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

Faizule Hassan[1], **M. Kamruzzaman**[1], **John J. Mekalanos**[2], and **Shah M. Faruque**[*,1]

[1]Molecular Genetics Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka-1212, Bangladesh

[2]Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115

Abstract

Bacterial chromosomes often carry integrated genetic elements (e.g., plasmids, transposons, prophages, and islands) whose precise function and contribution to the evolutionary fitness of the host bacterium are unknown. The CTXφ 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 characterization of a TLC-related element that corresponds to the genome of a satellite filamentous phage (TLC-Knφ1) which uses the morphogenesis genes of another filamentous phage (fs2φ) to form infectious TLC-Knφ1 phage particles. The TLC-Knφ1 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 (e.g., CTXφ) for chromosomal integration of their genomes. Bacterial cells defective in the dimer resolution often show an aberrant filamentous cell morphology3,4. We found that acquisition and chromosomal integration of the TLC-Knφ1 genome restored a perfect *dif* site and normal morphology to *V. cholerae* wild type and mutant strains that displayed *dif*⁻ filamentation phenotypes. Furthermore, lysogeny of a *dif*⁻ nontoxigenic *V. cholerae* with TLC-Knφ1 promoted its subsequent toxigenic conversion through integration of CTXφ 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 that displays sequence similarity to RstR, the

*Corresponding author: Mailing address: Shah M. Faruque, Molecular Genetics Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka-1212, Bangladesh., Phone: (+880 2) 8860523 through 8860532, Fax : (+880 2) 8812529, faruque@icddrb.org.

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repressor controlling lysogeny of the filamentous CTXφ and the target for anti-repression by the RstC product of satellite filamentous phage RS1φ1,2,5-9. For these reasons we hypothesized that the TLC element corresponds to the genome of a satellite filamentous phage that depended on another filamentous phage for its morphogenesis. As a prelude to the described study, we devised a screen for the postulated "TLC helper phage" and this effort identified filamentous phage fs2φ as such a helper10. In brief, our evidence (see supplementary information) that fs2φ is a TLC helper phage includes the following: 1) Strains encoding genetically marked versions of the TLC element (e.g., TLC-Kn1) inserted in their chromosome produce infectious TLC-Knφ1 phage particles only if also infected with fs2φ; 2) Such TLC-related phage particles carry ssDNA corresponding to a circularized variant of the TLC element; 3) TLC-Knφ1 phage infect only cells expressing mannose sensitive hemagglutinin (MSHA) pili, the known receptor of fs2φ11. Upon infection of MSHA+ vibrios, the TLC-Knφ1 ssDNA present in phage particles is converted to the double stranded replicative form (RF) that is detectable in infected cells as a plasmid or as a chromosomally integrated copy; 4) The RF form of the TLC-Knφ1 (designated pTLC-Kn1) was also shown to be sufficient for formation of TLC-Knφ1 phage in recipient cells provided that the cells are also infected with fs2φ. Thus fs2φ is a helper phage that provides essential gene products required for TLC-Knφ1 phage particle morphogenesis.

In order to better understand the biology of TLCφ, we sequenced pTLC-Knφ1 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 ORF (VC1471)(Fig. 1). Each of the two copies of chromosomally integrated TLC elements in AL33457 is comprised of 5 ORFs, spanning from VC1466 to VC1470 and VC1472 to VC1476, respectively. In strain AL33457-TLC-Kn1, the Kn^R determinant was located in VC1470 and thus, like ORF VC1471 was located between the duplicated copies of TLC. Nucleotide sequence analysis of pTLC-Kn1 indicated that this plasmid likely formed as a result of recombination between two directly repeated 25 base pair (bp) sequences (ACATAATGCGCACTAGGAACATTTT), which are located in the 3'end of VC1465 and within VC1471 (Fig. 1). Remarkably, this 25 bp sequence within VC1471 overlaps 18 bp (bold nucleotides) with the 28 base pair *dif1* sequence ATTTAACATA**ACATACATAATGCGCACT**14,15. *Dif1* is a site on the large chromosome of *V. cholerae* which is required for 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 recombination3,4,12-15. The *dif1* sequence is utilized by CTXφ and RS1φ for their chromosomal integration though XerC/XerD-mediated recombination with the corresponding *dif/attP* site formed by annealing of ssDNA derived from phage genomes14,15. The recombination event that formed pTLC-Kn1 looped out the entire region between the 25bp duplicated sequence in VC1465 and VC1471 including the 18bp identical to a part of the *dif1* sequence together with most part of the ORF defined as VC1471. Thus, TLC-Knφ1 and pTLC-Kn1 encode a 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. In order to test this hypothesis, we screened a collection of 97 clinical or environmental *V. cholerae* of both O1 or non-O1 serogroups and identified 18 strains which were negative for one or more chromosomal regions including TLC, VC1471 or the *dif* sequence[3,14,15] These included 12 non-O1, non-O139 strains and 6 nontoxigenic O1 strains. Sequencing of the relevant region in five such TLC negative CTX negative O1 strains (AO12682, AO7543, AV2684248, AN19908, and AN25049, see Table S1) revealed a gap between the *rtxA* gene (VC1451) and the gene designated VC1479 in all the five strains analyzed. In toxigenic strains of 7th 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 CTXφ are located in this space[16]. Notably in the intergenic region between *rtxA* and VC1479 in the TLC negative strains, a possible defective *dif*-like sequence, differing in two nucleotide pairs (G to A, and C to T) from the bona fide *dif* sequence, was identified (Fig. 2).

