Molecular Insights Into the Evolutionary Pathway of Vibrio cholerae O1 Atypical El Tor Variants

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Abstract

Pandemic V. cholerae strains in the O1 serogroup have 2 biotypes: classical and El Tor. The classical biotype strains of the sixth pandemic, which encode the classical type cholera toxin (CT), have been replaced by El Tor biotype strains of the seventh pandemic. The prototype El Tor strains that produce biotype-specific cholera toxin are being replaced by atypical El Tor variants that harbor classical cholera toxin. Atypical El Tor strains are categorized into 2 groups, Wave 2 and Wave 3 strains, based on genomic variations and the CTX phage that they harbor. Whole-genome analysis of V. cholerae strains in the seventh cholera pandemic has demonstrated gradual changes in the genome of prototype and atypical El Tor strains, indicating that atypical strains arose from the prototype strains by replacing the CTX phages. We examined the molecular mechanisms that effected the emergence of El Tor strains with classical cholera toxin-carrying phage. We isolated an intermediary V. cholerae strain that carried two different CTX phages that encode El Tor and classical cholera toxin, respectively. We show here that the intermediary strain can be converted into various Wave 2 strains and can act as the source of the novel mosaic CTX phages. These results imply that the Wave 2 and Wave 3 strains may have been generated from such intermediary strains in nature. Prototype El Tor strains can become Wave 3 strains by excision of CTX-1 and re-equipping with the new CTX phages. Our data suggest that inter-chromosomal recombination between 2 types of CTX phages is possible when a host bacterial cell is infected by multiple CTX phages. Our study also provides molecular insights into population changes in V. cholerae in the absence of significant changes to the genome but by replacement of the CTX phage that they harbor.

Introduction

Vibrio cholerae O1 serogroup strains have been categorized into 2 biotypes - classical and El Tor - based on microbiological properties and the CTX prophage that they harbor [1,2]. Classical biotype strains contain the classical CTX prophage (CTXcla), and El Tor strains are believed to contain the El Tor CTX prophage (CTX2

The CTX phage comprises 10 genes (rstR, rstA, rstB, psh, cpe, orfU, ace, tot, ctxA, and ctxB). Whereas rstR is phage type-specific, other genes differ between phages by several SNPs, except for ctxA, which is identical in the 2 phages [3]. The binding subunit of cholera toxin (CTB) is encoded by ctxB, and the CTBs between phages differ by 2 of their 125 amino acids (residues 39 and 68) [1]. An evolutionary model of pathogenic V. cholerae O1 strains, in which El Tor biotype strains acquired only the El Tor CTX phage, whereas the classical strains obtained the classical phage, has been widely accepted [4].

Atypical El Tor variants, defined as El Tor biotype strains that produce classical cholera toxin, were first recognized in 2006, and several atypical El Tor variants have since been reported [5–7]. Two atypical CTX phages that contain ctxB

Two atypical CTX phages that contain ctxB

This CTX phage contains the classical biotype-specific rstR

However, it was later found to contain other genes of CTX-1 and was renamed CTX-2 [10]. V. cholerae strains that contain CTX-2 harbor a tandem repeat of CTX-2 on
Author Summary

In this report, we suggest a genetic mechanism of how the V. cholerae atypical El Tor variants were generated from classical and prototype El Tor biotype strains. An intermediary strain, containing the CTX-1 and CTX-2 prophages, was identified among the clinical isolates that were collected in 1991, when the atypical strains emerged. This strain can be converted into various Wave 2 atypical El Tor strains by eliminating prototype components, CTX-1 and RS1. Further, new types of the CTX phage genome can be generated from the intermediary strain by inter-chromosomal recombination between CTX phages and recombination between the CTX phage and RS1. These new CTX phages can be transduced into other El Tor strains, transforming them into Wave 3 atypical strains. This is a demonstrated instance of how a single-segment-genome CTX phage re-organizes its genome through recombination between different types of phage, leading to generation of new phage variants and atypical El Tor strains.

chromosome 2. Based on the analysis of SNPs in the genome and the CTX phage they harbor, the El Tor strains have been categorized into 3 Waves [10]. The prototype El Tor strains that contain CTX-1 are considered Wave 1 strains. The strains contain CTX-2 constitute a phylogenetic subgroup among the seventh cholera pandemic strains by genome analysis and are therefore categorized as Wave 2 strains [10].

