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The *Vibrio cholerae* O139 Calcutta Bacteriophage CTXϕ Is Infectious and Encodes a Novel Repressor

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CTXϕ is a lysogenic, filamentous bacteriophage. Its genome includes the genes encoding cholera toxin (*ctxAB*), one of the principal virulence factors of *Vibrio cholerae*; consequently, nonpathogenic strains of *V. cholerae* can be converted into toxigenic strains by CTXϕ infection. O139 Calcutta strains of *V. cholerae*, which were linked to cholera outbreaks in Calcutta, India, in 1996, are novel pathogenic strains that carry two distinct CTX prophages integrated in tandem: CTX^{ET}, the prophage previously characterized within El Tor strains, and a new CTX Calcutta prophage (CTX^{calc}). We found that the CTX^{calc} prophage gives rise to infectious virions; thus, CTX^{ET}ϕ is no longer the only known vector for transmission of *ctxAB*. The most functionally significant differences between the nucleotide sequences of CTX^{calc}ϕ and CTX^{ET}ϕ are located within the phages' repressor genes ($rstR^{calc}$ and $rstR^{ET}$, respectively) and their RstR operators. $RstR^{calc}$ is a novel, allele-specific repressor that regulates replication of CTX^{calc}ϕ by inhibiting the activity of the $rstA^{calc}$ promoter. $RstR^{calc}$ has no inhibitory effect upon the classical and El Tor *rstA* promoters, which are instead regulated by their cognate RstRs. Consequently, production of $RstR^{calc}$ renders a CTX^{calc} lysogen immune to superinfection by CTX^{calc}ϕ but susceptible (heteroimmune) to infection by CTX^{ET}ϕ. Analysis of the prophage arrays generated by sequentially integrated CTX phages revealed that pathogenic *V. cholerae* O139 Calcutta probably arose via infection of an O139 CTX^{ET}ϕ lysogen by CTX^{calc}ϕ.

Cholera is a severe, infectious diarrheal disease caused by the gram-negative bacterium *Vibrio cholerae*. The principal virulence factor of *V. cholerae* is cholera toxin (CT), a potent, A-B-type exotoxin that ADP-ribosylates proteins within intoxicated intestinal epithelial cells (19). The CT produced by *V. cholerae* during the organism's colonization of its host's small intestine accounts for a majority of the symptoms that characterize the disease process (11). In 1996, Waldor and Mekalanos discovered that the genes encoding CT (the operon *ctxAB*) are not integral components of the *V. cholerae* genome, but instead are elements of the genome of a filamentous bacteriophage, CTXϕ, that specifically infects *V. cholerae* (22). Infection of *V. cholerae* by CTXϕ is frequently followed by integration of the phage genome into the *V. cholerae* genome, yielding a stable lysogen. Like the filamentous phages of *Escherichia coli*, CTXϕ can also replicate as a plasmid, and it does so in bacterial strains lacking appropriate integration sites; however, most if not all natural isolates of *V. cholerae* containing ***ctxAB*** contain integrated phage DNA (13).

Integration of the CTXϕ genome is site specific, but the integration sites and the prophage arrays they contain differ between the two biotypes of *V. cholerae* O1. Within El Tor biotype strains, which have been used for most analyses of phage genes, CTX prophages are found at a chromosomal site known as *attRS* (16). Integration of CTXϕ DNA into *attRS* occurs via recombination between an 18-bp sequence (originally designated the end repeat [ER]) in the phage genome and a nearly identical sequence in *attRS* (16). Some El Tor strains contain a single CTX prophage, while many others contain several in tandem (13). The length of this prophage array can fluctuate (generally expanding) both during the course of an infection and within laboratory cultures, in response to the bacterium's environment (6, 13). We have found that the CTX prophages in El Tor strains generally give rise to infectious phage particles (10).

V. cholerae strains of the classical biotype, which were the dominant cause of epidemic cholera until 1961 when they were replaced by El Tor strains, contain a more complex arrangement of CTXϕ genes. Classical strains have two integration sites, each of which contains a single CTX prophage (13). One site is identical to the *attRS* integration site found in El Tor strains. The second site has not been well characterized, but it has been localized to a different chromosome than *attRS* (21). Surprisingly, neither prophage within classical strains apparently gives rise to phage particles (unpublished data). In addition, the DNA of CTXϕ derived from El Tor strains does not integrate following CTXϕ infection of classical strains. Instead, phage DNA replicates as a plasmid in classical strains, rather than recombining into either of the two classical integration sites (22).

The CTXϕ genome is composed of two regions (Fig. 1) (6, 16). The core region contains the genes encoding CT and genes required for phage morphogenesis, including genes that are thought to encode major and minor phage coat proteins and a protein that aids in phage assembly and secretion (24). Some of these morphogenesis genes are similar to genes of *E. coli* filamentous phages, such as M13 and fd (22). In contrast, the three genes of the other CTXϕ region, RS2, are not similar to those of *E. coli* filamentous phages. Their products control phage replication and site-specific integration (16, 23). RstA is required for phage DNA replication, RstB is required for site-specific integration, and RstR is a repressor of *rstA* expression (9, 23). RS2 also contains two intergenic regions: ig-1 and ig-2. Ig-2 appears to encompass the *rstA* promoter and the RstR operator; no role has yet been established for ig-1. These three genes and the intergenic regions are also components of

