element(s) or an additional CTX prophage or RS1 element(s) on the large chromosome. Group II strains harbor RS1 and CTX prophage, which has an El Tor type *rstR* and classical *ctxB* on the large chromosome.

***Keywords*:** *V. cholerae*, biotype, altered strain, hybrid strain, CTX prophage, RS1 element

The *Vibrio cholerae* O1 serogroup is classified into two biotypes according to different phenotypic traits, the classical biotype and El Tor biotype (Kaper *et al.*, 1995). The classical biotype strains are believed to have been responsible for the fifth and sixth cholera pandemics in the 20th century, while the current seventh cholera pandemic is due to the El Tor biotype strains (Kaper *et al.*, 1995). The characteristics of El Tor and classical biotype *V. cholerae* strains differ by their ability to survive outside the human body, severity of symptoms, and case/carrier ratio (Kaper *et al.*, 1995; Sack *et al.*, 2004). El Tor biotype strains are known to produce El Tor type cholera toxin (especially B subunit, CT B) and classical biotype strains are believed to produce classical type cholera toxin which are carried by corresponding CTX phages. These two toxins differ by two out of 124 amino acids of *ctxB*, however, the exact physical manifestations they produce remain to be elucidated. From the late 1990s, El Tor biotype strains producing classical type CT B (hybrid and altered *V. cholerae* strains) began to emerge in South Asian countries (the Indian subcontinent) and lately these strains have entirely replaced the prototype El Tor biotype strains in the area (Nair *et al.*, 2006). In 2003, a new variant of El Tor biotype *V. cholerae* strain harboring a tandem repeat of classical CTX prophage was identified in Mozambique (Lee *et al.*, 2006); more recently a series of cholera outbreaks in Northern Vietnam were also caused by an altered El Tor strain (Nguyen *et al.*, 2009). In this study, we analyzed various hybrid and altered *V. cholerae* O1 strains that harbor classical *ctxB* and found many variants, which appeared to have existed at least since the early 1990's. They can be categorized into two main groups: Group I strains contain a tandem repeat of classical CTX prophage on the small chromosome and Group II strains contain RS1-El Tor CTX prophage encompassing classical type *ctxB* on the large chromosome.

Materials and Methods

V. cholerae strains

The strains analyzed in this study are shown in Table 1. B33, MJ1236, MG116926, 01.07VP are previously described (Nair *et al.*, 2002; Lee *et al.*, 2006; Safa *et al.*, 2006; Nguyen *et al.*, 2009). 12.02VP and 07.95VP are clinical isolates collected in Vietnam in 2002 and 1995, respectively. E1781 is an environmental isolate collected in Bangladesh in 2000. IB4322 and IB4642 are clinical isolates collected in Kolkata, India during cholera vaccine clinical trial conducted by IVI (Mahalanabis *et al.*, 2008).

Genetic analysis

We analyzed the genetic structures of CTX prophage and RS1 element of the strains by similar methods described previously with the primers shown in Table 2 (Nguyen *et al.*, 2009). Since CTX prophage and RS1 element share the same genes, each CTX prophage and RS1 element was PCR amplified individually after the array was determined and subjected for sequencing. Sequencing of the CTX prophage

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Table 1. Strains used in this study

	Strain	Year isolated	Country of origin and isolate type	Reference
Group I. Strains harboring a tandem repeat of classical CTX phage on the small chromosome	B33	2003	Mozambique, clinical	Lee *et al.* (2006)
	MJ1236	1994	Bangladesh, clinical	Nair *et al.* (2002) Safa *et al.* (2006)
	12.02VP	2002	Vietnam, clinical	This study
	07.95VP	1995	Vietnam, clinical	This study
	MG116926	1991	Bangladesh, clinical	Nair *et al.* (2002) Safa *et al.* (2006)
	E1781	2000	Bangladesh, environmental	This study
Group II. Strains harboring RS1-CTX phage on large chromosome	01.07VP	2007	Vietnam, clinical	Nguyen *et al.* (2009)
	IB4322	2004	India, clinical	This study
	IB4642	2006	India, clinical	This study

and the RS1 element was performed using primers listed in Supplementary Table 1.

