

CTX ϕ contains a hybrid genome derived from tandemly integrated elements

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CTX ϕ is a filamentous, temperate bacteriophage whose genome includes *ctxAB*, the genes that encode cholera toxin. In toxigenic isolates of *Vibrio cholerae*, tandem arrays of prophage DNA, usually interspersed with the related genetic element RS1, are integrated site-specifically within the chromosome. We have discovered that these arrays routinely yield hybrid virions, composed of DNA from two adjacent prophages or from a prophage and a downstream RS1. Coding sequences are always derived from the 5' prophage whereas most of an intergenic sequence, intergenic region 1, is always derived from the 3' element. The presence of tandem elements is required for production of virions: *V. cholerae* strains that contain a solitary prophage rarely yield CTX virions, and the few virions detected result from imprecise excision of prophage DNA. Thus, generation of the replicative form of CTX ϕ , pCTX, a step that precedes production of virions, does not depend on reversal of the process for site-specific integration of CTX ϕ DNA into the *V. cholerae* chromosome. Production of pCTX also does not depend on RecA-mediated homologous recombination between adjacent prophages. We hypothesize that the CTX ϕ -specific proteins required for replication of pCTX can also function on a chromosomal substrate, and that, unlike the processes used by other integrating phages, production of pCTX and CTX ϕ does not require excision of the prophage from the chromosome. Use of this replication strategy maximizes vertical transmission of prophage DNA while still enabling dissemination of CTX ϕ to new hosts.

CTX ϕ is a filamentous phage whose single-stranded DNA genome includes *ctxAB*, the genes that encode cholera toxin (CT) (1), the primary virulence factor produced by the cholera-genic bacterium *Vibrio cholerae* (2). Unlike the filamentous phages that infect *Escherichia coli*, CTX ϕ can give rise to lysogens, which contain the phage genome integrated into the bacterial chromosome (1, 3, 4). Integration of CTX ϕ DNA into the *V. cholerae* chromosome is a site-specific process that does not require RecA (5). Both O1 El Tor and O139 strains of *V. cholerae*, which have caused virtually all recent cholera epidemics, have just one chromosomal locus within which the phage genome is integrated (3, 6). The core of this integration site (*attB*) is a 17-bp sequence that is almost identical to an 18-bp sequence within the phage genome. Integration of phage DNA into the genome of an *attB*⁺, CTX ϕ ⁻ El Tor strain of *V. cholerae* yields single or tandem prophages flanked by these 17/18-bp sequences, which are known as end repeats (ERs) (5). If such a lysogen is infected by an additional phage or transformed with the replicative form (RF) (a plasmid) of phage DNA, the new phage DNA will integrate at an ER between tandem prophages or between a 3' end and chromosomal DNA; the ER between the 5' end of a prophage and adjacent chromosomal DNA is not used (7).

Toxigenic strains of *V. cholerae* frequently also contain a genetic element related to CTX ϕ , known as RS1 (4). This element is found on the chromosome adjacent (5' and/or 3') to CTX prophages (Fig. 1). Its genes include *rstR*, which encodes the phage repressor, and *rstA* and *rstB*, which are the phage genes needed for autonomous replication of phage DNA and for phage DNA integration. RS1 also contains an additional gene, *rstC*, not

found in the CTX ϕ genome; however, it lacks *ctxAB* and all other genes of the phage "core" region, which are thought to encode virion coat and assembly proteins (1, 4). Many different CTX ϕ /RS1 arrays have been observed among epidemic *V. cholerae* isolates (3). However, all of the arrangements are similar in that prophages and RS1s are immediately adjacent to each other; they are never separated by chromosomal loci. Furthermore, to our knowledge, natural O1 El Tor and O139 isolates harboring a solitary prophage, not flanked by either an RS1 or another CTX prophage, have not been identified.

Most prophage/RS1 arrays in O1 El Tor and O139 strains yield infectious virions at a low but measurable rate, even in the absence of inducing stimuli such as mitomycin C (7, 8). These virions are secreted, rather than released through bacterial lysis, and virion production is not known to limit growth of the host bacterium. It is likely that virion production by lysogens is preceded by formation of the RF of the phage genome (also known as pCTX). pCTX can be detected at low levels (fewer than 1 copy per 10 cells) within plasmid DNA prepared from most CTX lysogens (B.M.D. and H. H. Kimsey, unpublished data). This plasmid is apparently a preferred template for synthesis of viral proteins and for further rounds of extrachromosomal CTX replication, because strains engineered to contain at least one copy of the RF per bacterium produce a relatively high number of virions. In contrast, strains that harbor mutant RFs whose packaging depends on chromosome-encoded viral proteins yield far fewer virions (B.M.D., K. E. Moyer, E. F. Boyd, and M.K.W., unpublished work).

