Efficiency and specificity of CTXϕ chromosomal integration: *dif* makes all the difference

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Bacteriophage can convert their bacterial host from a nonpathogenic form to a pathogenic form by providing the bacterium with virulence genes, in a process called lysogenic phage conversion. *Vibrio cholerae* is a bacterium prevalent in marine environments that can infect humans to cause the devastating diarrheal disease cholera, which is endemic in much of Asia and Africa. Although the health and economic burdens of cholera are enormous, the disease is sometimes overshadowed by other diseases, but cholera's predilection for epidemic spread commands attention.

Cholera toxin, an A-B type exotoxin encoded by the *ctxAB* genes, is the main cause of the voluminous watery diarrhea that is characteristic of cholera (1–3). *V. cholerae* isolates that cause cholera encode the *ctxAB* genes in the genome of a filamentous bacteriophage CTXϕ (Fig. 1*A*) (4). CTXϕ is a small, positive, single-stranded DNA [(+) ssDNA)] virus that can be found either in a replicative form or, more commonly, integrated site-specifically in the host genome to form stable lysogens. Whether the ssDNA or dsDNA form of the virus is the substrate for recombination and integration between the dsDNA host genome and CTXϕ is open to debate (5, 6). Many different CTXϕ types and arrangements have been observed in the host *V. cholerae* genome (7). The basis of specificity and efficiency of integration of CTXϕ is not known, nor is the mechanism of emergence of strains with novel CTXϕ chromosomal arrangements. In PNAS, a report by Das et al. resolves many of the questions surrounding the mechanism of CTXϕ integration and the capacity of variant CTXϕ genomes to integrate at each of the known attachment sites (*att*) (8).

Like many bacteriophage, CTXϕ integrates its genome into the chromosome of a host *V. cholerae,* thereby ensuring stable vertical transmission within the bacterial host. Subsequent to CTXϕ particle adsorption to the *V. cholerae* cell wall, viral ssDNA is injected into the cell cytoplasm and forms a circular pCTX, which then integrates into the *V. cholerae* genome at a site-specific attachment site (4, 9). CTXϕ integration requires a number of phage-encoded and host-encoded factors (5, 9). A recombinase (integrase), which ordinarily catalyzes this integration in other phages, is not present in the CTXϕ genome; instead, it commandeers two host-encoded tyrosine recombinases, XerC and XerD (9). The XerCD proteins are conserved among eubacteria, as they serve to resolve chromosome dimers during cell division (10, 11). In *Escherichia coli*, XerCD proteins bind and catalyze recombination at homologous 28-bp *dif* sites, composed of two 12-bp binding sites for XerC and XerD separated by a 6-bp spacer or overlap region, which allows for XerC-XerD interactions that ensure stable synapsis (10, 11). Because *V. cholerae* harbors two distinct, nonhomologous circular chromosomes (chromosome I and II) (12, 13), two *dif* sites are present, *dif*1 in chromosome I and *dif*2 in chromosome II (9); similar to *E. coli*, the same FstK-dependent mechanism coordinates dimer resolution on each chromosome with cell division (14). The *dif*1 site differs from *dif*2 at four polymorphic sites, one of which is located in the XerC binding site and the other three sites are located in the 6-bp spacer region (9, 14).

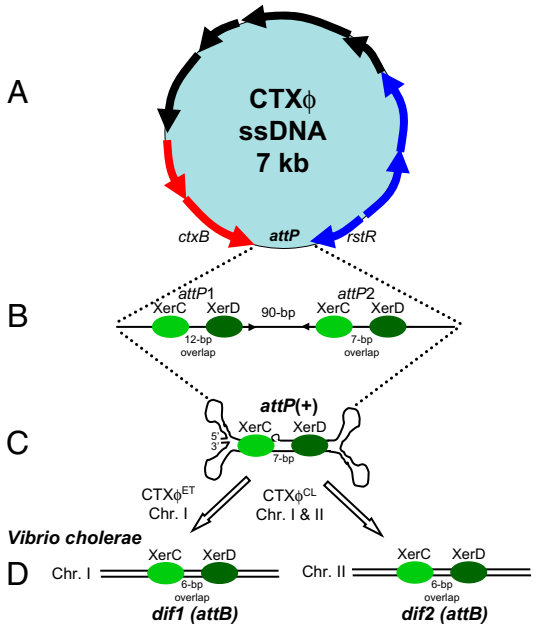

Fig. 1. (*A*) Schematic representation of the single-stranded CTXϕ genome. Arrows indicate ORFs identified and characterized in CTXϕ. Phage attachment site is indicated by *attP*. (*B*) Linear representation of ssDNA *attP* site and regions identified by Huber and Waldor (9) and by McLeod and Waldor (5) as essential for efficient integration of CTXϕ into chromosome I (ChrI) of *V. cholerae*. Green oval shapes represent XerC and XerD attached at their respective binding sites on *attP* sites 1 (*attP*1) and 2 (*attP*2), identified by McLeod and Waldor (5). Also shown are the spacer or overlap regions between XerC and XerD binding sites, 12 bp for attP1 and 7 bp for attP2. (*C*) Secondary fork and stem structure formed when attP1 and attP2 base pair, creating the new attP+ site, identified by Val et al. (6). (*D*) The bacterial attachment sites (attB), *dif*1 and *dif*2 on chromosome I and chromosome II, the primary function of which is to resolve chromosomal dimers during cell division.