Results and Discussion

The RS1 element(s) and CTX prophage(s) in the integration site of each chromosome are fully sequenced and the sequences of all strains in this study are deposited in GenBank. Whole genome sequencing results of strains B33 and MJ1236 are available at GenBank with accession number ACHZ00000000 and CP001485/CP001485, respectively. The CTX prophage and RS1 arrays of each strain are shown in Fig. 1, where they are compared with El Tor reference strain N16961 (Fig. 1A) and classical reference strain O395 (Fig. 1B).

Group I. Strains harboring a tandem repeat of the classical CTX prophage on the small chromosome.

i) B33, MJ1236, and 12.02VP carry a tandem repeat of the classical CTX phage on the small chromosome. They do not contain CTX prophage or RS1 element on the large chromosome and lack the toxin-linked cryptic (TLC) element (Fig. 1C).

ii) 07.95VP: This strain contains a tandem repeat of classical CTX prophage on the small chromosome and a solitary CTX phage on the large chromosome (Fig. 1D). We used primer pair Ch1F/Ch1R to amplify the whole CTX prophage on the large chromosome. A 7991 bp fragment was amplified as expected (Fig. 2A and B; lane 1) which corresponds to a solitary CTX prophage. This amplified fragment was digested with *Bgl*II and cleaved into 1,267 bp and 6,724 bp fragments (Fig. 2B; lane 2). *Bgl*II restriction digestion maps of a number of *V. cholerae* O1 strains are previously described (Maiti *et al.*, 2006).

iii) MG116926 is known to belong to Matlab variant III hybrid strain (Nair *et al.*, 2002; Safa *et al.*, 2006). This strain contains the tandem repeat of classical CTX prophage on the small chromosome and a tandem repeat of RS1 element on the large chromosome as shown in Fig. 1E. The TLC element is present in front of the RS1 repeat, a distinguishable characteristic among this group. To amplify the tandem repeat of the RS1 element from MG116926 by PCR, we used primer pair Ch1F/Ch1Rout. Ch1Rout; 5'-ACTATGTG GTGACTTCTGGC-3' was designed about 500 bp downstream of the last RS1 element to clarify size of *Bgl*II digestion fragments. A 7,061 bp fragment was amplified and this fragment was cleaved into 1172, 2726, and 3163 bp fragments by *Bgl*II digestion (Fig. 2B; lane 3 and 4).

iv) E1781 is an environmental isolate from Bangladesh in 2000. This strain has a structure similar to that of MG116926, but the TLC element in front of the RS1 element on the large chromosome is absent (Fig. 1F). Primer pair Ch1F0/ Ch1Rout2; 5'-CTCCCTAGGAAACTACAACTTCAACTGG GG-3' was used to amplify the tandem repeat of the RS1 element from E1781. Ch1Rout2 was designed at 287 bp further downstream of RS1Rout. A 7,192 bp fragment was amplified and was cleaved into 1016, 2726, and 3450 bp fragments by *Bgl*II digestion (Fig. 2B; lanes 5 and 6).

Group II. Strains harboring an RS1-CTX$^{El\ Tor}$ array on the large chromosome.

Only one array has been found with one variant in this group, however, these strains are prevalent in Asian countries and maybe the cause of current major epidemics in this area such as those in Vietnam in 2007 and 2008 (Nguyen *et al.*, 2009). To date, more than 400 clinical isolates collected from 2003 to 2007 in Kolkata, India, have been analyzed (Roychowdhury *et al.*, 2008). These isolates (representative strain IB4322) have the RS1 element fol-