Based on studies of the excision of lambda and other temperate phages that integrate into the chromosome in a site-specific fashion, we initially presumed that the CTX ϕ RF arises in lysogens through excision of the prophage via site-specific recombination between the flanking ER sequences. That is, we believed that the processes for generating CTX prophages and pCTX would be inversely related, as has been observed for integration/excision of lambdaoid phages and the phage P2 (9). However, we have discovered that a distinct process, apparently unrelated to phage DNA integration, usually underlies formation of CTX ϕ virions. This process, which does not require homologous recombination, depends on the presence of tandem prophages or of a prophage followed by an RS1. Engineered strains harboring solitary prophages rarely give rise to pCTX or to infectious virions. The RFs and virions produced from phage/RS1 arrays are hybrids, derived from the coding se-

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Abbreviations: Kn, kanamycin; Ap, ampicillin; RF, replicative form; CT, cholera toxin; ER, end repeat; ig-1 intergenic region 1.

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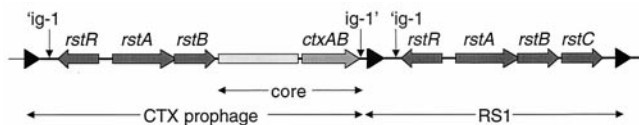


Fig. 1. RS1 is a genetic element frequently found adjacent to CTX prophages; it contains some but not all of the genes encoded within CTX ϕ . Black triangles represent end repeat (ER) sequences. The core region is not drawn to scale. Within the RF of CTX ϕ , intergenic region-1 (*ig-1*; composed of *ig-1'* and *'ig-1*) is a continuous sequence between *ctxAB* and *rstR*.

quences of the 5' prophage and an intergenic region from the 3' prophage or RS1. We hypothesize that replication of phage DNA from a chromosomal template, rather than prophage excision, is the first step in generating pCTX and ultimately CTX ϕ .

Materials and Methods

Bacterial Strains and Culture Conditions. All bacteria were cultured in Luria–Bertani broth at 37°C unless otherwise noted. Antibiotics were used at the following concentrations: ampicillin (Ap), 50 μ g/ml (*V. cholerae*); Ap, 100 μ g/ml (*E. coli*); kanamycin (Kn), 50 μ g/ml. Sucrose selection was performed as described (10).

Phage Transduction Assays. Donor cultures were grown to an OD₆₀₀ of \approx 1. Both marked and unmarked CTX phage transduction assays were performed as described (7).

Molecular Biology Methods. Southern hybridization was carried out by using horseradish peroxidase-labeled DNA probes, which were prepared and hybridized by using the ECl direct nucleic acid labeling and detection system (Amersham Pharmacia) according to the manufacturer's instructions. PCR products were cloned into the TA cloning vector pCRII-TOPO (Invitrogen) according to the manufacturer's instructions. Other techniques were performed by using standard protocols (11).

Plasmid and Strain Construction. Plasmid derivatives of pCTX and RS1 used in this study are described in Table 1. Strains are described in Fig. 4, Fig. 6, and/or below. AS207 CTX^{ET}::Kn was generated with the allele exchange vector pHK260 as described

Table 1. pCTX and RS1-derived plasmids used in this study

Plasmid	Description	Source
pCTX ^{ET} -Kn	Replicative form of CTX ^{ET} -Kn ϕ	Ref. 1
pCTX ^{ET} -Ap	Xba I fragment of pCTX ^{ET} -Kn (contains all CTX phage genes), inserted into pGP704	Ref. 7
pCTX ^{ETNF} -Kn	pCTX ^{ET} -Kn with <i>NotI</i> site filled in and religated	This study
pCTX ^{ETH1} -Kn	Hybrid pCTX ^{ET} -Kn from Kn ^R transductants of AS207 CTX ^{ET} ::Kn	This study
pGP704	<i>oriR6K mobR4</i> suicide vector, Ap ^R	Ref. 21
pBD275	PCR-amplified fragment (<i>rstC3-rig1-1</i>) of circular permutation of RS1 cloned into pCRII-Topo	This study
pBD276	PCR-amplified fragment (<i>rstC3-rstR9</i>) of circular permutation of RS1 cloned into pCRII-Topo	This study
pBD286	<i>EcoRI</i> insert of pBD275 in pGP704	This study
pBD287	<i>EcoRI</i> insert of pBD276 in pGP704	This study

