

Satellite phage TLC ϕ enables toxigenic conversion by CTX phage through *dif* site alteration

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Bacterial chromosomes often carry integrated genetic elements (for example plasmids, transposons, prophages and islands) whose precise function and contribution to the evolutionary fitness of the host bacterium are unknown. The CTX ϕ prophage, which encodes cholera toxin in *Vibrio cholerae*¹, is known to be adjacent to a chromosomally integrated element of unknown function termed the toxin-linked cryptic (TLC)². Here we report the characterization of a TLC-related element that corresponds to the genome of a satellite filamentous phage (TLC-Kn ϕ 1), which uses the morphogenesis genes of another filamentous phage (fs2 ϕ) to form infectious TLC-Kn ϕ 1 phage particles. The TLC-Kn ϕ 1 phage genome carries a sequence similar to the *dif* recombination sequence, which functions in chromosome dimer resolution using XerC and XerD recombinases³. The *dif* sequence is also exploited by lysogenic filamentous phages (for example CTX ϕ) for chromosomal integration of their genomes. Bacterial cells defective in the dimer resolution often show an aberrant filamentous cell morphology^{3,4}. We found that acquisition and chromosomal integration of the TLC-Kn ϕ 1 genome restored a perfect *dif* site and normal morphology to *V. cholerae* wild-type and mutant strains with *dif*⁻ filamentation phenotypes. Furthermore, lysogeny of a *dif*⁻ non-toxigenic *V. cholerae* with TLC-Kn ϕ 1 promoted its subsequent toxigenic conversion through integration of CTX ϕ into the restored *dif* site. These results reveal a remarkable level of cooperative interactions between multiple filamentous phages in the emergence of the bacterial pathogen that causes cholera.

The TLC element of *V. cholerae* encodes the Cri replicase with homology to filamentous phage replication proteins and TlcR, a protein with sequence similarity to RstR, the repressor controlling lysogeny of the filamentous CTX ϕ and the target for anti-repression by the RstC product of satellite filamentous phage RS1 ϕ (refs 1, 2, 5–9). For these reasons we speculated that the TLC element might correspond to the genome of a satellite filamentous phage that depended on another filamentous phage for its morphogenesis. As a prelude to the study described here, we devised a screen for the postulated ‘TLC helper phage’ and thus identified filamentous phage fs2 ϕ as this helper¹⁰. In brief, our evidence (see Supplementary Information) that fs2 ϕ is a TLC helper phage includes the following. First, strains encoding genetically marked versions of the TLC element (for example TLC-Kn1) inserted in their chromosome produce infectious TLC-Kn ϕ 1 phage particles only if also infected with fs2 ϕ . Second, these TLC-related phage particles carry single-stranded DNA (ssDNA) corresponding to a circularized variant of the TLC element. Third, TLC-Kn ϕ 1 phages infect only cells expressing mannose-sensitive haemagglutinin (MSHA) pili, the known receptor of fs2 ϕ (ref. 11). On infection of MSHA-positive vibrios, the TLC-Kn ϕ 1 ssDNA present in phage particles is converted to the double-stranded replicative form that is detectable in infected cells as a plasmid or as a chromosomally integrated copy. Fourth, the double-stranded replicative form of TLC-Kn ϕ 1 (designated pTLC-Kn1) was also shown to be sufficient for formation of TLC-Kn ϕ 1 phage in recipient cells provided that the cells are also infected with

fs2 ϕ . Thus, fs2 ϕ is a helper phage that provides essential gene products required for morphogenesis of TLC-Kn ϕ 1 phage particles.