Cultures of *V. cholerae* strains with deletions in the *dif* recombination site are known to contain a subpopulation of cells that display a filamentous morphology[3]. These filaments reflect aberrant cell division resulting from a defect in XerC/XerD-mediated chromosome dimer resolution[3]. We examined whether naturally occurring *V. cholerae* O1 strains that lack TLC, or the bona fide *dif* sequence, display filamentous morphology. As shown in Fig 3 and Table S3, these strains indeed display filamentous morphology for a noticeable sub-population of their cells. We next tested whether transduction with TLC-Knφ1 phage could correct this morphology defect. In each case, cell filamentation in these *V. cholerae* was found to be eliminated following transduction with TLC-Knφ1 (Fig. 3, Table S3). These results suggest that these filamentous strains are indeed *dif*-deficient and that lysogeny with TLC-Knφ1 apparently corrected this defect.

To further verify the natural formation of TLC-related phages and the role of the *dif*-like sequence encoded by VC1471 in correction of *dif*-deficient phenotypes, we used a set of chromosomal transposon insertion mutants of C6706[17]. We selected five strains carrying TnFGL3 insertions in the different ORF's 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 RF of TLCφ-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 replication of TLC-related plasmids[2]. When these TLC-related plasmids were introduced into each of the strains SA317, AO7543 and AO12682 (naturally occurring strains that showed filamentous morphology and were negative for VC1471), normal cellular morphology was restored except in one case. The RF plasmid derived from the mutant carrying a TnFGL3 insertion in VC1471 failed to complement the morphology defect in these strains (Fig. S4). Notably, the TnFGL3 insertion in VC1471 is located within the *dif*-like sequence (bold) encoded by VC1471 (insertion indicated by the * in the sequence: **ACATACA*TAATGCGCACT**AGGAACA). We conclude that the *dif*-like sequence present in VC1471 is required to correct morphological defects upon introduction of TLC-related plasmids into naturally occurring *dif*[+]/TLC[-] strains of *V. cholerae*.

We studied the integration of TLC-Knφ1 genome into the chromosome of naturally occurring TLC negative strains AO7543 and AO12682 after infecting these strains with TLC-Knφ1 phage particles. As expected, Southern blot hybridization (Fig S5) and PCR analysis indicated that the TLC-Knφ1 genome had inserted into the bacterial genome in the intergenic region between *rtxA* and VC1479. This was confirmed by PCR assays using two primers one of which was complementary to this chromosomal region and the other corresponding to pTLC-Knφ1. Notably, the correction of the cell filamentation phenotype of *dif*-deficient strains was observed only in strains in which the TLC-Knφ1 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 despite the fact that 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 utilized mutants of *dif*− strains with transposon insertions in the *XerC* or *XerD* genes. As expected, transduction of TLC-Knφ1 into these *XerC* or *XerD* defective strains did not cure their cell filamentation phenotype (Table S4). Furthermore, PCR based analyses as described above confirmed that the TLC-Knφ1 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 chromosomal integration of TLC-Knφ1 DNA, we sequenced the junction of several independent TLC-Knφ1 integration events. The sequence analysis showed that TLC-Knφ1 DNA had integrated into the intergenic region between *rtxA* and VC1479 in strains AO7543, AV2684248 and AO12682 using its defective *dif*-like site as a recombinational substrate (Fig 2A). Remarkably, the recombination event leading to the integration of the TLC-Knφ1 genome resulted in formation of a functional *dif* sequence identical to the bona fide *dif1* sequence reported for *V. cholerae*3 (Fig. 2B). This result also suggests that TLC-deficient *V. cholerae* strains contain alternative *dif*-like sequences that can still function in recombination with 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 CTXφ genome exists as a prophage inserted into the bacterial chromosome at the *dif* recombination site13-15. Because transduction with TLC-Knφ1 reconstructs a functional *dif* sequence in the recipient bacterium (Fig. 2), we tested whether TLC-Knφ1 transductants could be stably lysogenized by CTXφ. We chose test strains that were positive for the TCP locus (which encodes the receptor for CTXφ1), and utilized a CTXφ that was marked with a chloramphenicol resistance (CTX-Cmφ). As expected we found that TLC-Knφ1 transductants were readily superinfected with CTX-Cmφ and in these cases CTX-Cmφ was found integrated into the *dif* site generated through previous integration of TLC-Knφ1. In contrast, although natural TLC negative strains could also be infected with CTX-Cmφ, (Table S6) the CTX-Cmφ genome did not integrate, and the un-