(7). CTX ϕ lysogens were generated by transforming 2740-80 [an O1 El Tor, *attB*⁺, CTX ϕ ⁻ strain (5)] with the appropriate pCTX; prophage copy numbers were determined by Southern blotting. BD206 was generated with a derivative of the Δ *recA*::Kn targeting vector pGP84 (12) in which the *XbaI* fragment containing the Kn^R cassette was deleted. Mutants made with this new Ap^R, dominant streptomycin-sensitive, counterselectable vector (pBD176) lack a 2.8-kb *XbaI* chromosomal segment that includes most of *recA*. Δ *recA* clones were identified by their sensitivity to methyl methanesulfonate (Sigma) as described (13). The targeting vector (pBD570) used to generate BD578 contains a 1.09-kb *HpaI-XbaI* fragment of 2740-80 DNA that spans most of *recA*. The *recA643* mutation was introduced on PCR primers (K215QF: 5'GGTAACGCACTGCAATTCTACGC and K215QR: 5'CAGAAGCGTAGAATTGCAGTGC3') by using standard protocols, and the mutant fragment was cloned into the counterselectable (*sacB*) suicide vector pCVD442 (10). This allele was crossed onto the chromosome as described (10). *recA643* clones were identified by their inability to integrate, via homologous recombination, an unrelated Ap^R suicide vector. The presence of the A643C mutation in BD578 *recA* (which results in the amino acid change K215Q) was confirmed by DNA sequence analysis. The insert for pBD275 was amplified from a circularly permuted form of RS1 (B.M.D., unpublished results) by using *rstC3* (5'GCTTTATATCTGCGTTCAGGCG3') and *rig1-1* (5'CACGCTACGTCGCTTATGT3'). The insert for pBD276 was similarly amplified with *rstC3* and *rstR9* (5'CTTC-GACATCAAATGGCA3'). The related plasmids pBD286 and pBD287 were introduced into *V. cholerae* lysogens by mating; they could integrate into the *V. cholerae* chromosome by ER-mediated site specific recombination because the necessary proteins (RstB, perhaps RstA) were encoded by the resident prophage in the recipient strains. Strains with a single plasmid integrant downstream of the prophage were identified by Southern blotting.

Results

"Hybrid" CTX ϕ Virions Produced by a Clinical Isolate of *V. cholerae*. Studies of *V. cholerae* strain AS207 (14), a recent O139 clinical isolate from Calcutta, India, provided the first indication that CTX ϕ is not generated via ER-mediated excision of a CTX prophage. AS207 carries two distinct forms of CTX prophages: the prophage found in O1 El Tor strains of *V. cholerae*, CTX^{ET}, which was the first prophage to be identified as a producer of infectious virions, and a new prophage, CTX^{calc}, which also yields phage particles (7). These two prophages contain distinct *rstR* genes and distinct intergenic sequences flanking these genes (7, 15). To study production of virions by AS207, we created derivatives of this strain in which the various copies of *ctxAB* were replaced by a kanamycin resistance (Kn^R) cassette (7). Filtered supernatants from cultures of such marked strains can be used to transduce an O1 classical recipient strain, O395, to Kn^R. The CTX prophages in O395 encode a third form of RstR (RstR^{class}) and therefore do not interfere with replication of CTX^{ET} or CTX^{calc}. Copies of transduced DNA can subsequently be isolated by preparing plasmid DNA from the Kn^R clones because O395, like other classical strains (e.g., RV508, CA401) lacks a functional integration site for newly introduced CTX ϕ DNA (ref. 1; data not shown). Unexpectedly, AS207 CTX^{ET}::Kn (depicted in Fig. 2) yielded Kn-transducing virions that did not exactly correspond to the CTX^{ET}Kn prophage. Instead, AS207 CTX^{ET}::Kn generated virions that contained coding sequences, including *rstR*, derived from the El Tor prophage, but also included part of an intergenic sequence (*'ig-1*; see Fig. 1 and Fig. 2) derived from the downstream CTX^{calc} prophage. This "hybrid" phage, CTX^{ETH1}-Kn ϕ , was initially recognized because of the absence of an expected *NotI* restriction site in the corresponding pCTX^{ETH1}-Kn plasmid, and its structure was subse-

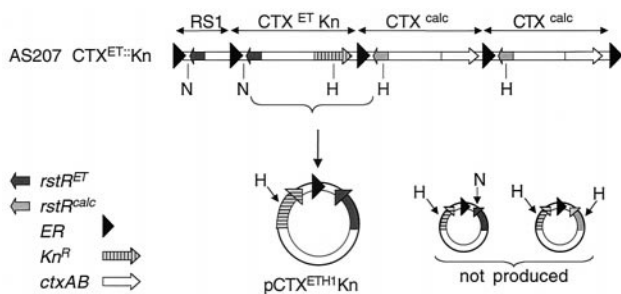


Fig. 2. Kn^R transductants derived from supernatants of AS207 $\text{CTX}^{\text{ET}}::\text{Kn}$ contain a plasmid with sequences from both the $\text{CTX}^{\text{ET}}::\text{Kn}$ prophage and the downstream CTX^{calc} prophage. A specific hybrid plasmid ($\text{pCTX}^{\text{ETH1}}::\text{Kn}$) is always produced; neither alternate hybrids nor the $\text{CTX}^{\text{ET}}\text{-Kn}$ RF are ever detected. H, *HindIII*; N, *NotI*.