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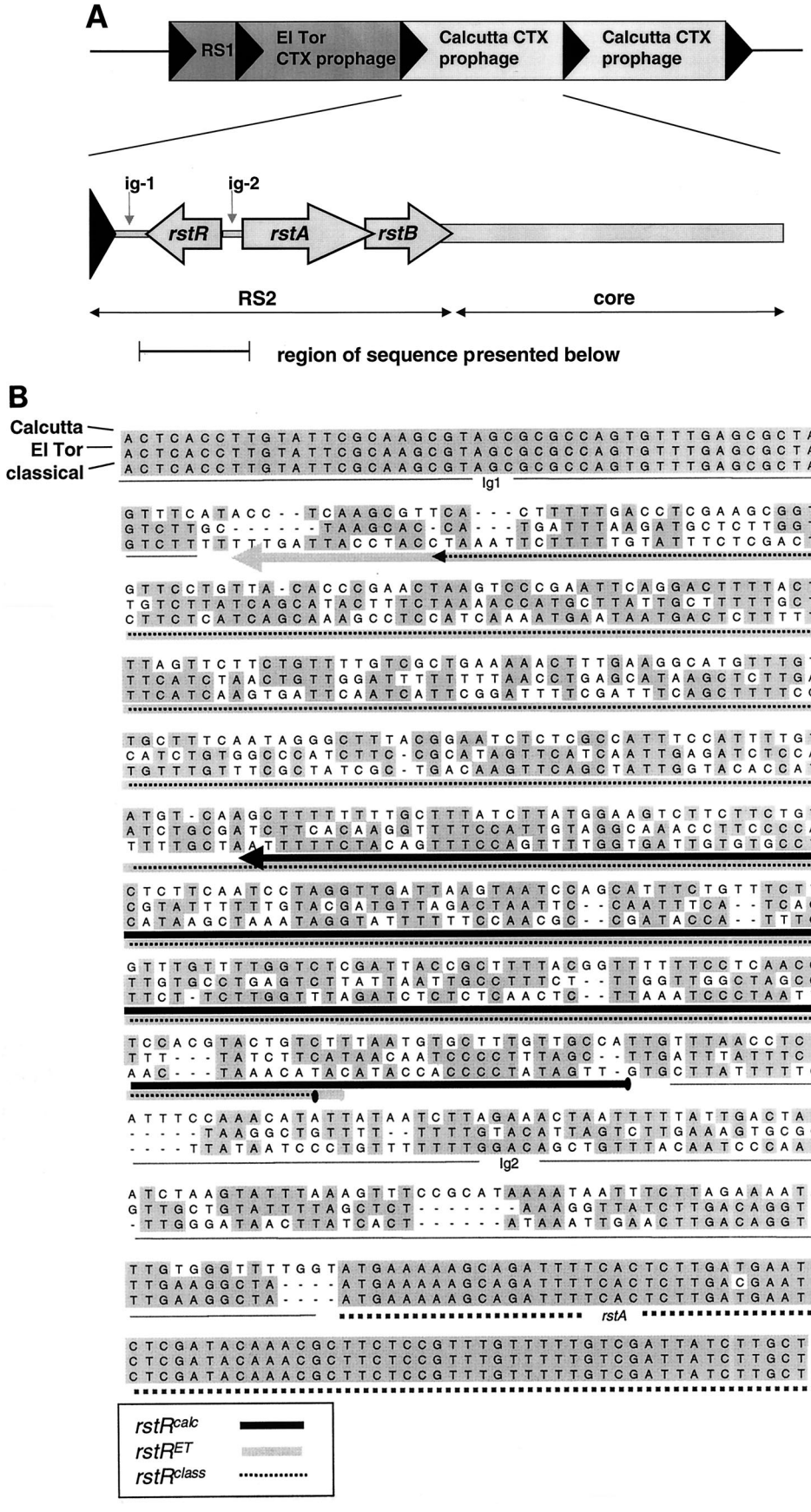

A
RS1
El Tor CTX prophage
Calcutta CTX prophage
Calcutta CTX prophage
ig-1
ig-2
rstR
rstA
rstB
RS2
core
region of sequence presented below
B
Calcutta
El Tor
classical
Ig1
Ig2
rstA
rstR^calc
rstR^ET
rstR^class

a related genetic element, RS1, which is found adjacent to CTX prophages in many *V. cholerae* strains (23).

We recently performed a detailed comparison of the RS2 regions from classical and El Tor CTX prophages (9). We found that *rstB* and the coding sequence of *rstA* are highly conserved between the biotypes (94% nucleotide identity), but that *rstR* and ig-2 (the *rstA* promoter) sequences diverge considerably (44 and 61% nucleotide identity, respectively). Due to the variations in the sequences and binding sites of both the repressor proteins, each RstR is a biotype-specific repressor of its cognate *rstA* (9). That is, expression of the classical *rstA* (*rstA*^{class}) reporter construct *rstA*^{class}-*lacZ* is repressed by classical, but not El Tor, RstR, and similarly, expression of the El Tor reporter construct *rstA*^{ET}-*lacZ* is repressed by El Tor, but not classical, RstR. This repression allows integrated phages to inhibit replication of newly infecting phages of the same biotype, thereby conferring immunity to secondary infection. However, the production of RstR^{class} by the prophages within classical strains of *V. cholerae* does not prevent infection of these strains by a Kn-marked El Tor CTXφ, suggesting that classical CTXφ lysogens are heteroimmune to the El Tor CTXφ (9).

Until 1997, only these two forms, El Tor and classical, of CTX prophages had been identified. However, analyses in 1997 of novel O139 strains responsible for severe outbreaks of cholera in Calcutta, India, revealed that they contained prophages with atypical restriction endonuclease sites (3, 20). We reported previously that these Calcutta strains contain sequences within RS2 quite dissimilar to both the classical and El Tor RS2s (8). In this study, we present further analyses of the Calcutta CTX prophage, especially of its RS2 domain. We show that RstR^{calc}, despite a size and structure dramatically different from previously described repressors, also functions as an allele-specific repressor, capable of repressing *rstA*^{calc} expression. In addition, we demonstrate that, unlike the classical prophages, the Calcutta prophage generates infectious phage particles. Thus, CTX^{calc}φ, as well as the El Tor CTXφ (denoted here as CTX^{ET}φ but in earlier works simply as CTXφ), can transmit *ctxAB* to nonpathogenic strains. Lysogens of CTX^{calc}φ have immunity to superinfection with CTX^{calc}φ, similar to the immunity provoked by CTX^{ET}φ. Finally, we have used CTX^{calc}φ and CTX^{ET}φ to investigate the potential genesis of multiply lysogenized *V. cholerae* strains, such as those identified in Calcutta.

MATERIALS AND METHODS

Nucleotide sequence of the CTX^{calc} prophage RS2 region. RS2 from the CTX^{calc} prophage was amplified from strain AS207 using PCR. In this PCR, a forward primer within *ctxB* (5′ GCGATTGAAAGGATGAAGGATAC 3′) and a reverse primer within *cep* (5′ AACCCCGAGTGAAAGCGTG 3′) allows amplification of the CTX^{calc} prophage RS2 region from strains such as AS207, which contain Calcutta prophages downstream of a single El Tor prophage (Fig. 1) (8). This PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.) to generate pHK268, which was used as a template for dye terminator cycle sequencing, using an Applied Biosystems 373A DNA sequencer. The BLAST programs (1) were used to compare the Calcutta RS2 nucleotide sequence to sequences in the GenBank databases. Potential repressor and helix-turn-helix (hth) DNA binding domains were evaluated by using the matrix of Dodd and Egan (4) and the motif analysis program fingerPRINTScan (5).