For a better understanding of the biology of TLC ϕ , we sequenced pTLC-Kn ϕ 1 and its chromosomally integrated form in strain AL33457-TLC-Kn1. Strain AL33457 was found to carry two copies of the TLC element that flank a unique open reading frame (ORF; VC1471; Fig. 1). Each of the two copies of chromosomally integrated TLC elements in AL33457 is composed of five ORFs, spanning from VC1466 to VC1470 and from VC1472 to VC1476, respectively. In strain AL33457-TLC-Kn1, the Kn^R determinant was located in VC1470 and thus, like ORF VC1471, it was located between the duplicated copies of TLC. Nucleotide sequence analysis of pTLC-Kn1 indicated that this plasmid probably formed as a result of recombination between two directly repeated 25-base-pair (bp) sequences (5'-ACATAATGCGCACTAGGAACATTTT-3'), which are located in the 3' end of VC1465 and within VC1471 (Fig. 1). This 25-bp sequence within VC1471 overlaps by 18 bp (bold nucleotides) with the 28-bp *dif1* sequence, 5'-ATTTAACATAACATACATAATGCGCACT-3' (refs 12, 13). *Dif1* is a site on the large chromosome of *V. cholerae* that is required for the XerC/XerD-mediated resolution of chromosome dimers, and similar sites are also exploited by various filamentous phages for integration of their genomes into the host chromosome using XerC/XerD-mediated recombination^{3,4,12–15}. The *dif1* sequence is used by CTX ϕ and RS1 ϕ for their chromosomal integration through XerC/XerD-mediated recombination with the corresponding *dif/attP* site formed by the annealing of ssDNA derived from phage genomes^{12,13}. The recombination event that formed pTLC-Kn1 looped out the entire region between the 25-bp duplicated sequence in VC1465 and VC1471, including the 18 bp identical to part of the *dif1* sequence together with most of the ORF defined as VC1471. Thus, TLC-Kn ϕ 1 and pTLC-Kn1 encode part of the *dif1* sequence (Fig. 1).

These observations suggest that naturally occurring TLC-related phages might be capable of reconstituting a functional chromosomal *dif* sequence by recombining its partial *dif* site with a defective *dif*-like sequence during lysogenic integration of its genome into the chromosome. To test this hypothesis, we screened a collection of 97 clinical or environmental *V. cholerae* strains of both O1 and non-O1 serogroups and identified 18 strains that were negative for one or more chromosomal regions including TLC, VC1471 and the *dif* sequence^{3,12,13}. These included 12 non-O1, non-O139 strains and 6 non-toxigenic O1 strains. Sequencing of the relevant region in five such TLC-negative CTX-negative O1 strains (AO12682, AO7543, AV2684248, AN19908 and AN25049; see Supplementary Table 1) revealed a gap between the *rtxA* gene (VC1451) and the gene designated VC1479 in all the five strains analysed. In toxigenic strains of the seventh pandemic El Tor biotype such as N16961, the CTX prophage and the TLC genes as well as the recombined *dif*-like sites formed by integration of CTX ϕ are located in this space¹⁶. In the intergenic region between *rtxA* and VC1479 in the TLC-negative strains we identified a possible defective *dif*-like sequence differing in two nucleotide pairs (G→A and C→T) from the genuine *dif* sequence (Fig. 2).

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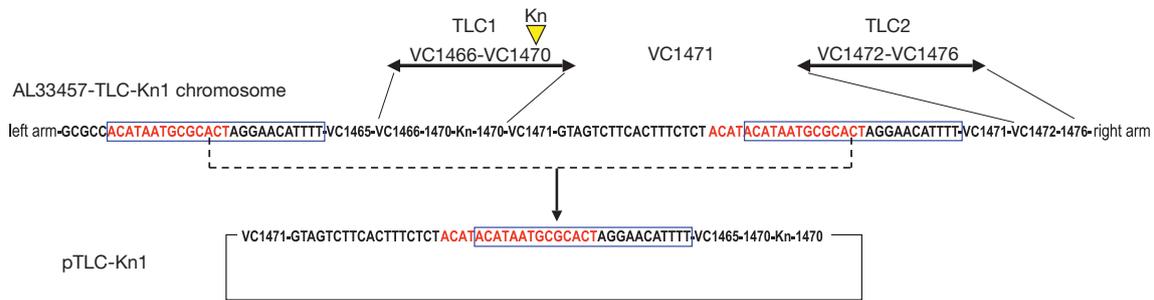


Figure 1 | Schematic diagram showing the formation of plasmid pTLC-Kn1. A conservative recombination event (dotted line and arrow) occurring between two identical 25-bp sequences (boxed nucleotides) located near the 3' end of VC1465 and within VC1471 excised the plasmid from the chromosome of the *V. cholerae* AL33457-TLC-Kn1 parental strain. This strain carries two chromosomal copies of the TLC element with a kanamycin resistance (Kn) insert located in VC1470 of the first TLC element (TLC1).