quently confirmed by DNA sequencing. The plasmid contains all of the CTX^{ET} prophage sequences except the first 357–473 bp, which have been replaced by the first 479–595 bp of the CTX^{calc} prophage. It is not currently possible to identify the junction between CTX^{ET} and CTX^{calc} -derived sequences more precisely because bp 358–473 of CTX^{ET} are identical to bp 480–595 of CTX^{calc} . (ig-1 of the CTX^{calc} prophage is slightly larger than the corresponding region of $\text{CTX}^{\text{ET}}\phi$; hence, the numerical boundaries for the regions of sequence identity differ between the two prophages. For this analysis, the first base pair after the 5' ER has been considered to be bp1.) Sequence analysis of AS207 $\text{CTX}^{\text{ET}}::\text{Kn}$ chromosomal DNA confirmed that the Kn -marked prophage in the chromosome still contained El Tor 'ig-1 sequences; these sequences had not been disrupted during generation of the Kn -marked AS207 derivative.

Analysis of four independently derived pools of Kn^R transductants, composed of >20 transductants, revealed that all contained plasmids with restriction maps corresponding to $\text{pCTX}^{\text{ETH1}}::\text{Kn}$ (described above and in Fig. 2). Alternate hybrid plasmids (e.g., containing the *HindIII* site within $\text{rstR}^{\text{calc}}$ as well as the 'ig-1 sequence of the CTX^{calc} prophage) were not detected. These data strongly suggest that homologous recombination between adjacent prophages to release a Kn^R -marked plasmid does not underlie formation of the $\text{CTX}\phi$ RF and virions, because such a mechanism would be expected to yield Kn^R -transducing virions containing $\text{rstR}^{\text{calc}}$ at least some of the time. Consistent formation of a specific hybrid is more likely to be the outcome of a replication and/or excision process with some sequence specificity.

We confirmed that formation of the hybrid phage was not an aberrant outcome induced by the presence of the Kn^R cassette by analyzing unmarked virions produced by AS207. Supernatants of AS207 were mixed with O395 and grown overnight at 30°C to maximize infection of the recipient, as described (7). Plasmid DNA was then prepared from these unselected potential transductants, and the DNA was analyzed by Southern blotting (Fig. 3). Hybridization of the blot with an rstR^{ET} probe revealed that all transduced DNA that included rstR^{ET} did not contain the *NotI* site found 5' to this gene in the prophage. Thus, AS207 appeared to produce unmarked, RstR^{ET} -encoding $\text{CTX}^{\text{ETH1}}\phi$ s with structures matching the marked hybrid virions produced by AS207 $\text{CTX}^{\text{ET}}::\text{Kn}$ and differing from the CTX^{ET} prophage from which they are principally derived. This finding is consistent with the hypothesis that virions regularly incorporate DNA from two, rather than just one, CTX prophage. This hypothesis was also supported by analyses of virions produced by additional lysogens containing tandem prophages that could be distinguished by their restriction maps. As with AS207 and AS207 $\text{CTX}^{\text{ET}}::\text{Kn}$, these lysogens produced virions in which

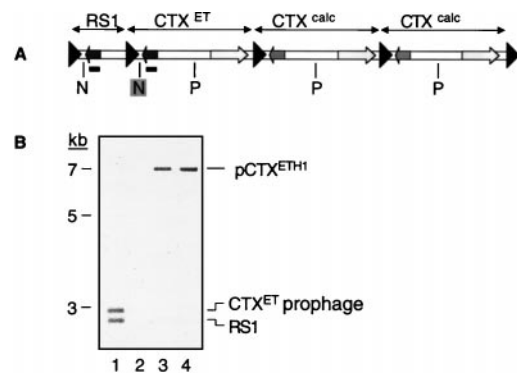


Fig. 3. The restriction map of the CTX^{ET} prophage in AS207 differs from the map of pCTX^{ET} found in transductants derived from AS207. (A) Restriction map of AS207 chromosomal DNA. N, *NotI*; P, *PstI*. (B) A Southern blot of donor chromosomal DNA and plasmid DNA from potential transductants probed with rstR^{ET} (underlined in A). This probe detected a 2.9-kb band in a *NotI/PstI* digest of AS207 chromosomal DNA (lane 1) but a 7-kb band in a *NotI/PstI* digest of plasmid DNA from a pool of potential transductants. Digestion of the plasmid DNA with *PstI* alone (lane 4) revealed that the *NotI* site (shaded in A), rather than the *PstI* site, was absent from the plasmid. No hybridization was observed to *NotI/PstI*-digested plasmid DNA isolated from the transduction recipient O395 grown without donor supernatant (lane 2).