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are described within Table 1. All bacteria were cultured in Luria-Bertani broth (14) at 37°C unless otherwise noted. Antibiotics were used at the following concentrations: ampicillin (AMP), 50 μg/ml (*V. cholerae*); AMP, 100 μg/ml (*E. coli*); KAN, 50 μg/ml; streptomycin (STR), 200 μg/ml; chloramphenicol (CMP), 15 μg/ml) (*E. coli*). Arabinose (ARA)-induced cultures contained 0.05% ARA, and sucrose-resistant (Sc^r) clones were selected on 10% sucrose.

Plasmid and strain construction. pBD40, which contains an *rstA*^{calc}-*lacZ* fusion, was constructed by first amplifying the *rstA*^{calc} promoter and part of the *rstA*^{calc} coding sequence with primers rstA^{calc} proF (5′ GATGTTTGTTTTGG TCTCGATTACCG 3′) and rstA proR (5′ TGAAGCATAAGGAACCGACC 3′). Next, the PCR product was cloned into the TA cloning vector pCRII-TOPO (Invitrogen). An *Xba*I/*Hin*dIII fragment containing the insert was then ligated to *Xba*I/*Hin*dIII-digested pCB192 (18) to generate pBD40. To construct pBD87, a PCR product containing *rstR*^{calc} was first amplified with primers rstR-11 (5′ AATAGGGCTTTACGGAATC 3′) and rstR-10 (5′ TGTTTGGAAATCAAG AGAGG 3′). Following subcloning of this product into pCRII-TOPO, a *Kpn*I/*Xba*I fragment containing the insert was ligated into *Kpn*I/*Xba*I-digested pBAD33 (7).

AS207 CTX^{calc}::Kn was made from AS207 with an allele exchange vector derived from the temperature-sensitive, *sacB*$^+$, counterselectable plasmid pCACTUS. This allele exchange vector, pHK260, was constructed by ligating the *Sph*I/*Bgl*II fragment of pCTX^{ET}-Kn (22), which spans the Kn^r cassette, to *Sph*I/*Bgl*II-digested pCACTUS. Following electroporation of AS207 with pHK260, plasmid integrants were isolated at 39°C. KAN-resistant colonies were subsequently screened for resistance to sucrose, which results from recombination and excision of the vector sequences. Sucrose- and KAN-resistant colonies were screened by Southern blotting to ascertain which *ctxAB* gene pair(s) had been replaced by the Kn^r cassette. In AS207 CTX^{calc}::Kn, the *ctxAB* gene pairs of both CTX^{calc} prophages were replaced. In addition, 408 bp of the Calcutta ig-1 were replaced by El Tor ig-1 sequences, which were present within the targeting vector. CTX^{calc}-Knφ produced by AS207 CTX^{calc}::Kn contains only Calcutta sequences for *rstR* and ig-2 and consequently is expected to have the same replicative and repressive properties as CTX^{calc}φ. As we have not yet generated a marked version of CTX^{calc}φ containing only ig-1^{calc}, we used this hybrid CTX^{calc}-Knφ in experiments requiring a selectable Calcutta phage.

Cell-free supernatant from an AS207 CTX^{calc}::Kn culture was used to transduce O395 to KAN resistance. pCTX^{calc}-Kn was then purified from these KAN-resistant O395 cells. Its structure was confirmed by restriction mapping and by sequencing of the ig-1 region. pCTX^{calc}-Ap was constructed by ligating the *Xba*I fragment of pCTX^{calc}-Kn (which lacks only the Kn^r cassette) to *Xba*I-digested pGP704 (17). pCTX^{ET}-Ap is an equivalent plasmid constructed from pCTX^{ET}-Kn and pGP704; pGP704 has the same orientation, relative to the CTXφ genes, in pCTX^{calc}-Ap and pCTX^{ET}-Ap.

Phage transduction assays. To transfer CTX^{calc}-Knφ from AS207 CTX^{calc}::Kn to O395, 50 μl of agglutinated O395 (grown at 30°C to induce expression of the CTXφ receptor, TCP [22]) was mixed with 50 μl of filtered supernatant from a log-phase culture of AS207 CTX^{calc}::Kn. The mixture was shaken gently at room temperature for 20 min, and transductants were selected on Luria-Bertani plates containing KAN. In order to transfer phages to O395 in the absence of antibiotic selection, 1 μl of agglutinated O395 was mixed with 1.5 ml of filtered log-phase-donor (e.g., AS207) supernatant. The mixture was grown overnight at 30°C, and phage transfer was subsequently detected by Southern blotting of plasmid DNA prepared from the culture.

Molecular biology methods. Southern hybridization was carried out using horseradish peroxidase-labelled DNA probes, which were prepared and hybridized using the ECL direct nucleic acid labelling and detection system (Amersham Pharmacia, Little Chalfont, Buckinghamshire, England) according to the manufacturer's instructions. The *rstR*^{calc} probe was a PCR product amplified with the primers rstR-10 and rstR-11; the *rstR*^{ET} probe was a PCR product amplified with the primers rstR-3 and rstR-8 (9). The PCR primers used for analysis of pCTX integration sites were TLCF1 (5′ TGTCGGAGCTGCTTGGATTAAG 3′) and RstR Rev (5′ CGACCAAGCAAGATAATCGAC 3′). Other techniques were performed using standard protocols (2).

Nucleotide sequence accession number. The sequence of the CTX^{calc}φ RS2 region has been assigned GenBank accession no. AF110029.