Cultures of *V. cholerae* strains with deletions in the *dif* recombination site are known to contain a subpopulation of cells with a filamentous morphology³. These filaments reflect aberrant cell division resulting from a defect in XerC/XerD-mediated chromosome dimer resolution³. We examined whether naturally occurring *V. cholerae* O1 strains that lack TLC, or the genuine *dif* sequence, have a filamentous morphology. As shown in Fig. 3 and Supplementary Table 3, these strains do indeed have a filamentous morphology for a noticeable subpopulation of their cells. We next tested whether transduction with TLC-Kn ϕ 1 phage could correct this morphology defect. In each case, cell filamentation in these *V. cholerae* strains was found to be eliminated after transduction with TLC-Kn ϕ 1 (Fig. 3 and Supplementary Table 3). These results suggest that these filamentous strains are indeed *dif*-deficient and that lysogeny with TLC-Kn ϕ 1 apparently corrects this defect.

For further verification of the natural formation of TLC-related phages and the role of the *dif*-like sequence encoded by VC1471 in the correction of *dif*-deficient phenotypes, we used a set of chromosomal

pTLC-Kn1 is therefore a circularized TLC-related element that carries most of VC1471 (a gene located between the 25-bp sequences). Within VC1471 there also exists an 18-bp region (red) that is identical to part of the known 28-bp *dif1* sequence. TLC1 is composed of genes VC1466, VC1467, VC1468, VC1469 and VC1470; TLC2 is composed of genes VC1472, VC1473, VC1474, VC1475 and VC1476. For simplicity, only a subset of these genes is shown in this diagram.

transposon insertion mutants of C6706 (ref. 17). We selected five strains carrying TnFGL3 insertions in the different ORFs of TLC (VC1466, VC1467, VC1468, VC1469, VC1470 and VC1471). With the exception of VC1469, for each strain we were able to recover a plasmid corresponding to the double-stranded replicative form of the TLC ϕ -related genome with a TnFGL3 insertion in the corresponding ORF. No plasmid recovery was actually expected for the insertion mutant in VC1469 because this gene encoded Cri, the protein required for the replication of TLC-related plasmids². When these TLC-related plasmids were introduced into each of the strains SA317, AO7543 and AO12682 (naturally occurring strains with a filamentous morphology and negative for VC1471), normal cellular morphology was restored except in one case. The double-stranded replicative-form plasmid derived from the mutant carrying a TnFGL3 insertion in VC1471 failed to complement the morphology defect in these strains (Supplementary Fig. 4). The TnFGL3 insertion in VC1471 is located within the *dif*-like sequence (bold) encoded by VC1471 (insertion indicated by the asterisk in the sequence 5'-ACATACA*TAATGCGCACTAGGAACA-3'). We conclude that

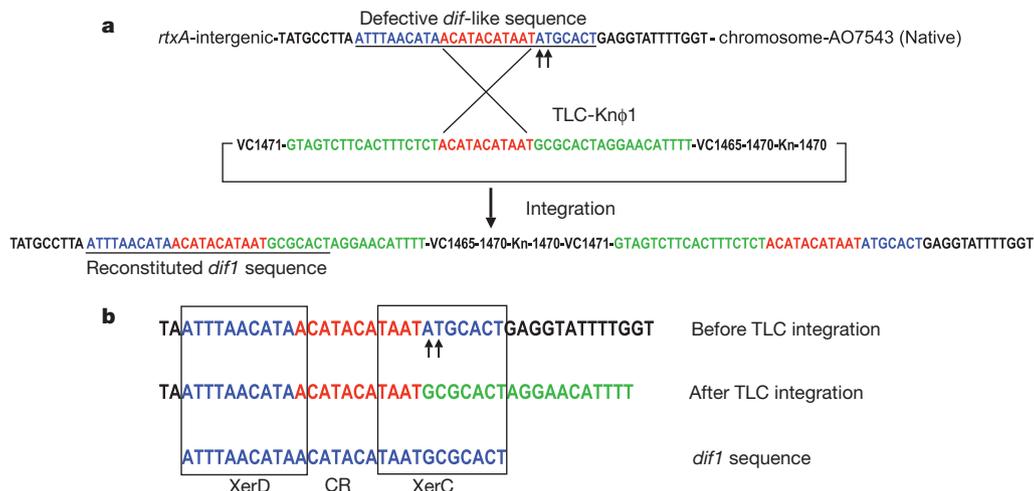


Figure 2 | Restoration of a perfect *dif* site in a *dif*-defective *V. cholerae* O1 strain through integration of the TLC ϕ genome. **a**, Schematic diagram showing site-specific integration of the TLC-Kn ϕ 1 genome into the chromosome of strain AO7543. The region in the vicinity of the phage attachment site is shown in green; the region in the vicinity of the chromosomal attachment site is shown in blue. The chromosomal attachment site corresponds to a defective *dif*-like sequence altered in an AT dinucleotide (arrows). Sequence analysis indicates that the recombination event that integrated the phage genome occurred in the central region of sequence identity (red), which also corresponds in part to the central region (CR) of a *dif1* site (see **b**). This recombination event was probably generated at least in part by the action of XerC and XerD on chromosomal and phage nucleic acid substrates in