Table 2. Primers used in this study

Primer	Sequence (5'→3')
Ch1F	GACCACTCAGGCCGCTGAAAT
Ch1F0	GCGAGTAATGTTGAGCATTTTCCTCAC
Ch1R	CCGCGCTCAAGTGGTTATCGG
Ch2F	AACAACAGGTTGCAAGAGAGCATT
Ch2R	TATTGCTTTTTTAATGGCCGTT
rstRclaF	TTTGCTACTTCTTCTTGGTT
rstRETF	TGAGCATAAGCTCTTGATTT
rstAR	CCGTGAAAGTCATCAACG
rstCF	GATGTTTACGATAGCCTAGAAGACTT
rstCR	TACAGTGATGGCTCAGTCAATGC
rstCF4	AAATCCGCAACTCAAGGCATTGA
rstCR4	TAAGCGCCTGAACGCAGATATAAAG
ctxBF	AGATATTTTCGTATACAGAATCTCTAG
cepR	AAACAGCAAGAAAACCCCGAGT
rstRCalF	TCAAGCTTTTTTTTGCTTTATCTTA
rstRCalR	TGGCAACAAAGCACATTAAAGA
rstREnvF	GCTTCATTTGTGTATTGGTCTATTAGGTAGTTA
rstREnvR	TCGAGTTGTAATTCATCAAGAGTGAAAA

* Primers were designed in this study based on the genome sequences of El Tor, classical, and environmental strains (Chun *et al.*, 2009).