RESULTS

Structure and sequence of CTX prophages within Calcutta strains of *V. cholerae*. The O139 strains of *V. cholerae* that emerged as a cause of widespread disease in Calcutta in 1996 were found by restriction mapping to contain two tandemly arranged copies of a novel CTX prophage (3, 8, 20). These prophages were integrated into the chromosome immediately

FIG. 1. Structure and sequence of CTX prophages within AS207, an O139 Calcutta strain of *V. cholerae*. (A) Within the AS207 chromosome, an RS1 element and a CTX^{ET} prophage are followed by two CTX^{calc} prophages (shown in light grey). The Calcutta prophage is structurally similar to the previously described CTX prophages within El Tor and classical strains, which contain two major domains known as RS2 and core. RS2 contains three genes (*rstA*, *rstB*, and *rstR*) whose transcriptional orientation is indicated by the arrows. The black triangles represent *attRS*-ER sequences. (B) Alignment of the nucleotide sequences of *rstR*, ig-2, and parts of *rstA* and ig-1 from Calcutta, El Tor, and classical prophages. The arrows underneath the sequences depict the ORFs that encode the three variants of RstR.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
V. cholerae		
AS207	O139 Calcutta strain	20
O395	O1 classical strain	15
E7946	1978 El Tor clinical isolate from Bahrain	13
2740-80	U.S. Gulf Coast isolate, *attRS*$^+$, CTXϕ$^-$	16
E. coli CC118	Δ*lacX74 recA1 phoA* F$^-$	12
Plasmids		
pHK101	pCB192 derivative, *rstA*^{class}-*lacZ* reporter	9
pHK102	pCB192 derivative, *rstA*^{ET}-*lacZ* reporter	9
pBD40	pCB192 derivative, *rstA*^{calc}-*lacZ* reporter	This study
pHK2	pBAD33 derivative, ARA-inducible RstR^{class}	9
pHK1	pBAD33 derivative, ARA-inducible RstR^{ET}	9
pBD87	pBAD33 derivative, ARA-inducible RstR^{calc}	This study
pCTX^{ET}-Kn	Replicative form of CTX^{ET}-Knϕ, previously called CTX-Knϕ	22
pCTX^{ET}-Ap	*Xba*I fragment of pCTX^{ET}-Kn inserted into pGP704	This study
pCTX^{calc}-Kn	Replicative form of CTX^{calc}-Knϕ prophage from AS207 CTX^{calc}::Kn, contains El Tor rather than Calcutta ig-1 region of CTXϕ	This study
pCTX^{calc}-Ap	*Xba*I fragment of pCTX^{calc}-Kn inserted into pGP704	This study
pCB192	β-Galactosidase reporter plasmid, Ap^r	18
pBAD33	ARA-inducible promoter vector, Cm^r	7
pGP704	*oriR6K mobRP4* suicide vector, Ap^r	17
pCACTUS	Allele-exchange vector, Temperature-sensitive ori, *sacB*$^+$, Cm^r	Chris Clark
pHK260	pCACTUS derivative containing the *Sph*I/*Bgl*II fragment of pCTX^{ET}-Kn	This study

downstream of an El Tor RS1 element and an El Tor CTX prophage (Fig. 1A). In order to sequence the RS2 region of the Calcutta prophage, we amplified and cloned a PCR product spanning this region from AS207, a representative O139 Calcutta strain (8). Comparison of the putative *rstR* and ig-2 from the Calcutta prophage with sequences from El Tor and classical prophages revealed striking differences (Fig. 1B). In contrast to *rstA* and subregions of ig-1, which are highly conserved among the three prophages, the putative Calcutta *rstR* and ig-2 share no extended sequence identity with the other prophages. In addition, the longest open reading frame (ORF) found within the *rstR* region of the Calcutta prophage is predicted to encode a protein that is significantly shorter (59 amino acids) than the El Tor and classical RstRs (113 and 112 amino acids, respectively). BLAST searches revealed no significant homology between the Calcutta *rstR* region and the sequences within the GenBank database, either at the nucleotide or amino acid level. In contrast, both RstR^{ET} and RstR^{class} show sequence similarity to a number of bacteriophage repressors (9), and both are predicted by the Dodd and Egan matrix to contain hth DNA binding motifs that start near their amino termini (4). We could not identify a similar hth domain within RstR^{calc} with the Dodd and Egan matrix. However, the protein motif analysis program fingerPRINTScan did identify RstR^{calc} as a repressor containing an hth DNA binding domain (hthrepressr fingerprint), but only if the stringency of analysis was reduced from the default value of 15% to 12%. Unlike in RstR^{ET} and RstR^{class}, the hth motif in RstR^{calc} is found near the carboxyl terminus of the protein.

Allele-specific repression of *rstA*^{calc} by RstR^{calc}. We have previously shown that the different RstRs expressed within classical and El Tor CTXϕ lysogens repress *rstA-lacZ* reporters in a biotype-specific manner (9). To assess whether Calcutta strains similarly encode a repressor specific for the novel *rstA*^{calc} promoter sequence, we coexpressed the putative RstR^{calc} with a panel of *rstA-lacZ* reporters in an *E. coli* K-12 strain, CC118 (12). In addition, each reporter, *rstA*^{ET}-*lacZ*, *rstA*^{class}-*lacZ*, and *rstA*^{calc}-*lacZ*, was coexpressed in CC118 with RstR^{ET} and RstR^{class}. Production of the RstRs was controlled by an ARA-inducible promoter (pBAD) (7) as described previously (9). We found that β-galactosidase activity produced from *rstA*^{calc}-*lacZ* was consistently high when this reporter was maintained alone, maintained with a pBAD33 vector control, or maintained with RstR^{ET}- and RstR^{class}-producing plasmids, both under inducing and noninducing conditions (Table 2 and data not shown). However, *rstA*^{calc}-*lacZ* expression decreased 50-

TABLE 2. The influence of different inducible RstRs on expression of *rstA-lacZ* reporters

Reporter[a]	Repressor construct[a]	β-Galactosidase activity[b]		Fold repression[c]
		No ARA	0.05% ARA	
rstA^{calc}-*lacZ*	pBAD33	890	882	NS
	pBADRstR^{calc}	1,868	39	48
	pBADRstR^{ET}	1,563	1,641	NS
	pBADRstR^{class}	1,256	2,315	NS
rstA^{ET}-*lacZ*	pBAD33	100	72	NS
	pBADRstR^{calc}	192	133	NS
	pBADRstR^{ET}	133	1.8	74
	pBADRstR^{class}	161	114	NS
rstA^{class}-*lacZ*	pBAD33	398	332	NS
	pBADRstR^{calc}	946	749	NS
	pBADRstR^{ET}	745	680	NS
	pBADRstR^{class}	616	8.8	70

[a] Reporter and repressor pairs were introduced into *E. coli* CC118. The *rstA*^{calc}-*lacZ* reporter was pBD40, the *rstA*^{ET}-*lacZ* reporter was pHK102, and the *rstA*^{class}-*lacZ* reporter was pHK101. The ARA-inducible Calcutta, El Tor, and classical RstRs were pBD87, pHK1, and pHK2, respectively.

[b] β-Galactosidase activity within overnight cultures is reported in Miller units (14).