that the TLC-Kn1 genome did not integrate chromosomally in XerC-defective and XerD-defective strains (see the text and Supplementary Information). Formation of the TLC phage by integration resulted in the formation of a functional *dif1* sequence (underlined) on its left border. The central sequence of identity is duplicated on the right border but the *dif1* sequence is not duplicated. **b**, Base-pair alignments between the defective *dif*-like sequence on the chromosome of strain AO7543 that is the target for TLC-Kn ϕ 1 integration, the resultant hybrid sequence found after integration of TLC-Kn ϕ 1 (left end) and the authentic *V. cholerae* *dif1* sequence. Colours of nucleotides correspond to those highlighted in **a**. The binding sequences for XerC and XerD recombinases are indicated by boxes.

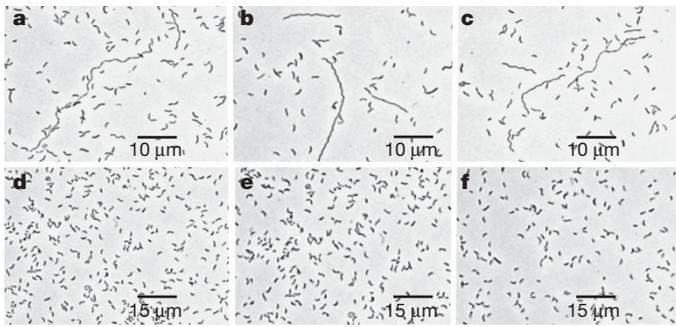


Figure 3 | Transduction with TLC-Kn ϕ 1 phage cures cell filamentation of *Vibrio cholerae* O1 strains that have probable defects in the resolution of chromosome dimers. a–c, Morphology of three strains (a, AO12682; b, AO7543; c, AV2684248) before infection with TLC-Kn ϕ 1; see Supplementary Tables 1 and 4 for details. d–f, Morphology of AO12682 (d), AO7543 (e) and AV2684248 (f) after infection and chromosomal integration of TLC-Kn ϕ 1.

the *dif*-like sequence present in VC1471 is required to correct morphological defects when TLC-related plasmids are introduced into naturally occurring *dif*[−]/TLC[−] strains of *V. cholerae*.

We studied the integration of the TLC-Kn ϕ 1 genome into the chromosome of the naturally occurring TLC-negative strains AO7543 and AO12682 after infecting these strains with TLC-Kn ϕ 1 phage particles. As expected, Southern blot hybridization (Supplementary Fig. 5) and polymerase chain reaction (PCR) analysis indicated that the TLC-Kn ϕ 1 genome had inserted into the bacterial genome in the intergenic region between *rtxA* and VC1479. The correction of the cell filamentation phenotype of *dif*-deficient strains was observed only in strains in which the TLC-Kn ϕ 1 genome had integrated into the chromosome. In contrast, introduction of a pUC18 clone of VC1471, designated pVC1471, into the defective cells did not cure the cell filamentation phenotype even though it carried the *dif*-like sequence. This finding indicated that the *dif*-like sequence present in VC1471 functions only *in cis* and not *in trans*. This is what one would expect if the *dif*-like sequence in VC1471 could indeed function in recombination with *XerC/XerD* to resolve chromosomal dimers only if it recombined into the chromosome. To verify this assumption, we used mutants of *dif*[−] strains with transposon insertions in the *XerC* or *XerD* genes. As expected, transduction of TLC-Kn ϕ 1 into these *XerC*-defective or *XerD*-defective strains did not cure their cell filamentation phenotype (Supplementary Table 4). Furthermore, PCR-based analyses as described above confirmed that the TLC-Kn ϕ 1 DNA did not integrate into the chromosome of the *XerC* or *XerD* mutant strains.