'ig-1 sequences were derived from the second prophage in the array whereas core sequences were derived from the upstream prophage (data not shown).

Negligible Production of Virions by Lone CTX Prophages. A corollary of the hypothesis that virions regularly incorporate DNA from two CTX prophages, rather than just one prophage, is that lysogens that contain only a single CTX prophage should not yield CTX virions. To test whether this is true, we constructed an isogenic set of strains that contain either a single or tandem antibiotic-marked prophage(s) by transforming *V. cholerae* 2740-80 with $\text{pCTX}^{\text{ET}}\text{-Kn}$. The resulting transformed strains were analyzed by Southern blotting to determine the number of prophages present in each (data not shown). Culture supernatants of these strains were then tested for the presence of Kn^R transducing particles (Fig. 4). Supernatants from strains containing tandem $\text{CTX}^{\text{ET}}\text{-Kn}$ prophages (e.g., BD193 and BD81) yielded more than 10^4 Kn^R transductants \times $\text{OD}_{600}^{-1} \times \text{ml}^{-1}$. In contrast, supernatants from a strain with a single $\text{CTX}^{\text{ET}}\text{-Kn}$ prophage (BD195) rarely yielded any transductants. Only one trial of BD195 supernatant yielded a single Kn^R colony. Similar data were obtained with single and tandem $\text{CTX}^{\text{ET}}\text{-Ap}$ prophages, which transduce recipients to ampicillin (Ap) resistance (Fig. 4). As expected, based on this data, pCTX could be detected within plasmid DNA isolated from cultures of lysogens with tandem prophages but not within DNA from lysogens with a single prophage (data not shown). These data indicate that production of virions from a solitary prophage is a very infrequent event.

Imprecise Excision of Solitary CTX Prophages. To gain insight into the mechanism by which virions occasionally arise from solitary CTX prophages, we used restriction mapping to analyze the plasmids within Ap^R transductants generated from supernatants of BD213, a strain with a single $\text{CTX}^{\text{ET}}\text{-Ap}$ prophage. In four of four transductants studied, the Ap^R plasmid did not correspond precisely to $\text{pCTX}^{\text{ET}}\text{-Ap}$ (Fig. 5, compare lanes 1–4 to lane 5 and lanes 6–9 to lane 10). None of the four contained sequences from the 3' end of the CTX prophage. Instead, restriction mapping and PCR analyses indicated that the plasmids all contained some chromosomal sequences from upstream of the $\text{CTX}^{\text{ET}}\text{-Ap}$ pro-

Source of donor supernatant		transductants
Strain	prophage array structure	(transductants \times O.D. ₆₀₀ ⁻¹ \times ml ⁻¹)
2740-80		N.A.
CTX ^{ET} -Kn lysogens		
BD195		<35
BD193		1.2 \times 10 ⁴
BD81		2.4 \times 10 ⁴
BD206 (Δ <i>recA</i>)		4.2 \times 10 ²
BD578 (<i>RecA</i> -K215Q)		1.0 \times 10 ⁴
CTX ^{ET} -Ap lysogens		
BD213		<36
BD137		2.0 \times 10 ⁴

Fig. 4. Production of CTX virions by lysogens with tandem arrays of prophages vs. solitary prophages. All strains were derived from strain 2740-80. BD 213, BD137, BD81, and its derivatives BD206 and BD578 are based on a *lac*⁻ 2740-80, BD22. Black triangles represent the ERs that flank each prophage after integration into *attB*. Gray lines represent chromosomal DNA. Transductants \times OD₆₀₀⁻¹ \times ml⁻¹ is normalized to the OD₆₀₀⁻¹ of the donor culture; the titer is the average result obtained in at least three experiments. Detection of one transductant/assay corresponds to a titer of \approx 35; the exact value depends on the donor OD₆₀₀⁻¹. When no transductants were obtained, the titer was, thus, less than the calculated threshold value. N.A., not applicable.

phage in BD213 as well as a portion of the CTX^{ET}-Ap ϕ genome (Fig. 5; data not shown). These data suggest that BD213, and presumably other strains with a solitary prophage as well, only produces virions after imprecise excision of the prophage from the chromosome. Thus, it appears that ER-mediated site specific recombination, which was initially believed to underlie all CTX ϕ virion production, is not even used to generate the rare subset of virions produced from solitary CTX prophages.