[c] Fold repression was calculated by dividing the β-galactosidase activity in the absence of ARA by the β-galactosidase activity in the presence of ARA. Changes in β-galactosidase activity of less than twofold were deemed not significant (NS).

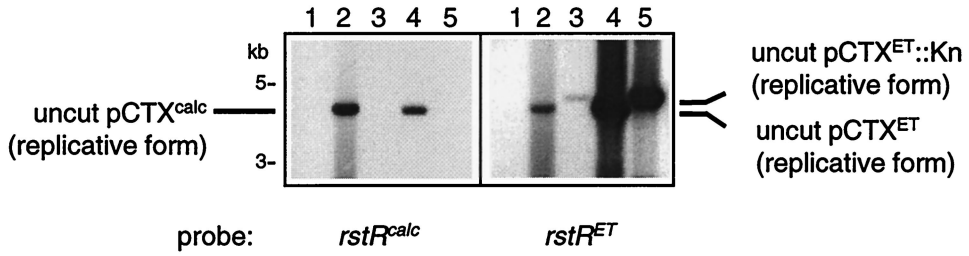


FIG. 2. Detection of transfer of CTX^{calc}φ and CTX^{ET}φ from supernatants of AS207 to O395 using Southern blot analysis of plasmid DNA. Undigested plasmid DNAs were run on agarose gels, transferred to a nylon membrane, and sequentially hybridized with probes for *rstR*^{calc} (left panel) and *rstR*^{ET} (right panel). Plasmid DNA was prepared from O395 (lanes 1), AS207 (lanes 2), 2740-80 (CTX^{ET}-Kn) (lanes 3), O395 cultured with AS207 supernatant (lanes 4), and O395 cultured with 2740-80 (CTX^{ET}-Kn) supernatant (lanes 5). The Kn^r cassette is slightly larger than the *ctxAB* genes it replaces in CTX^{ET}-Knφ, so pCTX^{ET}-Knφ migrates more slowly than pCTX^{ET}φ.

fold when production of RstR^{calc} was induced with ARA (Table 2). Thus, the 59-amino-acid polypeptide encoded by the ORF underlined in Fig. 1B is sufficient to repress *rstA*^{calc} expression. No additional repression was observed with a larger repressor construct containing an additional ORF found downstream of *rstR*^{calc}, nor was *rstA*^{calc}-*lacZ* expression repressed by the downstream ORF alone (data not shown). The RstR^{calc} repressor activity was specific; it did not reduce the β-galactosidase activity produced either from *rstA*^{class}-*lacZ* or *rstR*^{ET}-*lacZ* (Table 2). Specific repression of the reporter constructs was also observed when the reporter plasmids were transformed into *V. cholerae* strains producing various repressors from their endogenous prophages (data not shown). Thus, endogenous levels of RstR are sufficient to repress expression of RstA; repression is not an artifact of overexpressing the repressors in *E. coli*.

The CTX^{calc} prophage encodes an infectious bacteriophage. We next ascertained whether the CTX^{calc} prophage gives rise to transmissible bacteriophage particles, or if, like the classical CTX prophage, it lacks the capacity for independent replication. We hypothesized that CTX^{calc}φ transmission to the classical strain O395 would result in production of pCTX^{calc} (the plasmid, or replicative form [RF], of CTX^{calc}φ) as occurs following the infection of O395 with CTX^{ET}φ (22). Therefore, we incubated cell-free supernatants from AS207 with O395, then prepared plasmid DNA from potentially infected cells and used Southern hybridization analysis to assay for transmission of CTX^{calc}φ from AS207 supernatants to O395. Southern blots were probed sequentially with *rstR*^{calc} and then with *rstR*^{ET}. Control experiments revealed that no *rstR*^{calc}-hybridizing species could be detected in plasmid DNA prepared from O395 cultures (Fig. 2). However, an *rstR*^{calc}-hybridizing plasmid species was detected within plasmid DNA isolated from O395 cultured at 30°C in the presence of filtered supernatants from AS207. An equally sized *rstR*^{calc}-hybridizing species was detected in plasmid DNA prepared from AS207. Restriction digests revealed these plasmids to be the RF of CTX^{calc}, which probably forms in AS207 as a replication intermediate during CTX^{calc}φ production. Treatment of the AS207 cell-free culture supernatant with DNase I did not prevent the transfer of pCTX^{calc} to O395 from AS207 supernatants (data not shown), thereby suggesting that AS207 gives rise to a bacteriophage, CTX^{calc}φ, that is competent to infect and replicate within O395. Rehybridization of these Southern blots with an *rstR*^{ET} probe revealed that AS207 also is capable of transfer of CTX^{ET}φ, at a level matching or exceeding that of an El Tor strain with two tandemly arranged KAN-marked El Tor CTX prophages (2740-80 [CTX^{ET}-Kn]) (Fig. 2). Thus, the Calcutta strain AS207 of *V. cholerae* can give rise to two distinct infectious bacteriophages: CTX^{calc}φ and CTX^{ET}φ. Consequently, CTX^{ET}φ is not the sole phage capable of conveying the genes encoding CT to nonpathogenic *V. cholerae*.

Electroporation of the RF of an antibiotic marked version of CTX^{calc}φ (pCTX^{calc}-Kn) into CTXφ$^-$ strains yielded Kn^r transformants that produce CTX^{calc}-Knφ particles (data not shown). This result confirmis that CTX^{calc}φ is infectious; in addition, it demonstrates that the production of infectious CTX^{calc}φ particles by AS207 and other Calcutta strains is not dependent upon the CTX^{ET} prophage also present in these strains.

Immunity and heteroimmunity of a CTX^{calc}-Knφ lysogen. We previously found that strains harboring a CTX^{ET} prophage are significantly resistant to further infection with CTX^{ET}-Knφ. This immunity results from the repression of *rstA*^{ET} expression by RstR^{ET}, as RstA is essential for phage replication. The data presented above indicate that RstR^{ET} does not repress RstA^{calc} production, and similarly, RstR^{calc} does not repress RstA^{ET} production (Table 2). Therefore we tested, with marked CTX^{calc}φ derivatives, whether a lysogen harboring a CTX^{calc} prophage is immune to CTX^{calc}φ superinfection and heteroimmune to CTX^{ET}φ infection. Since immunity results from inhibition of phage replication following infection rather than from inhibition of the initial steps of infection, and since El Tor strains cannot be efficiently infected with CTX^{calc}φ in vitro (due to lack of expression of TCP, the phage receptor), we developed a transformation assay to study the immunity properties of the CTX^{calc} prophage. For these assays, 2740-80, an El Tor, CTXφ$^-$, *attRS*$^+$ strain, and 2740-80 lysogens of marked CTX^{calc}φ and CTX^{ET}φ were electroporated with differentially marked CTX^{calc} or CTX^{ET} plasmid DNA.