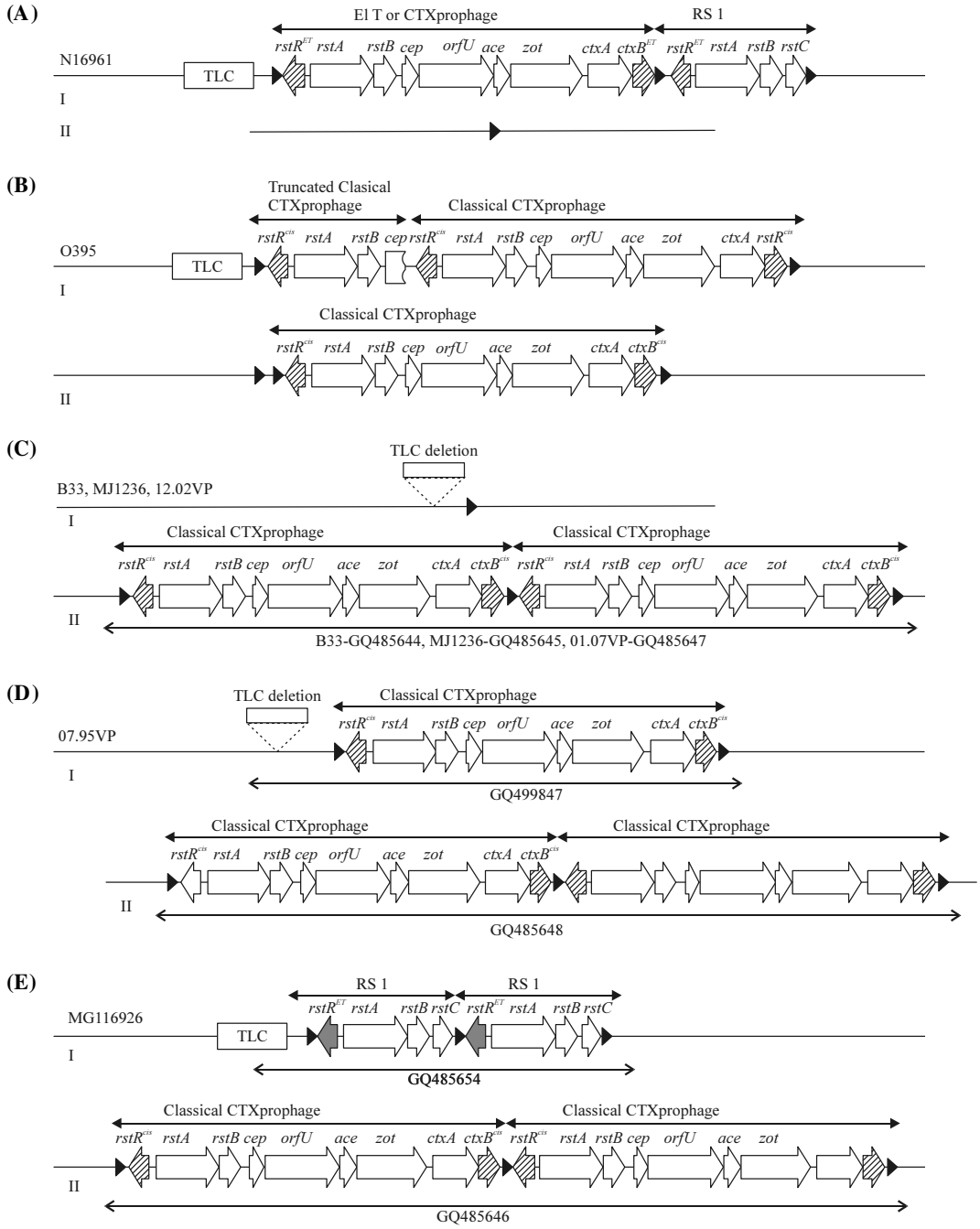

Fig. 1. Comparison of genetic structures and arrays of CTX prophage of *V. cholerae* strains. Block arrows indicate the transcription direction of each gene. Black triangles on the genome indicate the repeat sequence flanking the integrated phage DNA. (A) *V. cholerae* O1 El Tor strain N16961 contains a single El Tor CTX prophage and an RS1 after TLC element on the large chromosome (chromosome I). (B) *V. cholerae* O1 classical strain O395 contains truncated fused classical prophages after the TLC element in chromosome I and a solitary classical prophage on the small chromosome (chromosome II). (C) Strains B33, MJ1236, and 12.02VP contain a tandem repeat of classical CTX prophage on chromosome II and the TLC element is absent on the chromosome I. (D) Strain 07.95VP contains a classical CTX prophage on the chromosomes I and a tandem repeat of classical CTX prophage on the small chromosome and the TLC element is absent on chromosome I. (E) Strain MG116926 contains a tandem repeat of RS1 element after the TLC element in chromosome I and tandem repeated classical CTX prophages in chromosome II. (F) Strain E1781 contains a tandem repeat of RS1 element on the chromosome I and the tandem repeat of the classical CTX prophage on chromosome II. The TLC element is missing in chromosome I. (G) Strain IB4322 contains a single RS1 element and a single classical CTX prophage after the TLC element on chromosome I. (H) Strain IB4642 contains a single RS1 element and a single classical CTX prophage with variant classical *ctxB* after the TLC element on chromosome I. $rstR^{ET}$: El Tor type *rstR*, $rstR^{cla}$: classical type *rstR*. These abbreviations also indicate different types of *ctxB*. $ctxB^{variant\ cla}$: *ctxB* in strain IB4642 which has an additional amino acid change compared with classical *ctxB*. GenBank accession no. of DNA sequences of CTX prophage(s) and RS1 element(s) of each chromosome of each strain are shown.