The second atypical CTX phage (CTX-3) was first reported in Vietnam in 2007 and has the same genetic structure and sequence as CTX-1, with the exception of SNPs in rstA and ctxB (Figure 1) [11]. Strains that harbor CTX-3 typically contain a satellite phage, RS1, followed by a CTX-3 on chromosome 1 [6]. These strains have existed since the early 1990s on the Indian subcontinent [6]. A variant of CTX-3 that contains a new type of ctxB (ctxB genotype 7) emerged from India in 2006 (Figure 1). In addition to 2 SNPs in ctxB<sup>CTX-3</sup> of CTX-3 compared with ctxB<sup>CTX-1</sup> of CTX-1, CTX-3b has an additional SNP (nucleotide position 50) at amino acid residue 20 [12]. Most current global clinical isolates of V. cholerae are atypical El Tor variants that harbor CTX-3 or CTX-3b [6]. A recent surveillance study in India has shown that strains containing CTX-3b have been gradually replacing strains with CTX-3, as isolates that contain CTX-3b constitute 93.3% of all isolates that were collected in 2011 [13]. V. cholerae strains that contain CTX-3 or CTX-3b are distinguished from Wave 2 strains, based on the CTX phage and genomic variations, and are thus categorized as Wave 3 strains of the seventh cholera pandemic [10].

The catastrophic cholera outbreak in Haiti in 2010 was caused by a Wave 3 strain that contained ctxB genotype 7 [10,14,15]. We noted the CTX phage of Haitian strains differed from CTX-3b by several SNPs. We surveyed Wave 3 V. cholerae strains that were collected in Kolkata, India from 2003–2007 to verify that the Haitian-type CTX phage had existed earlier. We identified a CTX phage that contains identical SNPs as the Haitian-type CTX phage (except for ctxB) and other variant CTX phages. The SNPs of these variant CTX phages appeared to originate from the RS1 satellite phage, implying that the variant CTX phages were generated by recombination of homologous genes (rstR, rstA, and rstB) between RS1 and CTX.

V212-1, a strain that bears CTX-1 and CTX-2, was collected when atypical strains first appeared (Table 1) and was considered to be an intermediary between prototype and atypical El Tor strains [16]. In this study, we demonstrate that Wave 2 strains can be generated directly from V212-1 by progressive elimination of CTX-1 and RS1 through intra-strand recombination. Further, novel mosaic CTX phages can be produced by an inter-chromosomal recombination between 2 different CTX prophages in V212-1, and the mosaic phages can be transmitted to new host bacteria, converting them into Wave 3 strains. These results indicate that the intra-strand and inter-strand recombination between CTXs and RS1 element played an important role in the evolution of CTX phage and V. cholerae, as previously suggested [17].

Results

Variants of mosaic CTX phages in V. cholerae O1 El Tor Wave 3 atypical strains

The genome sequences of CTX prophages indicated that the CTX phage genome can be classified into several types, based on SNPs of rstA, rstB, and ctxB, as suggested by Mutreja et al. [10]. The CTX-3 prophages in V. cholerae isolates belonging to Wave 3 strains that were collected in Vietnam and India contained 3 SNPs (nucleotides 927, 933, and 942) in rstA compared with CTX-1 of N16961 and V212-1, as shown in Table 1 [8,18]. The CTX prophage in Haitian isolates that were collected during the 2010 cholera outbreak was believed to be CTX-3b, which contains ctxB genotype 7. However, SNPs (3-nucleotide [GTA] deletion of positions 74–76 and SNPs at positions 87, 93, 105, and 189) in rstB, in addition to the 3 SNPs of rstA, were identified by sequencing the CTX genome of Haitian strains (Table 1). The difference in SNPs between CTX-3 and Haitian-type CTX suggests that more CTX variants exist among Wave 3 V. cholerae strains and that the CTX phages in Wave 3 strains can be classified further.

Because the GTA deletion in rstB could be used as a marker for identifying the Haitian-type CTX phage genome, a PCR strategy was designed to detect the presence and absence of this deletion (Figure S1). rstB PCR and DMAMA-PCR for ctxB were performed on 365 clinical isolates that were collected in Kolkata, India between 2003 and 2007 [13,19]. Most clinical isolates contained CTX-3 or CTX-3b, whereas 11 had GATA-deleted CTX prophage (Table S1).
Table 1. Genetic variations in RS1 and CTX prophages aligned with CTX-1 of N16961.

| CTX type | Strain name | Origin   | rstA    | rstB    | ToxR repeat | ctxB | Sequence information |
|----------|-------------|----------|---------|---------|-------------|------|----------------------|
|          |             |          | 74-92   | 87      | 93          | 105  | 189                  | 285  | 341                  | 364  | 366-371 | 372 | 379 | 381 | 58 | 115 | 203 |
| RS1      | N16961      | Bangladesh, 1975 | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
|          | V212-1      | India, 1991   | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| CTX-1    | N16961      | Bangladesh, 1975 | C C C G G G A T C T C G GTA A T G A A C G A C A C C T T A | T A | A |      |                     |      |                     |      |        |     |    |     |    |
|          | V212-1      | India, 1991   | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| CTX-2    | B33         | Mozambique, 2004 | T T A C | T T | Δ |      |                      |      |                     |      |        |     |    |     |    |
| CTX-3    | IB4122      | Vietnam, 2004  | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| IB4322   | India, 2004  | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| IB4768   | Djibouti, 2007 | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| IB4710   | India, 2004  | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| CTX-3b   | IB4642      | India, 2006   | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| IB4712   | India, 2009  | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| CTX-4    | IB4563      | India, 2007   | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| CTX-5    | IB4247      | India, 2003   | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| IB4405   | India, 2005  | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| CTX-6    | IB4540      | India, 2007   | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| CTX-6b   | IB5230      | Haiti, 2010   | C T T Δ T C A G | G A Δ Δ A Δ |           |      |                      |      |                     |      |        |     |    |     |    |
| CTX-6a   | O395        | India, 1965   | T T A C T A G C C T | Δ T C G A G T |           |      |                      |      |                     |      |        |     |    |     |    |