To further examine the mechanism associated with the elimination of the *dif*[−] defect through the chromosomal integration of TLC-Kn ϕ 1 DNA, we sequenced the junction of several independent TLC-Kn ϕ 1 integration events. The sequence analysis showed that TLC-Kn ϕ 1 DNA had integrated into the intergenic region between *rtxA* and VC1479 in strains AO7543, AV2684248 and AO12682 by using its defective *dif*-like site as a recombinational substrate (Fig. 2a). The recombination event leading to the integration of the TLC-Kn ϕ 1 genome resulted in the formation of a functional *dif* sequence identical to the genuine *dif1* sequence reported for *V. cholerae*^{3,13} (Fig. 2b). This result also suggests that TLC-deficient *V. cholerae* strains contain alternative *dif*-like sequences that can still function in recombination with a TLC-encoded *dif*-like sequence but are not fully functional in chromosome dimer resolution. We conclude that the *dif*-like site in VC1471 recombines with the defective chromosomal *dif*-like sequence in these TLC-negative strains during the process of *XerC/D*-mediated TLC genome insertion and that the product of this integration event generates a *dif* sequence that is functional in chromosomal dimer resolution.

In toxigenic *V. cholerae*, the CTX ϕ genome exists as a prophage inserted into the bacterial chromosome at the *dif* recombination

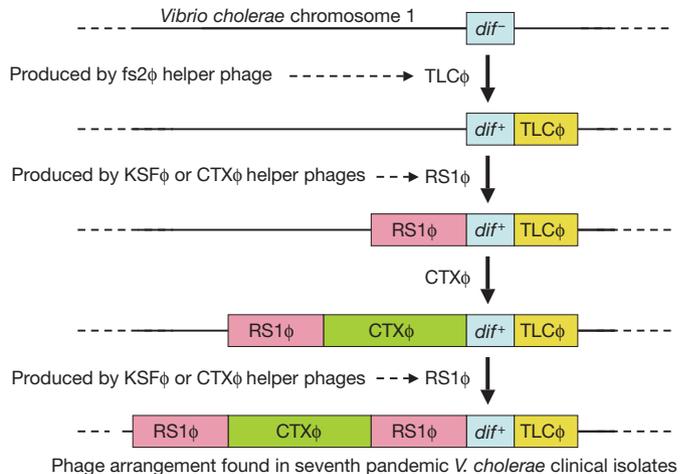


Figure 4 | Schematic diagram of interactions between phages and bacteria that probably led to the emergence of the seventh pandemic clone of *V. cholerae*. Boxes indicate the integrated prophages and *dif* sequence in the seventh pandemic strain, and the solid lines indicate the corresponding empty sites where these elements are absent in the precursor strains. Sequential lysogeny by three different filamentous phages (TLC ϕ , CTX ϕ and RS1 ϕ) and the role of two helper phages (fs2 and KSF-1) are shown. To generate the observed organization of RS1 prophages found in most seventh pandemic strains, it is postulated that two rounds of RS1 prophage integration would be needed if this prophage integrated into only a functional *dif* site and then reconstituted only one *dif* site after integration. For a more complete explanation of these hypothetical steps see Supplementary Fig. 6.

site^{12,13,15}. Because transduction with TLC-Kn ϕ 1 reconstructs a functional *dif* sequence in the recipient bacterium (Fig. 2), we tested whether TLC-Kn ϕ 1 transductants could be stably lysogenized by CTX ϕ . We chose test strains that were positive for the TCP locus (which encodes the receptor for CTX ϕ (ref. 1)), and used a CTX ϕ prophage that was marked with a chloramphenicol resistance marker (CTX-Cm ϕ). As expected, we found that TLC-Kn ϕ 1 transductants were readily superinfected with CTX-Cm ϕ and in these cases CTX-Cm ϕ was found integrated into the *dif* site generated through previous integration of TLC-Kn ϕ 1. In contrast, although natural TLC-negative strains could also be infected with CTX-Cm ϕ (Supplementary Table 6), the CTX-Cm ϕ genome did not integrate, and the unintegrated CTX-Cm ϕ genome was rapidly lost when inoculated into the intestinal loops of adult rabbits. Because the integrated form of the CTX ϕ genome is known to be more stably retained in *V. cholerae* than the unintegrated plasmid form is¹⁸, these data strongly argue that TLC ϕ is crucial to the natural, stable acquisition of CTX ϕ .

