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CTXϕ immunity: Application in the development of cholera vaccines

HARVEY H. KIMSEY AND MATTHEW K. WALDOR*

Division of Geographic Medicine and Infectious Diseases, Tupper Research Institute, Tufts–New England Medical Center 041, 750 Washington Street, Boston, MA 02111

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ABSTRACT **CTXϕ is a filamentous bacteriophage that encodes cholera toxin, the principal virulence factor of *Vibrio cholerae*. CTXϕ is unusual among filamentous phages because it encodes a repressor and forms lysogens. CTXϕ can infect the existing live-attenuated *V. cholerae* vaccine strains derived from either the El Tor or classical *V. cholerae* biotypes and result in vaccine reversion to toxinogenicity. Intraintestinal CTXϕ transduction assays were used to demonstrate that El Tor biotype strains of *V. cholerae* are immune to infection with the El Tor-derived CTXϕ, whereas classical strains are not. The El Tor CTXϕ repressor, RstR, was sufficient to render classical strains immune to infection with the El Tor CTXϕ. The DNA sequences of the classical and El Tor CTXϕ repressors and their presumed cognate operators are highly diverged, whereas the sequences that surround this "immunity" region are nearly identical. Transcriptional fusion studies revealed that the El Tor RstR mediated repression of an El Tor *rstA-lacZ* fusion but did not repress a classical *rstA-lacZ* fusion. Likewise, the classical RstR only repressed a classical *rstA-lacZ* fusion. Thus, similar to the mechanistic basis for heteroimmunity among lambdoid phages, the specificity of CTXϕ immunity is based on the divergence of the sequences of repressors and their operators. Expression of the El Tor *rstR* in either El Tor or classical live-attenuated *V. cholerae* vaccine strains effectively protected these vaccines from CTXϕ infection. Introduction of *rstR* into *V. cholerae* vaccine strains should enhance their biosafety.**

Vibrio cholerae is the cause of the severe diarrheal disease cholera. After oral ingestion of contaminated food or water, this Gram-negative rod colonizes the human small intestine. In the small intestine, *V. cholerae* secretes cholera toxin (CT), an A-B-type toxin that binds to GM_1 ganglioside on host intestinal epithelial cells (1). The activity of this ADP-ribosylating exotoxin largely accounts for the secretory diarrhea, which is characteristic of cholera (2). The classical biotype of *Vibrio cholerae* O1 gave rise to the fifth (1881–1896) and sixth (1899–1923) cholera pandemics (3). The ongoing seventh pandemic of cholera, which began in 1961 in Indonesia, is caused by the El Tor biotype of *V. cholerae* O1. The observation that cholera seems to engender long-lived immunity to repeat *V. cholerae* infection has led to efforts to develop an oral live-attenuated *V. cholerae* vaccine (4). In the past decade, both classical and El Tor *V. cholerae* strains have been used to construct several candidate live-attenuated *V. cholerae* vaccine strains that currently are undergoing clinical trials (4–6).

The genes encoding cholera toxin (*ctxAB*) are part of the genome of CTXϕ, a 6.9-kb single-stranded DNA filamentous bacteriophage (7). Whereas the DNA encoding most filamentous phages remains extrachromosomal as plasmids (8), the CTXϕ genome encodes a site-specific recombination system that catalyzes the integration of the phage DNA into the attRS site on the El Tor *V. cholerae* chromosome to form lysogens (7, 9). After infection of El Tor-derived live-attenuated vaccine strains that are deleted for attRS and all CTXϕ sequences (10) or classical-derived vaccine strains that are deleted for *ctxA* (which encodes the enzymatically active A subunit of cholera toxin) (4), CTXϕ remains extrachromosomal and replicates as a plasmid (7, 9). Thus, the discovery that *ctxAB* is transmissible as part of the CTXϕ genome suggests that CTXϕ infection could mediate the reversion of live-attenuated *V. cholerae* vaccine strains. In fact, we have found that *V. cholerae* vaccine strains derived from either biotype can be transduced efficiently by CTXϕ within the intestinal tract (7).