ToxR repeat sequence is TTTTGAT. SNPs of rstA, rstB and ctxB are indicated. The number of ToxR binding repeats between zot and ctxA is also shown. Dots indicate identical sequences as CTX-1, and Δ indicates deletion of nucleotide(s).

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CTX-3 was the most prevalent type; CTX-3b emerged in 2006, as previously reported [12]; and CTX-4, CTX-5, and CTX-6 were identified in 2004, 2003–2005, and 2007, respectively. The 20 clinical isolates from the cholera outbreak in Haiti in 2010 contained the same CTX prophage that could be categorized as CTX-6b, according to this scheme. However, strains that contained CTX-6b had not been identified in India by 2007.

Notably, the location of the SNPs in \textit{rstA} and \textit{rstB} in these CTX phages appear to be of RS1, implying that the CTX phage genomes are mosaics of CTX-1 and RS1 (Table 1).

\textbf{V212-1, an intermediary strain between prototype and atypical El Tor strains}

DNA sequence analysis of CTX-2 in Wave 2 strains and various CTX types was the origin of atypical strains. The infection of a classical strain with CTX-1, resulting in a single strain that harbors both CTX types is possible in vitro but, no clinical isolates or reference strains that contained both types of CTX in a single bacterial cell are available [17,21]. Instead, an intermediary strain, V212-1, that contains CTX-1 and CTX-2 has been reported [16]. We determined and verified the exact array of CTX and RS1 in V212-1—TLC:RS1:CTX-1:RS1 on chromosome 1 and CTX-2:CTX-2 on chromosome 2—by sequencing (Table 2). CTX-1 on chromosome 1 was identified as an authentic CTX-1, as the SNPs in \textit{rstA} and \textit{rstB} were identical to those of CTX-1 in N16961.

\textbf{V212-1 derivatives}

\begin{table}
\centering
\begin{tabular}{|l|l|l|l|}
\hline
\textbf{Strains and plasmids} & \textbf{Genetic structure} & \textbf{Chromosome 1} & \textbf{Chromosome 2} \\
\hline
\textbf{CTX array} & \textbf{GenBank accession} & \textbf{CTX array} & \textbf{GenBank accession} \\
\hline
\textbf{V212-1 derivatives} & & & \\
\hline
V212-1 & TLC:RS1:CTX-1:RS1 & KF664566 & CTX-2:CTX-2 & KF664567 \\
PM1 & TLC:CTX-1:RS1 & KF664568 & CTX-2:CTX-2 \\
PM2 & TLC:RS1:RS1 & KF664569 & CTX-2:CTX-2 \\
PM3 & TLC:RS1 & KF471410 & CTX-2:CTX-2 \\
PM4 & TLC & KF664570 & CTX-2:CTX-2 \\
PM5 & No TLC, No element & KF664571 & CTX-2:CTX-2 \\
PM6 & TLC:RS1:CTX-1:RS1 & CTX-2 & KF664572 \\
PM7 & TLC:RS1:CTX-1kan:RS1 & KF664573 & CTX-2:CTX-2 \\
PM8 & TLC:CTX-1kan:RS1 & KF664574 & CTX-2:CTX-2 \\
PM9 & TLC:RS1:CTX-1:RS1 & CTX-2kanCTX-2 & KF664575 \\
PM10 & TLC:RS1:CTX-1:RS1 & CTX-2:CTX-2kan & KF664576 \\
PM11 & TLC:CTX-3\textsuperscript{kan}:RS1 & KJ540266 & CTX-2kanCTX-2 \\
PM12 & TLC:CTX-5\textsuperscript{kan}:RS1 & KJ540267 & CTX-2kanCTX-2 \\
PM13 & TLC:CTX-6\textsuperscript{kan}:RS1 & KJ540268 & CTX-2kanCTX-2 \\
\hline
\textbf{pCTX} & & & \\
\hline
pCTX-1\textsuperscript{kan} & pCTX-Kan generated from PM7 & KF664579 & & \\
pCTX-1-1\textsuperscript{kan} & pCTX-Kan generated from PM8 & KF664580 & & \\
pCTX-1\textsuperscript{kan} & pCTX-Kan generated from PM9 & KJ540269 & & \\
pCTX-3\textsuperscript{kan} & pCTX-Kan generated from PM11 & KJ540270 & & \\
pCTX-5\textsuperscript{kan} & pCTX-Kan generated from PM12 & KJ540271 & & \\
pCTX-6\textsuperscript{kan} & pCTX-Kan generated from PM13 & KJ540272 & & \\
\hline
\textbf{N16961 derivatives} & & & \\
\hline
PM14 & TLC:RS1 & KJ540273 & No element & \\
PM15 & TLC:RS1:CTX1\textsuperscript{kan} & KJ540274 & No element & \\
PM16 & TLC:RS1:CTX1\textsuperscript{kan} & KJ540275 & No element & \\
PM17 & TLC:RS1:CTX3\textsuperscript{kan} & KJ540276 & No element & \\
PM18 & TLC:RS1:CTX5\textsuperscript{kan} & KJ540277 & No element & \\
PM19 & TLC:RS1:CTX6\textsuperscript{kan} & KJ540278 & No element & \\
\hline
\end{tabular}
\end{table}