Initially, we electroporated identical amounts of pCTX^{calc}-Ap and pCTX^{ET}-Ap in parallel into 2740-80. A similar number of AMP-resistant colonies was obtained with DNA from each phage, suggesting that these phages replicate and subsequently integrate with comparable efficiency within 2740-80 (Fig. 3). We then electroporated these AMP-marked phage DNAs into 2740-80 (CTX^{calc}-Kn) and 2740-80 (CTX^{ET}-Kn), which contain KAN-marked prophages integrated at the 2740-80 *attRS* site. Transformation of these strains with pCTX^{calc}-Ap and pCTX^{ET}-Ap did not yield comparable numbers of colonies; instead, the prophages conferred repressor allele-specific resistance to further transformation by CTXφ variants. Thus, pCTX^{calc}-Ap accounted for 58% of all 2740-80 transformants, but only 19% of 2740-80(CTX^{calc}-Kn) transformants. Similarly, pCTX^{ET}-Ap accounted for 42% of all 2740-80 transformants, but only 3% of 2740-80 (CTX^{ET}-Kn) transformants (Fig. 3). These data suggest that CTX^{calc}φ lysogens are im-

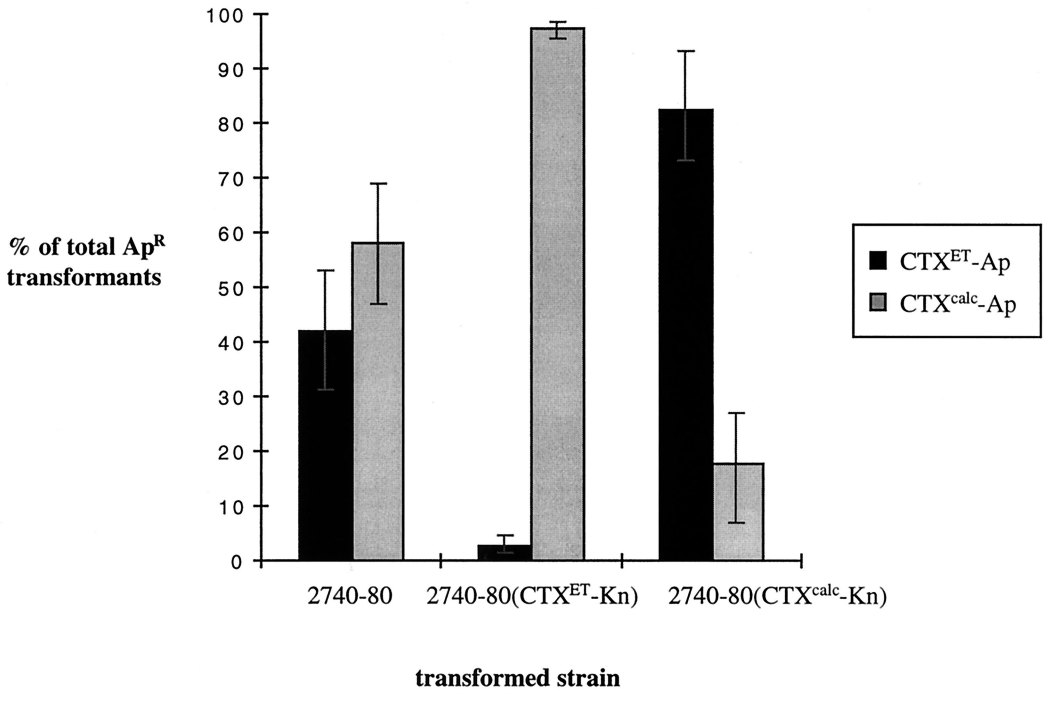


FIG. 3. Relative efficiency of transformation of pCTX^{calc}-Ap versus pCTX^{ET}-Ap into 2740-80, 2740-80 (CTX^{ET}-Kn), and 2740-80 (CTX^{calc}-Kn). Percentage of total Ap^r transformants = (pCTX^{calc}Ap or pCTX^{ET}Ap transformants)/(pCTX^{calc}Ap + pCTX^{ET}Ap transformants).

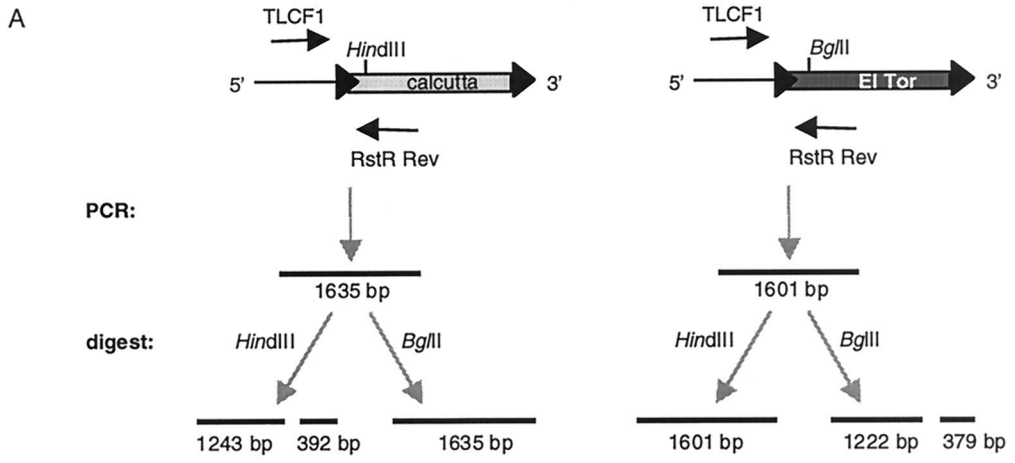
mune to further infection by CTX$^{calc}\phi$, just as CTX$^{ET}\phi$ lysogens are immune to further infection by CTX$^{ET}\phi$. These data also suggest that CTX$^{calc}\phi$ and CTX$^{ET}\phi$ lysogens are not immune to infection by CTX^{ET} and CTX^{calc} phages, respectively. Consequently, *V. cholerae* can be lysogenized by multiple distinct CTX phages; in fact, this process probably accounts for the development of Calcutta strains such as AS207, which contain both El Tor and Calcutta prophages.