The CTXϕ genome is divided into a 4.6-kb core region, which encodes cholera toxin as well as functions that are required for virion morphogenesis (7), and a 2.4-kb RS2 region, which encodes functions required for regulation, replication, and integration of CTXϕ (9). CTXϕ, similar to lysogen-forming (integrating), double-stranded DNA bacteriophages, and different from other filamentous phages, encodes a repressor (9). This repressor, RstR, has been shown to repress expression of a *rstA*, a gene that is divergently transcribed from *rstR* and is required for CTXϕ replication (9). In lambdoid phages, repressors provide immunity to secondary infection by an identical phage (11). Because lysogeny is unusual among filamentous phages, in the current study we explored whether *V. cholerae* CTXϕ lysogens exhibit immunity to infection by CTXϕ and the role of RstR in CTXϕ immunity. El Tor lysogens were found to be immune to infection by El Tor-derived CTXϕ whereas classical strains were not. RstR was sufficient to render classical strains immune to CTXϕ infection. The sequences of the classical and El Tor CTXϕ encoded repressors (*rstR* genes) and their cognate operators were found to be highly diverged and repression mediated by these *rstR* alleles was biotype-specific. The use of *rstR* as a means to protect classical and El Tor live-attenuated *V. cholerae* vaccine strains from reversion to toxinogenicity mediated by CTXϕ infection is described.

MATERIALS AND METHODS

Plasmid Constructions. pHK1 contains the El Tor *rstR* gene cloned into the arabinose-inducible promoter vector pBAD33 (12) and was constructed as follows: oligonucleotide primers rstR-3 (GGGAGCTCAAAGGGGATTGTTAT) and rstR-2 (CCTCTAGATAGTATTACGGGGGT) were used to amplify *rstR* from CTXϕ replicative form (RF) DNA with PCR.

Abbreviations: Kn, kanamycin; CFU, colony-forming units; RF, replicative form.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF055890).

*To whom reprint requests should be addressed at: Division of Geographic Medicine and Infectious Diseases, NEMC 041, 750 Washington Street, Boston, MA 02111. e-mail: matthew.waldor@es.nemc.org.

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PCR products were purified by using QIAquick PCR purification kit (Qiagen, Chatsworth, CA), digested with *Sac*I and *Xba*I (New England Biolabs), and ligated to *Sac*I–*Xba*I-digested pBAD33 plasmid DNA. pHK2, containing the *rstR*^{CL} gene, was constructed similarly by using oligonucleotides rstR-5 (GGGAGCTCGTTCAAAAATAAGCACAA) and rstR-6 (CCTCTAGAGATTACCTACCTAAATTTC). Chromosomal DNA from the classical strain 0395 was used as the template DNA for PCR. To construct pHK3 and pHK4, which contain the El Tor *rstR* gene cloned into pBR322 (13) and pACYC184 (14), respectively, primers rstR-8 (AACGGCCGCTAAGCACCATGATTT) and rstR-9 (GGGGATCCTTCGACATCAAATGGCA) were used to amplify *rstR*. The purified PCR product then was digested with *Eag*I and *Bam*HI and ligated into *Eag*I- and *Bam*HI-digested pBR322 and pACYC184.

The classical *rstA*^{CL}-*lacZ* reporter plasmid pHK101 contains the ig-2^{CL} region and the 5′ end of *rstA*^{CL} cloned into the β-galactosidase (*lacZ*) reporter plasmid pCB192 (15) and was constructed by PCR cloning by using oligonucleotide primers crstR-1 (GGAAGCTTGTTTAGATCTCTCTCAAC), crstR-2 (GGTCTAGACCAGATAAGCGAGGACAA), and classical strain 0395 DNA as template. PCR products were purified, digested with *Hin*dIII and *Xba*I, and ligated to *Hin*dIII- and *Xba*I-digested pCB192 plasmid DNA. The El Tor *rstA-lacZ* reporter plasmid, pHK102, contains the El Tor ig-2 region and the 5′ end of *rstA* and was constructed by digesting CTXϕ RF DNA with *Nhe*I and *Kas*I. DNA fragments were blunt-ended with T4 DNA polymerase, and the 290-bp fragment containing ig-2 and the 5′ end of *rstA* was gel-purified and ligated to *Eco*RV-digested pBluescript II-KS(+) (Stratagene), generating pHK100. Finally, pHK100 was digested with *Hin*dIII and *Xba*I, and the 300-bp fragment containing ig-2 was cloned into *Hin*dIII- and *Xba*I-digested pCB192 plasmid DNA yielding pHK102.

Intraintestinal CTX-Knϕ Transduction Assay. The suckling mouse *V. cholerae* colonization model (16) was used to detect intraintestinal CTX-Knϕ transduction of recipient strains. Five-day-old CD-1 mice were intragastrically inoculated with 2×10^5 cells of a CTX-Knϕ donor strain along with 1×10^5 cells of different potential recipient strains. The *lacZ*$^-$ classical strain LAC-1 (pCTX-Kn) (16), which harbors the replicative form of CTX-Knϕ, was used as the CTX-Knϕ donor strain. All the recipient strains were lacZ$^+$. Thus, transductants of the different recipient strains were identified as kanamycin-resistant (Kn^r) LacZ$^+$ colonies. The different inocula mixtures were plated to verify that there were no Kn^r LacZ$^+$ cells in any of the inocula. After 20 hr of intraintestinal growth, homogenates of the small intestines were plated to determine the total number of recipient cells as well as the number of recipient cells that were transduced to Kn resistance with CTX-Knϕ within the intestine. There were at least six mice in each group.