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\textbf{V212-1 derivatives}

\textbf{pCTX} derivatives

\textbf{N16961 derivatives}

We determined and verified the exact array of CTX and RS1 in V212-1—TLC:RS1:CTX1:RS1 on chromosome 1 and CTX-2:CTX-2 on chromosome 2—by sequencing (Table 2). CTX-1 on chromosome 1 was identified as an authentic CTX-1, as the SNPs in \textit{rstA} and \textit{rstB} were identical to those of CTX-1 in N16961.

\textbf{Generation of Wave 2 strains from V212-1}

Whole-genome sequence analysis and the presence of a tandem repeat of CTX-2 on chromosome 2 indicate that V212-1 belongs to the Wave 2 strains [10]. However, V212-1 could be considered as an intermediary strain between Wave 1 and Wave 2 strains, because it also contained CTX-1 on chromosome 1.

\textbf{Table 2. Genetic information on V. cholerae strains and pCTXs generated in this study.}
The excision of RS1 and/or CTX-1 from chromosome 1 seemed to be mainly by a homologous recombination, since the recombination frequency was reduced 2-fold, 5-fold, and 50-fold in recA− background depending on the recombination position (Table 3). However, the frequency of recombination was not reduced evenly, indicating the deletion of RS1 and/or CTX might be mediated also by other mechanisms.

 Whereas the CTX prophage of strain PM1 was identified to be the authentic CTX-1 by DNA sequencing, 3 other variant CTX phages were obtained among the derivatives that contain the CTX:RS1 array (Figure 3 and Table 2). These CTX variants can be generated by various recombination events between the RS2 region (rstR, rstA, and rstB) of CTX-1 and RS1 (Figure 3). The SNPs in rstA and rstB of these CTX variants were identical to the CTX-3, CTX-5, and CTX-6 prophages of clinical isolates, respectively, and were thus designated CTX-3*, CTX-5*, and CTX-6* to clarify that they contained ctxB El Tor (Table 2). These variant strains were used later to produce pCTX-3kan, pCTX-5kan, and pCTX-6kan.

 Two additional recombinant strains of V212-1, in which pCVDrstRET was inserted into rstR in each RS1, were constructed to examine the excision of CTX-1 and RS1 further. From a recombinant strain that contained pCVDrstRET in the first RS1, the overall excision rate of RS1 and/or CTX-1 was 1/(4.4 × 10^3). Of the resulting 3 arrays (RS1:CTX-1:RS1, CTX-1:RS1, and RS1) from this strain, CTX-1:RS1 array was the most frequently produced. In a strain that contained the pCVDrstRET in the second RS1, the overall excision rate was 1/(4.7 × 10^4) and the solitary RS1 array was primarily generated.

 No strain that had lost the entire array of chromosome 1 was produced directly from V212-1; therefore, stepwise excision of the entire array was tested in strain PM3. pCVDrstRET was inserted into rstR of RS1 to construct PM3CVD. A strain that had lost RS1 (PM4) and 4 strains (one of them was analyzed as PM5) that had lost RS1 and the TLC (toxin-linked cryptic) were obtained among 110 recombinants that were screened (Figure S3 and Table S2). After the removal of RS1 (PM4) and TLC:RS1
Figure 3. The generation of new mosaic CTX phages from V212-1 by inter-strand recombination between CTX phages and intra-strand recombination between CTX-1 and RS1 on chromosome 1. A double crossover recombination event between 2 prophages on each chromosome of V212-1 (indicated by arrows) results in the generation of the CTX-1* prophage which contains ctxBB on chromosome 1. Intra-strand recombination between CTX-1* and RS1 generates a mosaic CTX prophage. Depending on the recombination position (shown as R1, R2, and R3), the generation of kanamycin-resistant CTX virions was approximately 10^4 virions/ml and 10^5 virions/ml in the kanamycin-resistant O395 transductant. The titer of CTX virions from PM7 and PM8 was confirmed by generation of the kanamycin cassette to construct strains PM7 and PM8, respectively, and the generation of kanamycin-resistant CTX virions (PM6).