Production of CTX ϕ from Prophage Arrays Does Not Depend On Homologous Recombination. The elevated phage yields from lysogens with tandem prophages (relative to single prophage strains; Fig. 4) is consistent with the model that virions incorporate genetic material from two distinct prophages. However, the uniformity of hybrids produced by AS207 suggests that unrestricted homologous recombination between adjacent CTX prophages is not the dominant mechanism for producing pCTX from such arrays. Consequently, we assessed whether homologous recombination is necessary for production of CTX virions by strains with tandem prophages, using two *recA*⁻ derivatives of

BD81. One derivative, BD206, has a 2.8-kb deletion encompassing most of *recA* whereas the other, BD578, has a point mutation (K215Q) expected to impair homologous recombination but not RecA-mediated protease activity (16). We found that supernatants from BD206 yielded significantly fewer transductants than the isogenic wild type strain, although Kn^R colonies were still obtained with an average frequency of 420 transductants \times OD₆₀₀⁻¹ \times ml⁻¹ (Fig. 4). In contrast, BD578 yielded 10⁴ transductants \times OD₆₀₀⁻¹ \times ml⁻¹, similar to the wild-type strain BD81, despite the fact that its capacity for homologous recombination was equivalent to that of the *recA* deletion strain (data not shown). pCTX DNA was detectable within plasmid DNA prepared from cultures of both strains (data not shown). These data suggest that homologous recombination between adjacent prophages is not required for efficient production of virions but that RecA-mediated proteolysis does contribute to formation of CTX ϕ . As with other temperate phages, RecA may mediate cleavage of the CTX ϕ -specific repressor, RstR, and thereby facilitate viral replication. Interestingly, CTX ϕ lysogens still yield a low level of virions even in the absence of RecA (i.e., BD206), indicating that the generation of CTX ϕ does not entirely depend on RecA-mediated cleavage of RstR.

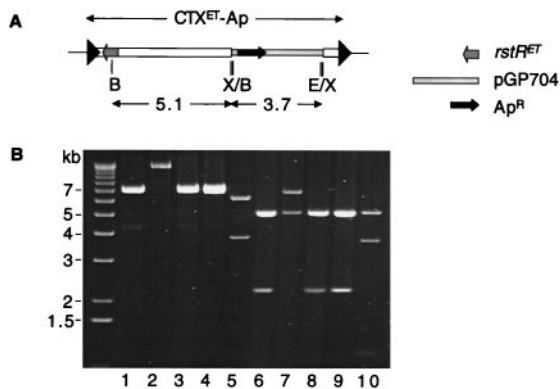


Fig. 5. Plasmids isolated from Ap^R transductants derived from the supernatant of BD213, which contains a solitary CTX^{ET}-Ap prophage, are not equivalent to pCTX^{ET}-Ap. (A) Restriction map of BD213 chromosomal DNA. B, *Bgl*III; X, *Xba*I; E, *Eco*RI. (B) pCTX^{ET}-Ap (lanes 5 and 10) yielded different restriction fragments than did plasmid DNA from four transductants (lanes 1–4 and 6–9) after digestion with *Xba*I (lanes 1–5) and *Eco*RI/*Bgl*III (lanes 6–10). All of the new Ap^R plasmids contained the 5.1-kb *Bgl*III fragment and the adjacent Ap^R cassette; all lacked the *Eco*RI and *Xba*I sites at the 3' end of the prophage.

“Rescue” of a Solitary Prophage by Downstream Insertion of RS1 Derivatives. Because the CTX^{ETH1}-Kn ϕ produced by AS207 CTX^{ET}::Kn consistently contain only a short sequence derived from the 3' prophage in the array, we speculated that a complete prophage followed by just a fragment of a prophage might still efficiently give rise to virions. We therefore generated derivatives of 2740-80 in which fragments of RS1 were inserted downstream of a solitary prophage. Like the sequentially integrated prophages in BD315 and BD550, the new downstream fragments were introduced into the chromosomal DNA by ER-mediated recombination. The fragments do not encode the proteins needed for CTX ϕ replication and integration (RstA and RstB), but these were provided by the prophage already present on the chromosome. The new prophage arrays generated by using this strategy are diagrammed in Fig. 6; the structure of each was confirmed by Southern blotting (data not shown).