Although the TLC element was known to be invariably present in all CTX-positive strains and notably absent in CTX-negative strains², the role of this element in the evolution of toxigenic *V. cholerae* was not clear until the present study. Here we show that the TLC element can give rise to infectious phage particles (TLC ϕ) when its morphogenesis is supported by another filamentous phage, previously designated fs2 ϕ (ref. 10). Furthermore, infectious forms of TLC ϕ that encode a *dif*-like sequence can be easily isolated. These specialized TLC ϕ -related transducing phages can, after chromosomal integration, generate a functional *dif* sequence and correct aberrant filamentous morphology present in TLC-negative cells that apparently show defective *dif*/*XerC/XerD*-mediated chromosome dimer resolution. Lysogeny by these TLC phages leads to the restoration of a functional *dif* site, which is also essential for the stable integration of CTX ϕ and the conversion of *V. cholerae* to a toxigenic form.

The most common strains of *V. cholerae* causing cholera in the world today are all highly related to the seventh pandemic clone of *V. cholerae*, which emerged as a human pathogen in 1971 in the Celebes Islands^{19,20}. The arrangement of the TLC prophage, and the *dif* site used by CTX ϕ in these highly successful pandemic strains, is

virtually the same as the one that we produced experimentally in this study by lysogeny of *dif*⁻ strains such as AO7543 sequentially with TLC-Kn ϕ 1 followed by CTX-Cm ϕ . It therefore seems highly likely that the precursor of the seventh pandemic clone was a *dif*⁻ strain that emerged as a pandemic pathogen after sequential lysogeny by three filamentous phages, namely TLC ϕ , CTX ϕ and RS1 ϕ (Fig. 4). Because *dif*⁻ defects are deleterious to growth, it is possible that the precursor of the seventh pandemic clone may be rare in the environment or that the *dif*⁻ genotype confers an as yet undetermined advantage for non-toxicogenic O1 strains in the context of its environmental niche. Nonetheless, our data suggest that the evolutionary emergence of the toxigenic seventh pandemic clone of *V. cholerae* probably involved molecular interactions between two satellite filamentous phages (TLC ϕ and RS1 ϕ), three helper filamentous phages (fs2 ϕ , CTX ϕ and KSF ϕ (ref. 21)), and two type IV pilus-based phage receptors (MSHA and TCP) (Supplementary Fig. 6). Accordingly, our results provide a model for understanding the cooperative interactions of multiple genetic elements in the evolution of pathogenic bacteria from non-pathogenic environmental progenitors.

METHODS SUMMARY

A genetic marker encoding kanamycin resistance (Kn^R) was introduced into the TLC element carried by multiple *V. cholerae* strains followed by screening of the marked strains for the production of TLC-related Kn^R transducing particles in the culture supernatants. Digestion with mung bean nuclease and also hybridization analysis with strand-specific oligonucleotide probes corresponding to the (+) and (-) strands of the TLC element were conducted to test whether the DNA carried by putative TLC-related phage particles present in filter-sterilized culture supernatants was single-stranded. The role of fs2 ϕ as a helper of TLC satellite phage was established by demonstrating the formation of TLC-Kn ϕ 1 phage in recipient cells that contained pTLC-Kn1, provided that the cells were also infected with fs2 ϕ .

For transduction assays, recipient *V. cholerae* strains were mixed with genetically marked phage preparations⁵, and transductants were selected by using Luria-Bertani agar medium containing appropriate antibiotics. Integration of the TLC-Kn ϕ 1 genome was detected by Southern blot hybridization and PCR assays using two primers, of which one was complementary to the chromosomal region and the other corresponded to pTLC-Kn ϕ 1. DNA sequencing was conducted for further confirmation of the integration event and to detect the generation of the *dif* sequence. Subsequently, a chloramphenicol resistance (Cm^R)-marked CTX phage was used to study the susceptibility and chromosomal integration^{5,18} of CTX phages into the restored *dif* site. The Cm^R-marked CTX phage genome (pCTX-Cm) was constructed by replacing the Kn^R marker in pCTX-Km (ref. 1) derived from strain SM44 with a Cm^R cassette.

The full list of strains and plasmids is available as Supplementary Table 1. Full methods and associated references are provided in Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions F.H., M.K. and S.M.F. conducted the experiments and performed analyses of bacterial strains and phages. S.M.F. and J.J.M. designed the studies, analysed data and wrote the manuscript.

Author Information The sequences described in the article are deposited in GenBank under accession numbers HM134797, HM134798, HM134799 and HM134800. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.M.F. (faruque@icddr.org).