The CTX arrays of some V212-1 variants that we generated were identical to those in the clinical isolates of Wave 2 strains (PM2). The excision of RS1 of V212-1 was tested in the PM9 and PM10 strains, as no virions were produced from strain PM9 (Table 2 and Figure 3). These CTX-1*kan virions were transduced from a recipient strain, from which pCTX-1*kan was obtained. The CTX-1*kan virion titer was 50 virions/ml in the presence of mitomycin C. pCTX-1*kan virions that were generated in PM7 (10^4 virions/ml); thus, the efficiency of excision of entire array from chromosome 1, generation of CTX-1*kan virions was verified by the generation of CTX-1*kan virions on chromosome 2 in V212-1, generating new mosaic phage genomes, was verified by the generation of CTX-1*kan virions from strain PM9 (Table 2 and Figure 3). These CTX-1*kan virions were transduced into a recipient strain, from which pCTX-1*kan was generated only from strain PM9, which has a kanamycin cassette in the first CTX-2 on chromosome 2 (PM6).

The CTX arrays of some V212-1 variants that we generated were identical to those in the clinical isolates of Wave 2 strains. PM2 has the TLC:RS1:RS1 array on chromosome 1 and CTX-2 on chromosome 2 and has the same structure as B33 and MJ1236 [8]. PM5 has the same structure as B33 and MJ1236 [23]. Our results suggest that the intermediary strains such as V212-1 might be the immediate ancestor of Wave 2 strains.

Infectious CTX-1 virions can be generated from chromosome 1 of V212-1

CTX virions can be generated from chromosome 1 of V212-1 and PM1, based on the replication mechanism of CTX phage [24]. ctxAB of CTX-1 of V212-1 and PM1 was replaced by a kanamycin cassette to construct strains PM7 and PM8, respectively, and the generation of kanamycin-resistant CTX virions from PM7 and PM8 was confirmed by generation of the kanamycin-resistant O395 transducing strain. The titer of CTX virions was approximately 10^4 virions/ml and 10^5 virions/ml in the culture supernatant of PM7 and PM8, respectively, in the presence of mitomycin C. pCTX-1kan generated from PM7 and pCTX-1-1kan generated from PM8 were extracted from the recipients and analyzed by sequencing. pCTX-1kan and pCTX-1-1kan had identical DNA sequences, because they were replication products from the same CTX-1 prophage genome of chromosome 1. No CTX-2 virions were generated from the tandem repeat of CTX-2 on chromosome 2 of PM9 and PM10, as no virions were produced from B33 [22].

Generation of new mosaic CTX phages from V212-1

An inter-chromosomal recombination event between ctxAB_El-Tor of CTX-1 on chromosome 1 and ctxAB_El-Tor of CTX-2 on chromosome 2 in V212-1, generating new mosaic phage genomes, was verified by the generation of CTX-1*kan virions from strain PM9 (Table 2 and Figure 3). These CTX-1*kan virions were transduced into a recipient strain, from which pCTX-1*kan was generated in PM7 (10^4 virions/ml); thus, the efficiency of generation of CTX-1*kan virions from V212-1 was estimated to be 1 per 200 progeny virions. CTX-1* can be defined as a CTX phage that is identical to authentic CTX-1, except that it contains ctxBB.
recombination event happens between two prophages (CTX-1 and CTX-2) or, between the replicative pCTX generated from chromosome 1 and the CTX-2 prophage. Perhaps, the recombination occurs more frequently between pCTX and the CTX-2 prophage than between two prophages.

PM11, PM12, and PM13 were generated similarly from strains that contained CTX-3*:RS1, CTX-5*:RS1, and CTX-6*:RS1, respectively, by replacing the ctxABcl of CTX-2 with a kanamycin resistance cassette (Table 2). pCTX-3kan, pCTX-5kan, and pCTX-6kan were generated from PM11, PM12, and PM13, respectively, by inter-strand recombination like pCTX-1*kan (Table 2 and Figure 3).

These results demonstrate that new mosaic phages are generated through combination of inter-chromosomal recombination event between 2 phages and intra-strand recombination event between the resulting recombinant CTX phage genome and RS1 in an intermediary strain.