integrated CTX-Cmφ genome was rapidly lost when inoculated in the intestinal loops of adult rabbits. Because the integrated form of the CTXφ genome is known to be more stably retained in *V. cholerae* compared to the un-integrated plasmid form18 these data strongly argue that TLCφ plays a critical role in the natural, stable acquisition of CTXφ.

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 (TLCφ) when its morphogenesis is supported by another filamentous phage, previously designated as fs2φ10. Furthermore, infectious forms of TLCφ which encode a *dif*-like sequence can be easily isolated. These specialized TLCφ-related transducing phages can, upon chromosomal integration, generate a functional *dif* sequence and correct aberrant filamentous morphology present in TLC-negative cells that apparently exhibit defective *dif*/XerC/XerD-mediated chromosome dimer resolution. Lysogeny by such TLC phages leads to the restoration of a functional *dif* site which also is essential for stable integration of CTXφ and 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 7th pandemic clone of *V. cholerae* that emerged as a human pathogen in 1971 in the Celebes Islands19,20. The arrangement of TLC prophage, and the *dif* site utilized by CTXφ in these highly successful pandemic strains is virtually the same as the one we experimentally produced in this study by lysogeny of *dif⁻* strains such as AO7543 sequentially with TLC-Knφ1 followed by CTX-Cmφ. Thus, it seems highly likely that the precursor of the 7th pandemic clone was a *dif⁻* strain which emerged as a pandemic pathogen after sequential lysogeny by three filamentous phages TLCφ, CTXφ, and RS1φ (Fig 4). Because *dif⁻* defects are deleterious to growth, it is possible that the precursor of the 7th pandemic clone may be rare in the environment or that the *dif⁻* genotype confers a yet-to-be-determined advantage for nontoxigenic O1 strains in the context of its environmental nitch. Nonetheless, our data suggest that the evolutionary emergence of the toxigenic 7th pandemic clone of *V. cholerae* likely involved molecular interactions between two satellite filamentous phages (TLCφ, and RS1φ), three helper filamentous phages (fs2φ, CTXφ and KSFφ21), and two type IV pilus-based phage receptors (MSHA and TCP) (Fig. S6). Accordingly, our results provide a paradigm for understanding the cooperative interactions of multiple genetic elements in the evolution of pathogenic bacteria from nonpathogenic environmental progenitors.

Methods Summary

A genetic marker encoding kanamycin resistance (KnR) was introduced into the TLC element carried by multiple *V. cholerae* strains followed by screening of the marked strains for production of TLC-related KnR transducing particles in the culture supernatants. Mung bean-nuclease digestion as well as hybridization analysis with strand-specific oligonucleotide probes corresponding to the (+) and (-) strand of TLC element was 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 fs2φ as a helper of

TLC satellite phage was established by demonstrating the formation of TLC-Knφ1 phage in recipient cells which contained pTLC-Kn1 provided that the cells were also infected with fs2φ.

For transduction assays, recipient *V. cholerae* strains were mixed with genetically marked phage preparations5, and transductants were selected using Luria-Bertini agar medium containing appropriate antibiotics. Integration of TLC-Knφ1 genome was detected by Southern blot hybridization, and PCR assays using two primers one of which was complementary to the chromosomal region and the other corresponding to pTLC-Knφ1. DNA sequencing was conducted to further confirm the integration event and detect the generation of the *dif* sequence. Subsequently, a chloramphenicol resistance (Cm^R)-marked CTX phage was used to study the susceptibility and chromosomal integration5,18 of CTX phage into the restored *dif* site. The Cm^R-marked CTX phage genome (pCTX-Cm) was constructed by replacing the Kn^R-marker in pCTX-Km1 derived from strain SM44 with a Cm^R cassette.