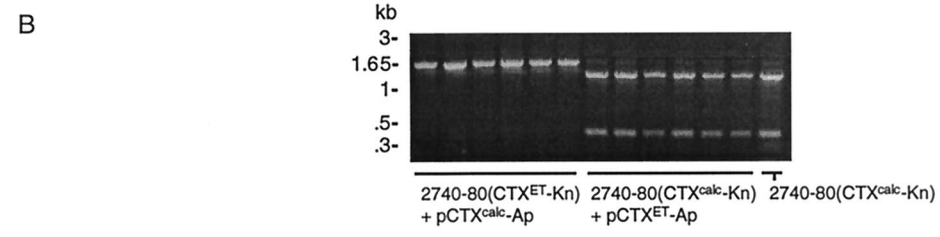
Integration site preference and evolution of *V. cholerae* O139 Calcutta. We used 2740-80 harboring either marked El Tor or Calcutta CTX prophages to explore potential steps in the evolution of Calcutta strains such as AS207. In these experiments, we determined where the DNA of CTX$^{ET}\phi$ and CTX$^{calc}\phi$ integrates on the chromosome in strains harboring either the CTX^{calc} prophage or the CTX^{ET} prophage, respectively. We have found that CTX phage DNA reliably integrates into *attRS* following infection of *attRS*$^+$, CTX$^-$ El Tor strains, such as 2740-80. However, integration regenerates the 18-bp core of *attRS* or the very similar ER sequence on both ends of the prophage (16). Thus, a subsequently infecting phage has two or more potential integration targets; the precise number of integration targets is determined by the length of the prophage array. To determine the site of CTXϕ integration following infection of a CTXϕ lysogen, we developed a PCR and restriction-digest-based assay. For the PCR reaction, one primer was complementary to DNA 5′ of *attRS* on the 2740-80 chromosome and the other primer was complementary to a conserved sequence within *rstA* (Fig. 4). PCR products were digested with enzymes that cleave either the El Tor or the Calcutta *rstR*, thereby allowing us to identify the furthest 5′ prophage within an array of prophages. We found that the initial phage integrated on the 2740-80 chromosome always retained the most 5′ position on the chromosome (Fig. 4); thus, CTX^{calc}-Kn, CTX^{ET}-Kn, CTX^{calc}-Ap, and CTX^{ET}-Ap were each maintained as the most 5′ prophage if their DNA was the first in a series to be electroporated into 2740-80. Subsequent integrations, regardless of which particular phage's DNA was tested, occurred 3′ of an integrated prophage (Fig. 4 and data not shown). In other words, DNA from the second antibiotic-marked phage always integrated either between tandem prophages or between an integrated prophage and 3′ chromosomal DNA. This unambiguous insertion site preference in sequentially transformed strains (and presumably also in sequentially infected strains) strongly suggests that Calcutta strains such as AS207 arose by infection of a *V. cholerae* CTX^{ET} lysogen by CTX$^{calc}\phi$.

DISCUSSION

We have investigated the repressive and replicative capabilities of a novel variant of CTXϕ, which was found as a prophage within epidemic-linked strains of O139 *V. cholerae* isolated from Calcutta. This prophage gives rise to CTX$^{calc}\phi$ virions that infect and/or lysogenize both relatively nonpathogenic (lacking *ctxAB*) *V. cholerae* strains, such as 2740-80, as well as classical and El Tor strains that already contain CTX prophages. These findings demonstrate that CTX$^{ET}\phi$ is not the sole viable, CT-encoding, filamentous phage capable of transmitting *ctxAB* within *V. cholerae* populations. CTX$^{calc}\phi$ lysogens are immune to infection by CTX$^{calc}\phi$ due to the production by these lysogens of a new repressor, RstR^{calc}, that inhibits expression from the adjacent *rstA*^{calc} promoter. Like RstR^{ET} and RstR^{class}, the transcriptional repressor activity of RstR^{calc} is sequence specific; RstR^{calc} does not inhibit the activity of either the *rstA*^{class} or the *rstA*^{ET} promoter. Similarly, the *rstA*^{calc} promoter is not repressed by RstR^{class} or RstR^{ET}.

Despite the functional similarity of the three CTXϕ repressors, the RstR^{calc} amino acid sequence is unrelated to the sequences of RstR^{class}, RstR^{ET}, and the known repressor proteins of other lysogenic phages. In fact, BLAST searches using