Generation of Wave 3 strains

Because new mosaic CTX phages were generated from V212-1, we examined their transmission to novel El Tor strains to generate Wave 3 strains. Construction of the Wave 3 strains required a strain that contained only RS1 on chromosome 1, but no such strains were available among clinical isolates. PM14, which contains only RS1 on chromosome 1, was generated by the excision of CTX-1 from N16961. PM14 was transformed with various pCTXs that were extracted from transductants. pCTXs could be maintained in PM14 in plasmid form under selective antibiotic pressure or as an integrated prophage form. PM15, PM16, PM17, PM18, and PM19, which contained only the integrated pCTX-1kan, pCTX-1*kan, pCTX-3kan, pCTX-5kan, and pCTX-6kan next to the RS1 on chromosome 1, respectively, were screened in the transformants (Table 2).

These results demonstrate that the mosaic CTX phages that are produced from an intermediary strain such as V212-1 can be transmitted to an El Tor strain that contains an RS1 and can be integrated into the genome of a new host bacterial cell, enabling it to develop into a Wave 3 strain.

Discussion

The emergence of atypical El Tor strains traces back to 1991 on the Indian subcontinent, where prototype and atypical El Tor strains began to co-existed for several years [6]. It is assumed that prototype El Tor strains have been extinguished, because only atypical El Tor strains have since been isolated from cholera patients globally [6]. Phylogenetic analyses that are based on SNPs in the V. cholerae genome have indicated that prototype and atypical El Tor strains originated from a common ancestor in the 1950’s [10,25]. Although the genome of El Tor V. cholerae strains has modulated gradually, the changes of their CTX phage have occurred stepwise manner. In addition, strains that harbor various CTX phages constitute phylogenetically distinct subgroups, implying that the acquisition of a new CTX phage is independent of evolution of the bacterial genome [10,26].

Although several studies have proposed models for the generation of atypical El Tor strains, suggesting that the atypical variants originated through recombination events and lateral gene transfer, the exact process by which the mosaic CTX phages and atypical El Tor strains were generated remains unknown [16,17,22,27]. In this study, we propose a genetic mechanism by which atypical El Tor variants are generated. We demonstrated how a single-segmented CTX virus reorganized its genome through recombination of 2 prophages and or between a CTX prophage and RS1 in a host bacterial cell, resulting in a novel mosaic CTX virus, which can be transduced to a new host.

Homologous recombination between the common genes (rstRE, rstA, and rstB) between RS1 and CTX-1 or between any gene, except rstR, of the CTX-1 and CTX-2 (or, CTXcla), mediates generation of mosaic CTX phage genomes. Although the creation of CTX-2 was not shown directly—because no V. cholerae strains have been shown to contain CTX-1 and CTXcla—we report that the existence of an ancestral strain that harbors CTX-1 and CTXcla in nature. Further, V. cholerae O141 strains have been shown to be a reservoir of CTXcla, and an El Tor strain has been shown to take up CTXcla from them [17].

Intra-strand recombination between CTX-1 and RS1 is essential not only for the generation of mosaic CTX phages but

![Figure 4. A model of the generation of Wave 2 and Wave 3 strains.](Image)
also for the emergence of Wave 3 *V. cholerae* strains that harbor these mosaic phages. CTX and/or RS1 can be excised from chromosome 1 if the array contains a CTX repeat or a CTX:RS1 repeat. When the array ends with RS1, the ultimate product of the excision is a strain that contains a lone RS1. Depending on the structure of the array, the excision rate can reach 1/(4.4×10^3), indicating that a substantial proportion of the *V. cholerae* population tends to shed the CTX phage they harbor and is competent to acquire a new CTX phage. Thus, the Wave 3 atypical strains perhaps arose from prototype strains that had lost CTX-1 and obtained mosaic CTX phages that were generated from an intermediary strain.

Based on these results, we propose a pathway by which atypical El Tor variants were generated from prototype El Tor strains (Figure 4). A prototype El Tor strain was infected by a CTX-2 virion, and the CTX-2 genome was integrated as a tandem repeat in the small chromosome—thus, the prototype strain turned into an intermediary strain. The prototype components were removed from the intermediary strain stepwise, and the resulting strains from each step developed into a group of Wave 2 strains. The intermediary strain was also a source of new mosaic phages of Wave 3 strains. New mosaic CTX phages were generated from the intermediary strain, and the progeny mosaic CTX phages were transduced to progenitor strains of Wave 3 strains.

We are interested in examining the dynamics of the emergence of Wave 2 and Wave 3 atypical strains. Classical biotype and prototype El Tor biotype strains coexisted for approximately 2 decades after the prototype El Tor strains first appeared in 1962 in South Asian countries [6]. Yet, inexplicably, the initial appearance of atypical strains was not documented until 1991—30 years after prototype El Tor strains originated. Mosaic CTX phages were most likely generated during the early seventh cholera pandemic period. Whether they were generated in the 1960s—when prototype El Tor strains and classical strains coexisted—and took 30 years to spread, or in the 1990s, shortly before dissemination into the human population, has not been determined. Further study is required to establish when the atypical strains were generated in their natural habitat and disseminated throughout the human population. Coincidentally, had the first atypical strains appeared around the early 1990s, their emergence would have concurred with that of the new O139 serogroup, which is also responsible for epidemic cholera.