The full list of strains and plasmids is available as Table S1. Full methods and associated references are available in the supplementary information linked to the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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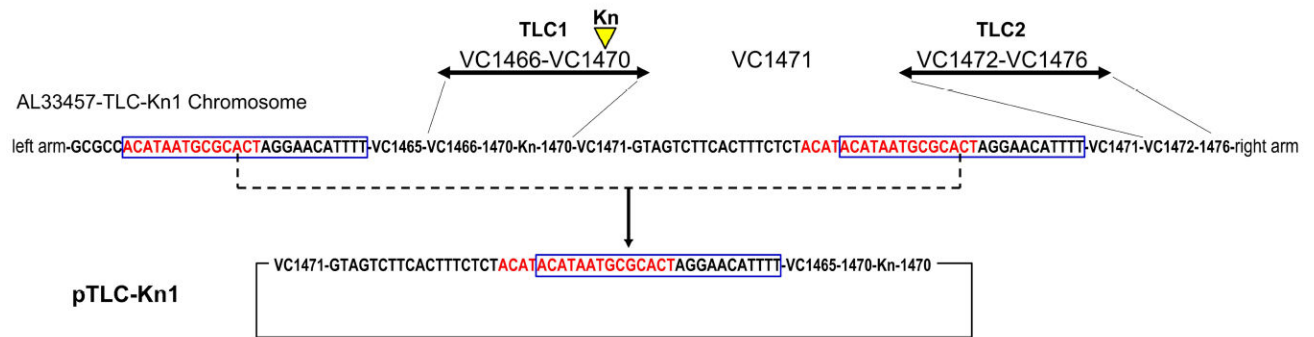
Figure 1.
Schematic diagram showing the formation of plasmid pTLC-Kn1. A conservative recombination event (dotted line and arrow) occurring between two identical 25 bp sequences (boxed nucleotides) located near the 3' end of VC1465 and within VC1471 excised the plasmid from the chromosome of the *V. 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). Thus pTLC-Kn1 represents a circularized TLC-related element that carries VC1471 (a gene that was located between the 25 bp sequences. Within VC1471 there also exists an 18-bp region (red font) which is identical to a part of the known 28 bp *dif1* sequence. TLC1 is composed of genes VC1466, VC1467, VC1468, VC1469, and VC1470 while TCL2 is composed of genes VC1472, VC1473, VC1474, VC1475, and VC1476. Only a subset of these genes are shown for simplicity in this diagram.
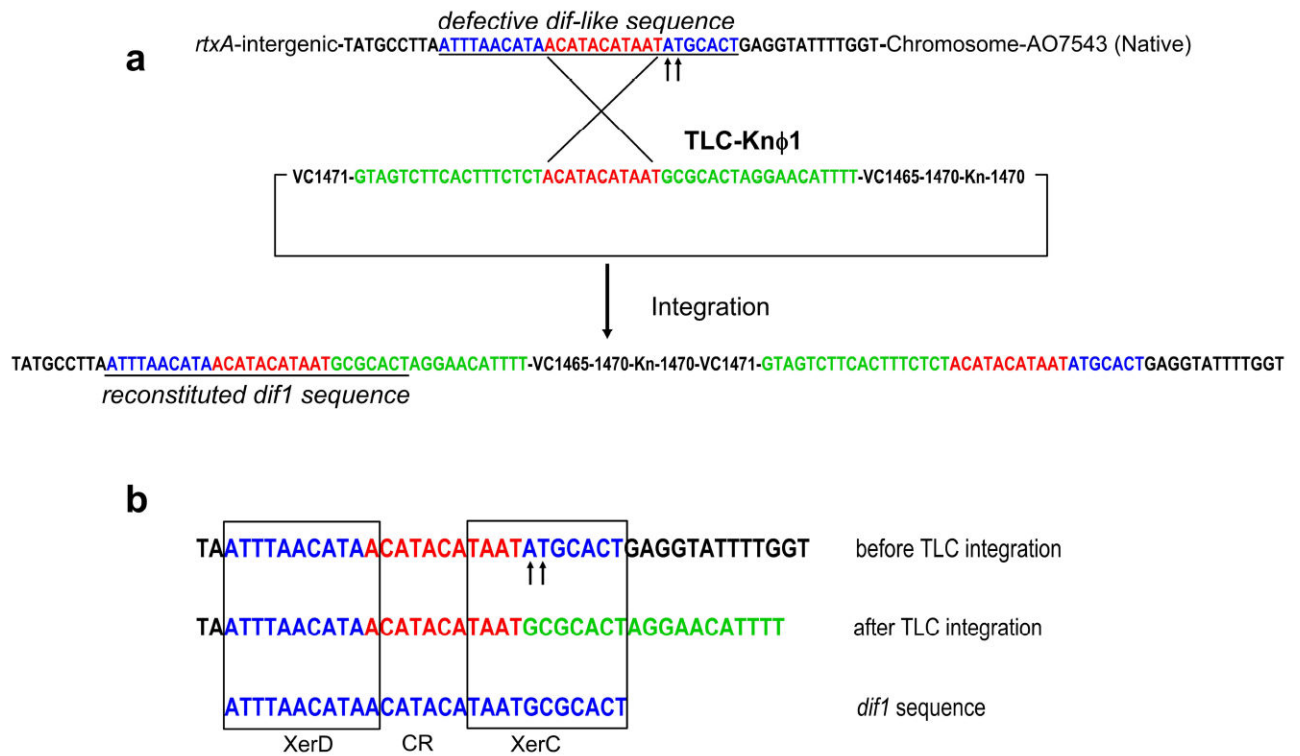
Figure 2.