Supernatants from these new strains were used in transduction assays to determine whether insertion of a prophage fragment could rescue a solitary prophage (i.e., enhance its capacity to yield virions). We found that insertion of a 1,280-bp sequence (from ER through the beginning of *rstA*) downstream of a single

Source of donor supernatant		transductants
Strain	prophage array structure	(per O.D. ₆₀₀ ⁻¹ × ml ⁻¹)
BD193		1.2 × 10 ⁴
BD305		2.5 × 10 ⁴
BD555		2.7 × 10 ⁴
BD328		94
BD195		<35

Fig. 6. A solitary prophage with a fragment of RS1 inserted downstream can efficiently produce CTX virions. BD555 is identical to BD305 except that it contains a CTX^{ETNF}-Kn prophage (derived from pCTX^{ETNF}-Kn), not a CTX^{ET}-Kn prophage. Kn^R transductants derived from BD555 always contain pCTX^{ET}-Kn. The inserted fragment in BD328 is too small to rescue the 5' prophage.

prophage was as effective as insertion of a complete prophage in rescuing virion production by the 5' prophage. Supernatants from strains containing the 1,280-bp insertion (e.g., BD305, BD555) yielded at least 10⁴ transductants × OD₆₀₀⁻¹ × ml⁻¹, a titer equivalent to the tandem prophage strain BD193 (Fig. 6). Furthermore, the RF of virions produced by BD555 always contained a *NotI* site (pool of 15 assayed), although the 5' prophage in BD555 lacks this site, suggesting that virions had again incorporated sequences from the 3' element within the array. These data also demonstrated that a defect in the solitary prophage in BD195 (which was used to construct BD305) was not responsible for its failure to yield virions.

Insertion of a shorter fragment of RS1 was much less effective at rescuing virion production by a solitary prophage. Introduction of a 340-bp fragment (which included all but the final 17 bp of the region known to be derived from the 3' prophage; e.g., in BD328) resulted in production of 96 transductants × OD₆₀₀⁻¹ × ml⁻¹. Thus, the presence of the 340-bp fragment downstream of the solitary prophage enhanced virion production relative to the yield from the prophage alone; however, the effect from this fragment was far below that of a larger fragment or a complete prophage.

Discussion

We have shown that the “unit prophage” from which CTX ϕ virions are generally derived is in fact a hybrid, composed from two adjacent prophages or prophage-related elements. Virions derive all of their coding sequence from the 5' prophage in an array, but their DNA for an intergenic region ('ig-1) is primarily from the 3' genetic element. The 3' element can be a second prophage, an RS1, or a derivative of RS1. Formation of virions from tandem elements does not depend on RecA-mediated homologous recombination between adjacent prophages.

Virions are rarely produced by CTX lysogens that contain only a solitary prophage, even if such strains are treated with Mitomycin C (Fig. 4; data not shown). Analysis of the few transductants derived from these strains suggests that virions were produced after imprecise excision of the single CTX prophage from the chromosome. Similar imprecise excision of a CTX precursor phage is thought to have enabled a *ctxAB*⁻ precursor phage to acquire the genes encoding cholera toxin (E. F. Boyd and M.K.W., unpublished work). We have never detected virions that appear to have arisen from site specific recombination between the ERs flanking the prophage, a process that could theoretically also result in excision of prophage DNA. Many mobile genetic elements, including lambdoid phages, phage P2, and Tn916, use such a process to generate extrachromosomal

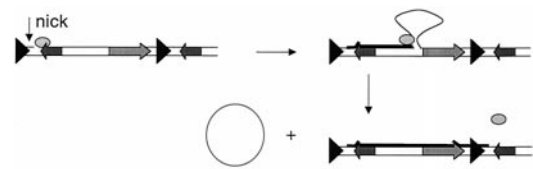


Fig. 7. Model for generation of the replicative form of CTX ϕ from a prophage array. The phage replication protein RstA (oval) introduces a nick into 'ig-1 of the 5' prophage. DNA synthesis (thick black line) originates at the nick and proceeds along the prophage, displacing one strand of DNA but keeping it tethered to the replication complex, until the replication machinery encounters the origin within the 3' prophage. A new nick is introduced, generating a 3' end that can be joined to the 5' end of the displaced single strand to yield a circular, single stranded copy of the phage genome. Second strand synthesis is equivalent to synthesis after infection by CTX ϕ .

forms of DNA (9, 17). However, these elements depend on an element-encoded excisionase (e.g., Xis) to reverse the integration pathway, and such a protein is not known to be encoded within CTX ϕ . The absence of an Xis could account for the failure of solitary CTX prophages to yield virions with intact genomes.