The whole-genome analysis of the Haitian strains and a group of Nepalese strains showed that their genomes differ by 1 or 2 base pairs, indicating that the Haitian *V. cholerae* strains originated from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28]. The strain that was isolated from the 2010 Haiti cholera outbreak contained CTX-6b and the proportion of strains from Nepal [28].

A link between the increase in severe cholera cases and atypical *V. cholerae* strains has been proposed, but the precise mechanism by which the clinical symptoms and genetic changes in the bacterial strains correlate remains unknown [29]. Although our results demonstrate the mechanism of the genesis of atypical strains, further studies are required to explain how new variants differ in pathology and how they dominate earlier prevalent strains. It is unclear whether population changes in clinically isolated *V. cholerae* strains reflect those of *V. cholerae* in the natural habitat. In addition to bacterial and environmental factors, human activity and, perhaps, the human immune response against temporarily prevalent strains influence population changes in clinically isolated *V. cholerae* strains.

**Materials and Methods**

**Bacterial strains and genetic structure analysis**

The bacterial strains and pCTX are shown in Tables 1 and 2. The CTX and RS1 arrays of the *V. cholerae* strains were determined and analyzed by sequencing. The DNA sequences of the CTX arrays of bacterial strains and pCTXs that were generated in this study were deposited into GenBank (Table 2).

**Discrimination PCR of SNPs of rstB**

A PCR primer set was designed for detection of the trinucleotide GTA (nt position 74–76) in rstB, similar to MAMA PCR for distinguishing classical, El Tor, and Haitian type ctxB [13]. For the Wave 3 strains that contained the RS1:CTX array, the forward primer, rstBFw (CTC ATT CTG AAG GGG TGA GTA A) was designed to anneal to rstB of RS1 (Figure S1), and the reverse primer, GTARev (GGT GCA CCA GTC TTA CAA C) was designed to detect GTA with rstBFw (annealing temperature at 63°C). The primer DelRev (GGC CGA GTG GCA GAC GA) was designed to verify the absence of GTA with rstBFw (annealing temperature at 58°C).

**Surveillance of various CTX prophage genomes in Wave 3 strains**

The GTA trinucleotide was examined in rstB in 365 clinical isolates of Wave 3 strains that were collected from 2004 to 2007 in Kolkata, India and 20 isolates that were gathered in Haiti in 2010 [14,19]. DMAMA (Double-Mismatch-Amplification Mutation Assay)-PCR for ctxB was also performed with these isolates. CTX phages that contained GTA-deleted rstB were confirmed by DNA sequencing of the entire RS1:CTX array.

**Excision of the CTX and RS1 from chromosome 1 and chromosome 2 of V212-1**

A DNA fragment (690 bp) that contained rstRE (339 bp) and the first 226 bp of rstA was inserted into pCVD442 to generate a recombinant plasmid, pCVDrstRET. The recombinant plasmid was inserted into rstRRE of CTX-1 and RS1s of V212-1 to construct V212-1CVD [30]. The excision of CTX-1 and/or RS1 was verified by analyzing the genetic structure of strains that were selected on LB plates with 15% sucrose. In addition, pCVDrstRET was inserted into rstRE of PM3 to construct PM3CVD, and excision of the RS1 element was confirmed as described. Excision of CTX-2 from the tandem repeat of CTX-2 on chromosome 2 was examined similarly by inserting rstRRe fragment-containing pCVD442 into each rstRRe of CTX-2s.
Replacement of ctxAB in V212 with a kanamycin cassette

The entire ctxA gene and the first 166 bp (of 375 bp) of ctxB of CTX-1 on chromosome 1 and CTX-2s on chromosome 2 of V212-1 were individually replaced by a kanamycin resistance cassette by allele exchange method [21]. The residual ctxB fragment on each recombinant strain contained the second SNP (nucleotide 203) of ctxB(T) and ctxB(C) on chromosomes 1 and 2, respectively. This SNP was used to distinguish pCTX-1kan and pCTX-1*Ckan. Similarly, a kanamycin cassette was introduced into CTX-1 on chromosome 1 of PM1, resulting in strain PM8. Strains PM11, PM12, and PM13 were constructed similarly by replacing the ctxAB of the first CTX-2 on chromosome 2 with a kanamycin cassette.