Part a, Schematic diagram showing site specific integration of TLC-Knφ1 genome into the chromosome of strain AO7543. The region in the vicinity of the phage attachment site is shown in green and the region in the vicinity of the chromosome attachment site is shown in blue. The chromosomal attachment 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 Fig 2, Part b). This recombination event is likely generated at least in part by action of the XerC and XerD on chromosomal and phage nucleic acid substrates in that the TLC-Kn1 genome did not chromosomally integrate in XerC-defective and XerD-defective strains (see text and supplement). Formation of the TLC prophage by integration resulted in 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. **Part b**, Base pair alignments between the defective *dif*-like sequence on the chromosome of strain AO7543 that is the target for TLC-Knφ1 integration, the resultant hybrid sequence found after integration of TLC-Knφ1 (left end) and the authentic *V. cholerae dif1* sequence. Colors of nucleotides correspond to those highlighted in Part A of this figure. The binding sequences for XerC and XerD recombinases are indicated with filled boxes.
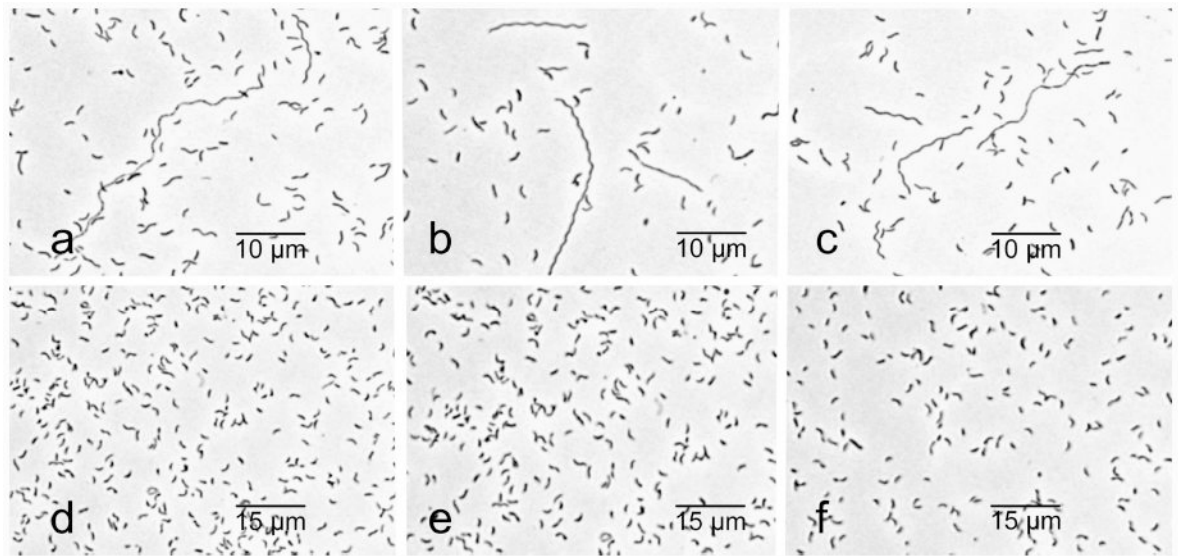
Figure 3.