Interestingly, clinical isolates of O1 El Tor and O139 *V. cholerae* rarely, if ever, harbor solitary CTX prophages. In fact, all of our strains with solitary prophages were engineered in the laboratory. Some O1 El Tor and O139 clinical isolates do contain a single prophage, but to our knowledge all of these prophages are flanked by at least one RS1. This raises the possibility that virion-producing lysogens cause more disease and thus are more frequently detected in patients than lysogens that produce CT but few, if any, virions. Selection for production of virions might account for the widespread distribution of RS1 among O1 El Tor lysogens. RS1 is found in most epidemic strains along with CTX prophages, despite the fact that it has not been shown to play a direct role in bacterial survival or pathogenesis. Selection for virion production might also account for the fact that O1 strains of the classical biotype, whose prophages do not yield virions, have been supplanted by O1 El Tor and O139 strains as the dominant cause of epidemic disease.

The precise mechanism by which prophage arrays yield CTX ϕ has not been determined, and it could be very difficult, using currently available reagents, to analyze this process and characterize bacterial chromosomal DNA after virion production. Virion yields from lysogens are so low (maximum 10⁵ × OD₆₀₀⁻¹ × ml⁻¹, which corresponds to approximately 1 virion per 1,000 cells) that most cells in a culture have not produced virions. Consequently, identifying individual virion producers within a population is very challenging. Currently, there is no evidence that production of virions by a lysogen induces any changes within chromosomal DNA; indeed, Southern blot analysis suggests that the structures of prophage/RS1 arrays are quite stable, at least in the absence of extreme selective pressures. Furthermore, our data indicates that homologous recombination between adjacent prophages does not underlie formation of pCTX. We therefore suspect that production of pCTX and subsequently CTX ϕ relies on the mechanism described below, which is consistent with the results presented in this paper, the observed stability of prophage arrays, and previous analyses of plasmid and virion replication.

We hypothesize that the phage-replicative machinery, which probably comprises RstA and several *V. cholerae* encoded proteins (4), can use chromosomal as well as plasmid templates for DNA synthesis. Thus, instead of commencing phage DNA replication at a nicked site within pCTX [a site possibly produced by RstA, which shows some similarity to rolling circle initiator proteins (4)], phage replication could originate at an RstA-

induced chromosomal nick within the prophage (Fig. 7). As in rolling circle replication (18), DNA synthesis could then proceed along the prophage, displacing the original DNA, until a sequence and/or secondary structure equivalent to the initial nick site was encountered. In plasmid replication, this would in fact be the site of initiation; in a prophage array, this site would be the corresponding site in the 3' prophage (or RS1). In either case, this site could then prompt production of a new nick and circularization of the DNA displaced by the synthesis, which would be equivalent to a single strand of the CTX RF. Second strand synthesis, presumably identical to that after CTX ϕ infection of a new host, would then ensue to yield pCTX.

Site-specific nicking/initiation within *ig-1* of the CTX prophage would account for the uniform composition of the hybrids produced by each of the clinical and lab-derived lysogens that we studied. Furthermore, this model is consistent with independent analyses of the origin of pCTX replication, which suggest that the double-stranded origin is near the 3' end of *ig-1* (K. Moyer, personal communication). Thus, we propose that production of pCTX from an array of prophages occurs without excision of a prophage from the chromosome. In this respect, replication of the CTX genome may resemble propagation of replicative transposons or phage Mu more closely than it resembles that of phages such as λ or P2. It may be particularly similar to transposition of *IS91*, which also depends on a protein with homology to rolling circle replication proteins (19). However, although we favor the replicative process outlined above as the explanation for generation of pCTX from a prophage array, our current data does not exclude the possibility that pCTX production could result from a conservative process in which hybrid genomes are excised from the chromosome. Still, regardless of

whether the process yielding pCTX is replicative or conservative, it is clearly distinct from the typical excision mechanisms of other prophages, because it depends on sequences within tandem elements rather than a solitary prophage. Tandem lambdoid prophages can also yield hybrid virions (20); however, lambdoid prophages are generally found as solitary, rather than tandem, elements.

A replicative strategy in which production of virions and maintenance of proviral DNA are not mutually exclusive pathways could have significant evolutionary advantages for the prophage. If virions can be produced for horizontal transmission of phage DNA (transduction) without disrupting the vertical transmission of the prophage (via chromosomal replication), then virion production does not need to be solely an escape mechanism used to release prophage DNA from a damaged or replication-deficient host. Instead, constitutive (albeit at low levels) virion production under normal growth conditions is feasible. The fact that CTX ϕ is secreted, rather than released by host lysis, is a complementary feature of CTX ϕ dissemination. In summary, the CTX genome appears to replicate by a strategy that maximizes stable propagation of the lysogen while still enabling spread of the phage to new hosts.

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