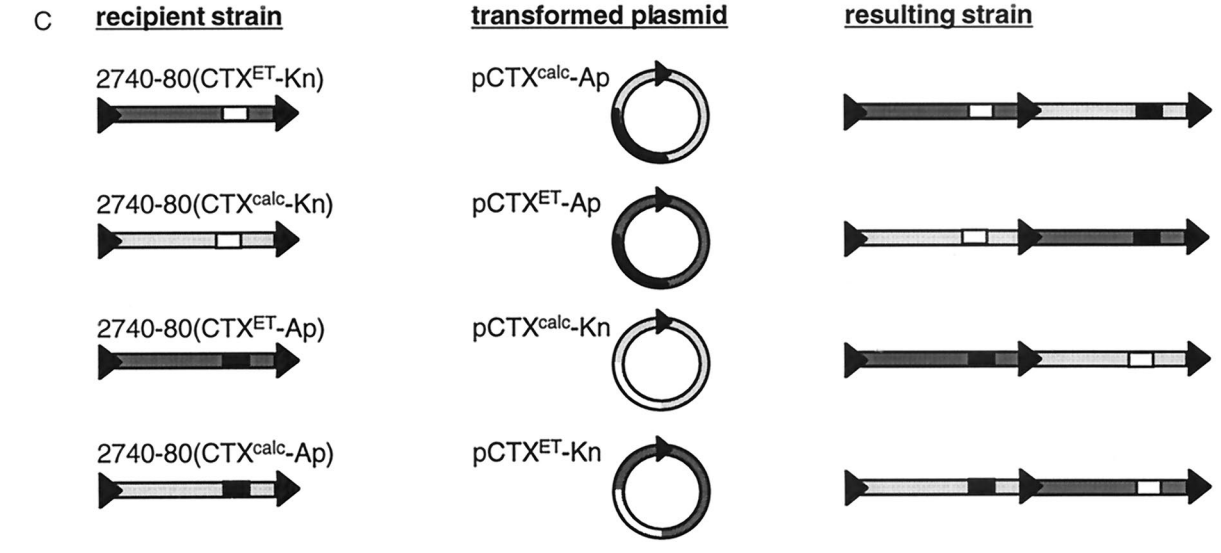


FIG. 4. Integration site selection by sequentially integrated plasmids. (A) PCR followed by restriction digestion was used to determine the order of prophages within the chromosome following sequential integration of CTX^{ET} and CTX^{calc} antibiotic-marked plasmids. When the CTX^{calc} prophage is the furthest 5′, the TLCF1-RstR Rev PCR product contains a *Hin*dIII site but not a *Bgl*II site. Conversely, when CTX^{ET} is upstream, the PCR product contains a *Bgl*II site but not a *Hin*dIII site. (B) A representative agarose gel containing PCR products digested with *Hin*dIII. Template DNA is as follows: 2740-80(CTX^{ET}-Kn) transformed with $pCTX^{calc}$-Ap (lanes 1 to 6), 2740-80 (CTX^{calc}-Kn) transformed with $pCTX^{ET}$-Ap (lanes 7 to 12), and 2740-80 (CTX^{calc}-Kn) (lane 13). (C) Summary and model of integration site selection following sequential CTXϕ integration. Phage DNA does not integrate into the 5′ ER (black triangle) if the chromosome already contains a CTX prophage. Instead, the new phage DNA inserts into the 3′ ER.

the nucleotide and predicted amino acid sequences of this repressor revealed no significant homology to any sequence within the GenBank databases. It is therefore not surprising that most protein analysis algorithms that we tested did not predict any repressor or DNA binding activity for RstR^{calc}. However, the motif-detecting program fingerPRINTScan does detect a low level of similarity between RstR^{calc} and the hth domain found in lambdoid repressors and in a subset of homeotic proteins (hthrepressr fingerprint). Interestingly, fingerPRINTScan assigns RstR^{class} and RstR^{ET} to different hth categories (homeobox and hthLysR fingerprints, respectively), suggesting that the three *V. cholerae* repressors may be more closely related to proteins from other species than they are to each other. The genetic mechanism(s) by which the unrelated RstR-RstR operator pairs became associated with otherwise very similar CTXϕs has not been explored. However, recombination between a CTXϕ containing a particular repressor-operator pair and repressor-operator sequences within other temperate bacteriophages or even within other genetic elements is clearly one possibility.

Production of RstR^{calc} enables the CTX^{calc} prophage both to control its own replication and to inhibit RstA-mediated replication of any newly introduced DNA that relies upon the *rstA*^{calc} promoter. This confers upon a lysogen a degree of immunity to secondary infections by identical phages, similar to the immunity produced by λ prophages within *E. coli*. Also like lambdoid prophages, CTX^{calc}ϕ lysogens are susceptible to infection (heteroimmune) by CTXϕ with different immunity regions (*rstR*/RstR operator sequences). In addition, results from our transformation efficiency assay suggest that CTX^{ET}ϕ lysogens are heteroimmune to CTX^{calc}ϕ infection. Integrated CTX^{calc}-Kn does not completely inhibit replication of the related replicon, CTX^{calc}-Ap; however, it does significantly diminish CTX^{calc}-Ap replication relative to that of CTX^{ET}-Ap. In the transformation efficiency assay system, CTX^{calc}-Kn is less effective at providing immunity than is CTX^{ET}-Kn. This finding could reflect the relative weakness of RstR^{calc} as a repressor; alternatively, it may indicate the strength of the *rstA*^{calc} promoter. When assayed in CC118, an *rstA*^{calc}-*lacZ* reporter fusion has higher activity than either *rstA*^{class}-*lacZ* or *rstA*^{ET}-*lacZ* reporters, so even if repressed to the same degree as the other promoters, *rstA*^{calc} may maintain higher residual activity. Residual production of RstA, either from the prophage or from the RF, presumably enables some plasmids to replicate and subsequently integrate. Although our assay measures immunity indirectly, by monitoring transformation of lysogens with the RF of the CTXϕ genomes, these experiments yielded results similar to those obtained when CTX^{ET}ϕ immunity was assayed directly, using an intraintestinal transduction assay (9).

Our results support the hypothesis that AS207 and related Calcutta strains arose via infection of an O139 CTX^{ET}ϕ lysogen by the previously unknown CTX^{calc}ϕ. Epidemic O139 *V. cholerae* strains isolated prior to 1996 contain only El Tor CTX prophages but otherwise are very similar to Calcutta O139 strains (3, 20) and thus are likely AS207 progenitors. Our experiments indicate that CTX^{calc}ϕ infection and lysogenization of such a progenitor strain would result in the same arrangement of CTX prophages as is seen in Calcutta O139 strains such as AS207. A recent survey of environmental *V. cholerae* strains in Calcutta detected a prophage encoding RstR^{calc} (presumably integrated CTX^{calc}ϕ) in a non-O1, non-O139 strain of *V. cholerae* (3a). This may indicate that CTX^{calc}ϕ is transmitted within the estuarine environment as well as within the laboratory; it also reveals a potential source of CTX^{calc}ϕ. Thus, it seems probable that AS207 and other Calcutta strains arose via infection of an earlier, epidemic-linked O139 strain with CTX^{calc}ϕ. It is also possible that recombination between a CTX^{ET} prophage within an O139 strain and a repressor-operator from an unrelated genetic element gave rise to O139 Calcutta strains, but such a mechanism seems less likely to account for the origin of these strains.

When we proposed using *rstR*^{ET} to protect classical and El Tor live-attenuated *V. cholerae* vaccine strains from reversion to toxigenicity mediated by CTXϕ infection (9), the only described infectious CTXϕ was CTX^{ET}ϕ. Nucleotide sequence analysis of the *rstR*/ig-2 immunity regions in multiple El Tor clinical isolates from around the world had revealed that they are identical (9), lending credence to this approach. However, the current description of the infectious CTX^{calc}ϕ, which encodes the novel RstR^{calc}, suggests that *rstR*-mediated immunity to CTXϕ infection may not constitute a useful method of enhancing the biosafety of live-attenuated *V. cholerae* vaccines. Besides *rstR*^{calc}, two additional putative *rstR*s have been identified recently in environmental, non-O1/O139 *V. cholerae* isolates (3a, 3b), and more alleles probably remain to be detected. Thus, introduction of a comprehensive immunizing library of *rstR*s into vaccine strains may not be practical. Ongoing studies are exploring alternative mechanisms for preventing CTXϕ-mediated transfer of *ctxA* and *-B* to vaccine strains.

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