Transduction with TLC-Knφ1 phage cures cell filamentation of *Vibrio cholerae* O1 strains which have likely defects in resolution of chromosome dimers. Panels **a** through **c** (**a**,strain AO12682, **b**, strain AO7543, and **c**, strain AV2684248, see Table S1 and Table S4 for details) show the morphology of three different strains before infection with TLC-Knφ1 whereas panels **d** through **f** show the same strains respectively after infection and chromosomal integration of TLC-Knφ1.
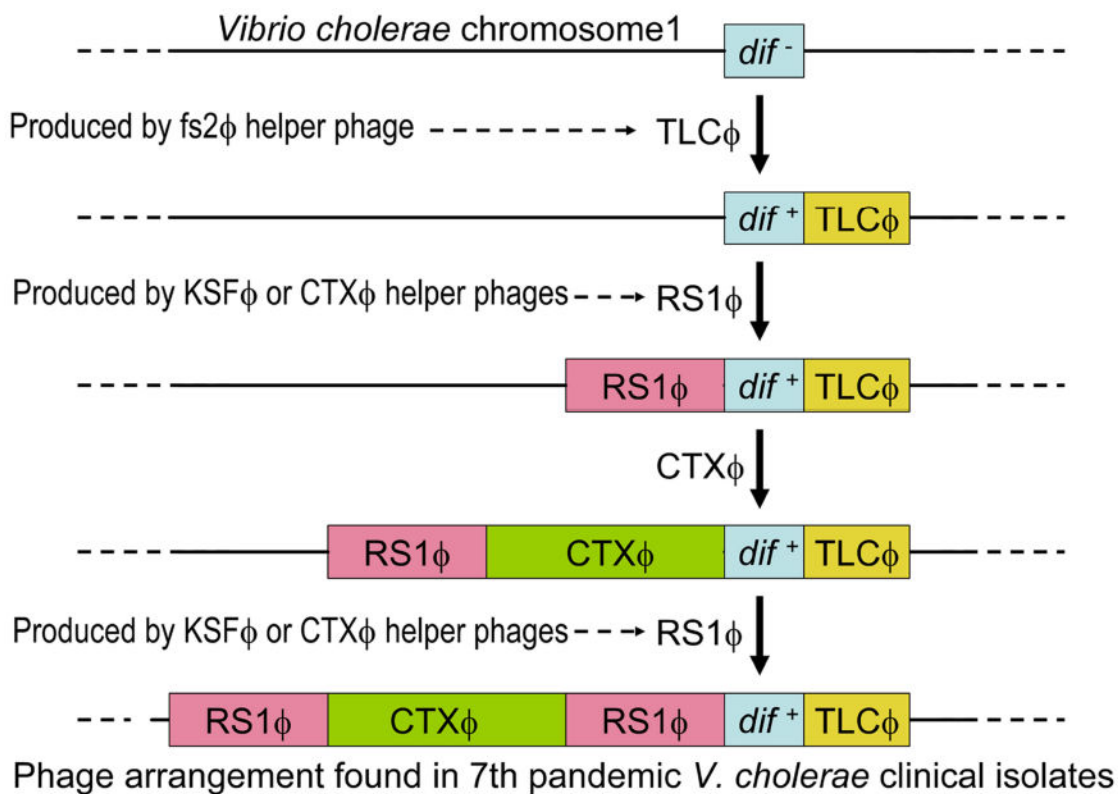
Figure 4.
Schematic diagram of phage-bacterial interactions that likely led to the emergence of the 7th pandemic clone of *Vibrio cholerae*. Boxes indicate the integrated prophages and *dif* sequence in the 7th pandemic strain, whereas the solid lines indicate the corresponding empty sites where these elements are absent in the precursor strains. Sequential lysogeny by three different filamentous phages TLCφ, CTXφ, and RS1φ, and the role of two helper phages fs2 and KSF-1 are shown. In order to generate the observed organization of RS1 prophages found in most 7th pandemic strains, it is postulated that two rounds of RS1 prophage integration would be needed if this prophage integrates into only a functional *dif* site and then reconstitutes only one *dif* site after integration. For a more complete explanation of these hypothetical steps see Fig. S6 in the supplementary information.