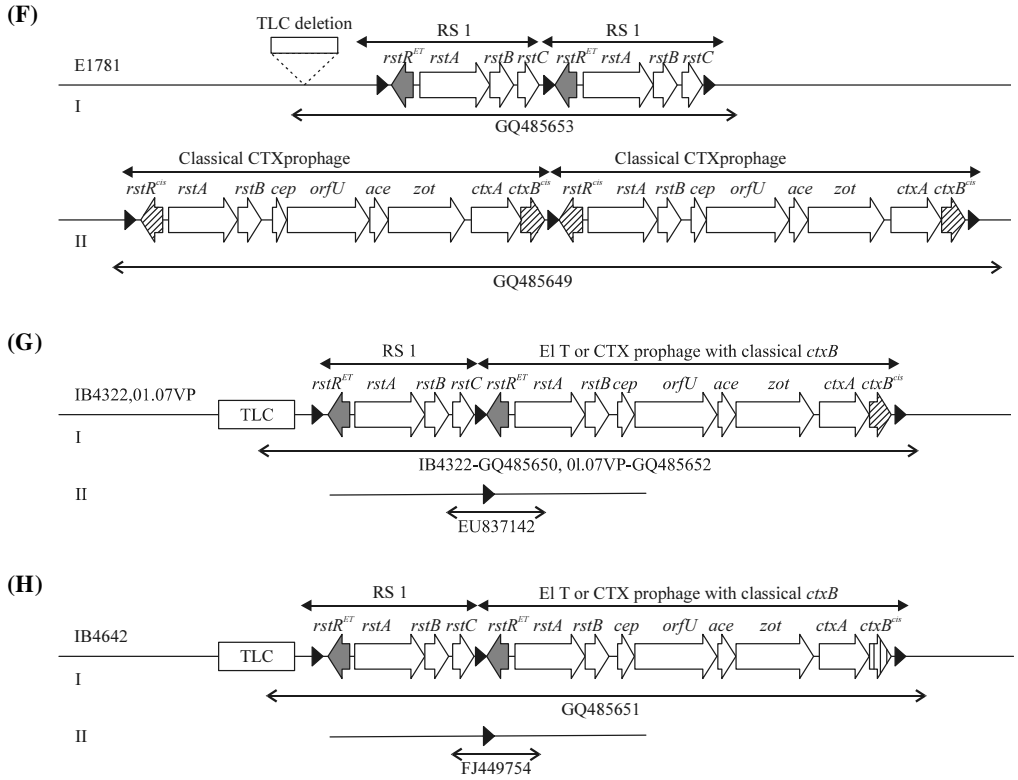

Fig. 1. Continued

lowed by the CTX prophage that contains the El Tor type ig-1 element and *rstR*, but the *ctxB* is of classical type (Fig. 1G). A variant was found in the group of Indian isolates (strain IB4642) with an amino acid change on the *ctxB* (Fig. 1H). The 20th amino acid histidine is substituted by asparagine but the rest of the gene is the same as classical *ctxB*. A similar strain was reported in eastern India (Goel *et al.*, 2008). None of these strains contained O139 type *rstR* or environmental type *rstR*.

As noted, there are many variants among the *V. cholerae* O1 strains that contain the classical *ctxB*, especially in the first group. Perhaps there may be more variants yet to be identified, namely a strain co-harboring El Tor and classical CTX prophages that could be considered the transient strain between the prototype El Tor strain and hybrid/altered strains.

Although Group I strains have a similar genetic CTX phage array, their behavior in diverse geographic areas differs. B33 was isolated from a cholera epidemic in Mozambique while MJ1236 and 12.02VP (which have the same CTX array) and other similar strains were isolated from sporadic cases or from the environment in Bangladesh and Vietnam. Interestingly, Group I strains (12.02VP and 07.95VP) were isolated between 1995 and 2004 in Northern Vietnam from only sporadic cases, and Group II strain (01.07VP) has been the cause of epidemics since October 2007 in the same area. Group II strains continue to cause major epidemics and perhaps are now endemic in Asian countries, but they have not yet been reported in Africa.

Recently, the generation and evolution of the 7th pandemic strains by the lateral gene transfer have been well documented and perhaps hybrid and altered strains in this report are some examples of the evolution of the 7th pandemic strains (Chun *et al.*, 2009). Until recently, classical *V. cholerae* strains were considered incapable of producing progeny CTX phages due to a unique phage replication mechanism and the truncated CTX array in the classical strains (Davis *et al.*, 2000). However, the finding of uptake of the classical CTX phage by El Tor strains suggests these hybrid and altered strains are changing continuously (Udden *et al.*, 2008). The generation of strain B33 from the seventh pandemic strains has been suggested and some of the strains we described in this report may be precursor of B33 or derived from it (Faruque *et al.*, 2007). A detailed genomic analysis of the strains regarding relationship among these strains, whether the Group I and Group II strains are generated independently or if they are related to each other is being planned. Ongoing monitoring of the spread of these strains is required to determine if these altered strains are in the process of replacing the prototype El Tor strains globally, and if the Group II strain will reach the African continent.