The arrangement of CTXϕ in the *V. cholerae* genome depends on whether it is integrated at one or two of the chromosome dimer resolution sites, *dif*1 and *dif*2, and the number of copies present at each site. For example, in many El Tor strains, the cause of the seventh and ongoing cholera pandemic, the El Tor phage $CTX\phi^{ET}$ is arranged in tandem and interspersed with a related element, RS1, to give an RS1-CTX^{ET}-RS1-CTX^{ET}-RS1 arrangement on chromosome I. The El Tor Strain C6709, isolated in Peru in 1991, encodes a CTX^{ET}-RS1 arrangement on chromosome I (7, 15). In *V. cholerae*

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classical biotype isolates, which were the cause of earlier pandemics and are now extinct, the classical type phage CTXϕ^{CL} is integrated on both chromosomes and never contains an RS1 element (16, 17). Interestingly, recent El Tor strains isolated in Mozambique and India have been found to contain a single CTXϕ^{CL} integrated on chromosome I (18); but how these novel arrangements and strains emerge remains an open question.

Huber and Waldor (9) proposed that the integration of CTXϕ resulted from recombination between a 200-bp intergenic region (*attP*) of the replicative double-stranded form of CTXϕ and *dif1* (*attB*) on chromosome I of *V. cholerae* (Fig. 1*A*). These authors demonstrated that the recombination reaction was catalyzed by XerC and XerD; but here, unlike in *E. coli* chromosomal dimer resolution, the cell division protein FtsK was not required. Unlike other phage site-specific integration mechanisms, CTXϕ integration was irreversible (9). CTXϕ virons are generated from a chromosomally integrated phage by a process analogous to rolling circle replication (19). Further detailed molecular characterization of the CTXϕ *attP* and chromosome I *dif*1 *(attB)* interaction was carried out using purified recombinases to show XerC and XerD binding to *attP* and subsequent single-strand exchange (5), but surprisingly, the mechanism did not follow that executed by other known tyrosine recombinases.

McLeod and Waldor found that XerC and XerD also bound to a second site ≈90-bp downstream of the first binding site (Fig. 1*B*) (5). The precise role of second site was undetermined, although it was required for integration (5). In addition, the XerC cleavage on *attP* was separated from XerD cleavage by 12-bp, which is wholly unexpected given the 6–8 bp spacing of XerC and XerD binding at two homologous *dif* sites, which is essential for interaction between the two recombinases that control synapse formation and catalysis (10, 11). McLeod and Waldor's data suggested that synapsis of the two duplexes performed by XerCD at the *attP* and *dif*1 sites are potentially less stable than recombination between homologous *dif* sites. The reasons why CTX integration was irreversible were also unresolved.

Following from these data, an alternative model for CTXϕ integration suggested that the (+) ssDNA genome is the form exploited for integration at *dif*1 by XerC and XerD, which is further supported by Das et al. (Fig. 1*C*) (6, 8). Val et al. (6) uncovered a double-forked hairpin structure within the region encompassing *attP* in the (+) ssDNA of form of CTXϕ that creates an alternative *attP*+ site in the stem of the secondary structure (Fig. 1*C*). Val et al. further demonstrated that XerC can catalyze a single pair of strand exchanges between this target, *attP* +, and *dif*1 in the presence of XerD, resultant in CTXϕ integration upon conversion of the ensuing Holliday junction by repair and/or replication, similar to the earlier findings by McLeod and Waldor (5, 6). However, at the time, neither the ssDNA nor the dsDNA integration models explained how CTXϕ integrates at *dif*2 on chromosome II, given the lack of complementary base pair interactions that would result, which are required to stabilize the exchange of strands catalyzed by XerC. Das et al. have clarified this issue, showing how and why there is specific integration between CTXϕ^{ET} at *dif*1 and none at *dif*2, in contrast to the integration of CTXϕ^{CL} at both *dif*1 and *dif*2 (8). The authors demonstrate that this altered integration behavior of CTXϕ^{CL} is due to two base changes in the overlap region of *attP*2 in this phage, which allows XerCD recombination between CTXϕ^{CL} *attP*+ with *dif*1 and *dif*2. Indeed, they show that alteration of these two bases in the El Tor CTXϕ *attP*+ site results in efficient integration into both *dif*1 and *dif*2. Their data determine that the specificity of integration of the different CTXϕ variants is governed by the potential of the ssDNA CTXϕ to form base pair interactions that stabilize strand exchanges. Furthermore, the authors show that complementary base pairing may account for recent El Tor isolates that contained a *dif*-like site (*dif*G) with an overlap region different from *dif*1 and *dif*2. The authors further observe that neither the El Tor nor the classical CTXϕ *attP*+ could recombine with *difG*, but they identified a variant *attP*^{G}+ site that allowed recombination to take place with *difG*.

The wide distribution of the XerCD recombinase system among bacteria and the prevalence of *dif* sites among filamentous phages suggest that this system may be the paradigm for filamentous phage integration into the bacterial genome. One question that is still puzzling is how CTXϕ^{CL} are transferred between natural isolates. Although the data explain the specificity of the integration mechanism, they do not account for the recent emergence of *V. cholerae* El Tor strains containing CTXϕ^{CL}. Recent studies have shown that CTXϕ^{CL} isolated in a range of strains cannot produce CTXϕ virons; the infectious form of CTXϕ^{CL} is only ever present integrated in the genome (17, 18, 20). It is possible that these novel strains acquired CTXϕ^{CL} via a mechanism alternative to lysogenic conversion, such as lytic phage and/or transformation mechanisms, although the in vitro efficiencies of these events have been shown to be quite low (20, 21).

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