Production of CTX-1 phage from chromosome 1 of V212-1

Transduction of kanamycin-resistant CTX virions was monitored as described [21,31]. Briefly, PM7 culture supernatant (LB containing 20 ng/ml of mitomycin C) was mixed with a classical strain, O395, as the recipient. The mixture was incubated for 30 min for phage infection and plated on LB plates that contained kanamycin. pCTX-1-kan, a replicative form of the CTX genome containing 20 ng/ml of mitomycin C, was mixed with a classical strain, O395, as the recipient. The mixture was incubated for 30 min for phage infection and plated on LB plates that contained kanamycin. pCTX-1-kan, a replicative form of the CTX genome in the transductants, was extracted and analyzed by sequencing. CTX-1-kan virions were generated similarly from PM9 and were determined to have the same DNA sequence as CTX-1-kan (Table 2).

Generation of Wave 3 strains

PM14 was generated from N16961 by excision of CTX-1. The recombinant plasmid pCVDrstRET was inserted into CTX-1 of N16961, and strain PM14 was screened as described above. PM14 was transformed by the replicative form of CTXs that were generated in this study (pCTX-1-kan, pCTX-1*Ckan, pCTX-3-kan, and pCTX-6-kan). Integration of pCTXs next to RS1 on chromosome 1 of PM14 was confirmed by DNA sequencing (Table 1) [8].

recA- mutants construction

The internal 600 nt DNA fragment (nucleotide position 119–720) of recA was amplified with primers recASacIF: GGC GCG GAG CTC CGA CCC GAT TTC GAC ACA and recAEcoRIR: GAG CTC CGA CCC GAT TTC GAC ACA and recAEcoRIR: by using PCR. This fragment was inserted into a suicide plasmid pSW23.oriT to construct a recombinant plasmid pSWrecA [32]. The recombinant plasmid was individually transferred by conjugu- tation to V212-1CVD, PM3CVD, and PM9. The recA of each strain was disrupted by insertion of pSWrecA.

Accession numbers

The nucleotide sequences of CTX prophages and pCTXs were deposited in GenBank under accession numbers KF471410, KF664566–KF664576, KF664579, KF664580, and KJ540257–KJ540278.

Supporting Information

Figure S1 Discriminatory PCR of rstB in CTX-3 and CTX of Haitian strain. [A] rstB-discriminating PCR primers. The common rstB forward primer is shown on top. DNA sequences of rstB of CTX-3 (nucleotides 65–88, shown in bold) and Haitian CTX aligned with reverse primers. Three nucleotides, GTA (nt position 74–76, underlined), are absent in rstB of CTX of the Haitian strain. (B) Agarose gel electrophoresis of PCR product using rstBFW/GTARev (left panel) and rstBFW/DelRev (right panel), Lane 1: IB4122, lane 2: Haiti strain, lane 3: IB4247, lane 4: IB4712. (C) Primer annealing location on RS1-CTX array of Wave 3 strains. The size of amplicon is approximately the same as the RS1 element.

(TIF)

Figure S2 Excision of CTX-1 and RS1 from chromosome 1 of V212-1. (A) RS1:CTX-1:RS1 array on chromosome 1 of V212-1. (B) pCVDrstRET (red bar) was inserted into the rstR of CTX-1 of V212-1, generating a recombinant strain, V212-1CVD. Excision of the recombinant plasmid was screened by inoculating the strain V212-1CVD on LB agar plates containing 15% sucrose. Potential recombination positions are indicated (the dotted line also shows a potential recombination position that is not detected during the screening). (C) The CTX and RS1 arrays generated from each recombination shown in B. Generation of RS1:CTX-1:RS1 and RS1 array is mediated by recombination between rstR, but the generation of CTX-1:RS1 and RS1 arrays occurs through recombination between the entire RS2 region (rstR, rstA, and rstB) of RS1 and CTX-1. TLC is not shown in this figure. The overall excision rate and frequencies of generation of each array are described in Table 3.

(TIF)

Figure S3 Generation of PM4 and PM5 from PM3. pCVDrstRET (red bar) was inserted into the rstR of RS1 in PM3 to construct PM3CVD, and the recombinant strain was inoculated on LB plates containing 15% sucrose to screen for excision of pCVDrstRET. Most strains (105 strains) had the same array as PM3, but one strain (PM4) that had lost the RS1, and 4 strains that had lost RS1 and TLC were obtained (PM5).

(TIF)

Table S1 Distribution of CTX phages in Wave 3 strains collected in India between 2003 and 2007.

(DOCX)

Table S2 Frequency of generation of each array from PM3CVD and PM5CVD recA-.

Each array can be generated by recombination events shown in Figure S3. (DOCX)

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Author Contributions

Conceived and designed the experiments: EJK DWK. Performed the experiments: EJK DL SHM CHL SJK JHL. Analyzed the data: EJK DWK. Contributed reagents/materials/analysis tools: JOK MS BD JDC JWP GBN. Contributed to the writing of the manuscript: EJK BD GBN DWK.

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