Nucleotide sequence accession numbers

The nucleotide sequences of the genome fragments of the strains described in this report are deposited in GenBank under accession numbers GQ485644 – GQ485654, GQ499847, EU837142, and FJ449754.

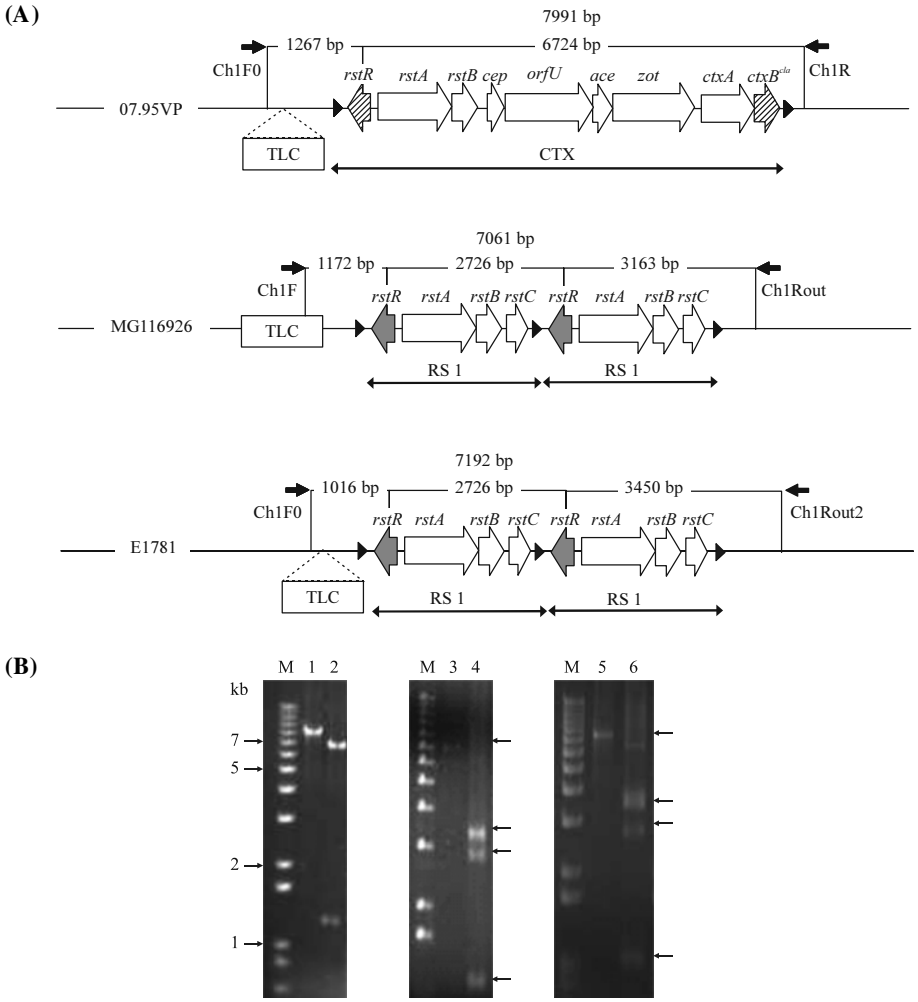

Fig. 2. CTX prophage and RS1 element array determination of 07.95VP, MG116926, and E1781. CTX prophage and RS1 element integration region of the large chromosome of the strains were PCR-amplified and the analyzed by *Bgl*II restriction enzyme digestion. (A) Genetic map of 07.95VP, BG116926, and E1781. PCR amplified fragment and restriction enzyme digestion pattern of the PCR-amplified fragments of (B) 07.95VP, (C) MG116926, (D) E1781. Lanes: M, DNA size marker 1 kb ladder; 1, 3, and 5, PCR amplified product from 07.95VP using primer pair Ch1F/Ch1R, MG116926 using primer pair Ch1F/Ch1Rout, and E1781 using primer pair Ch1F0/Ch1Rout, respectively. 2, 4, and 6; *Bgl*II digestion fragment analysis of PCR-amplified fragments from 07.95VP, MG116926, and E1781, respectively.

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