Sequencing the Classical CTXϕ RS2 Region. Plasmid subclones containing the two chromosomal copies of the classical strain 569B RS2 region, pGP2 and pGP19 (17), were used as templates for dye terminator cycle sequencing of the classical RS2 region. DNA sequences were determined with an Applied Biosystems 373A DNA sequencer. The BLASTP (18) program was used to detect similarities of RstR^{CL} to other bacteriophage repressors, and GENEWORKS (Oxford Molecular Group, Oxford) was used to align the classical and El Tor RS2 nucleotide and protein sequences. The classical RS2 region has been assigned GenBank accession number AF055890.

RESULTS AND DISCUSSION

Immunity of El Tor Strains to CTXϕ Infection. An essential *V. cholerae* intestinal colonization factor, the toxin coregulated pilus (TCP) (19), is the receptor for CTXϕ (7). In El Tor strains, TCP is not expressed efficiently during *in vitro* growth; therefore, we used an intraintestinal transduction assay to study whether El Tor CTXϕ lysogens exhibit immunity to CTXϕ infection. In this assay, a donor strain harboring the RF of CTX-Knϕ [the El Tor CTXϕ RF, which contains a Kn resistance gene replacing *ctxAB* (7)], was coinoculated with either an El Tor lysogen (E7946) or its CTXϕ$^-$ attRS$^-$ vaccine derivative (Bah-2) (10) into the gastrointestinal tracts of suckling mice. These two potential CTX-Knϕ recipient strains colonized the suckling mouse small intestine approximately equally well (Fig. 1). Comparison of the numbers of CTX-Knϕ transductants of E7946 or Bah-2 in intestinal homogenates revealed that although nearly 1 in 10 Bah-2 cells were transduced to Kn^R with CTX-Knϕ in the intestine, only 1 in 10^4 E7946 cells were transduced within the intestine (Fig. 1). This three-order-of-magnitude difference in the frequency of recovery of transductants between these strains indicates that El Tor CTXϕ lysogens exhibit immunity to further CTXϕ infection.

RstR Is Sufficient for CTXϕ Immunity. Strains of the classical biotype of *V. cholerae* are not immune to infection with the El Tor-derived CTXϕ. Classical strains readily express TCP either *in vitro*, by using appropriate growth conditions, or within the intestine and can be efficiently transduced with the El Tor-derived CTX-Knϕ both *in vitro* and *in vivo* (7). In these transductants, CTXϕ does not integrate but replicates as a plasmid (7, 9). In lambdoid phages repressors are known to mediate phage immunity (11). Although CTXϕ is unrelated to lambdoid phages, we investigated whether El Tor CTXϕ RstR could render a classical strain immune to transduction with the El Tor CTXϕ. To test this possibility, a plasmid (pHK1) that contains the El Tor *rstR* under the transcriptional control of P_{BAD}, an arabinose-inducible promoter (12), was introduced into classical strain O395 (20). After growth of O395(pHK1) in the presence or absence of inducer, the cells were infected with the El Tor CTX-Knϕ. Induction of *rstR* expression with arabinose resulted in an approximately 900-fold reduction in

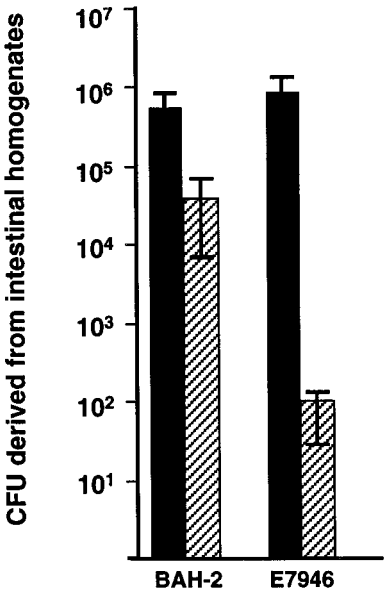

FIG. 1. Intraintestinal transduction of recipient strains. Donor *V. cholerae* strain LAC-1(pCTX-Kn) was mixed with each of the different *V. cholerae* recipient strains and then gastrointestinally inoculated into suckling mice. After 20 hr of intraintestinal growth, intestinal homogenates were plated to determine the total number of CFU of each recipient strain (solid bars) and the number of recipient cells that were transduced to Kn resistance by CTX-Knϕ (hatched bars). There were at least eight mice in each group. For each group, the median number of CFU along with the range are depicted in the graph.

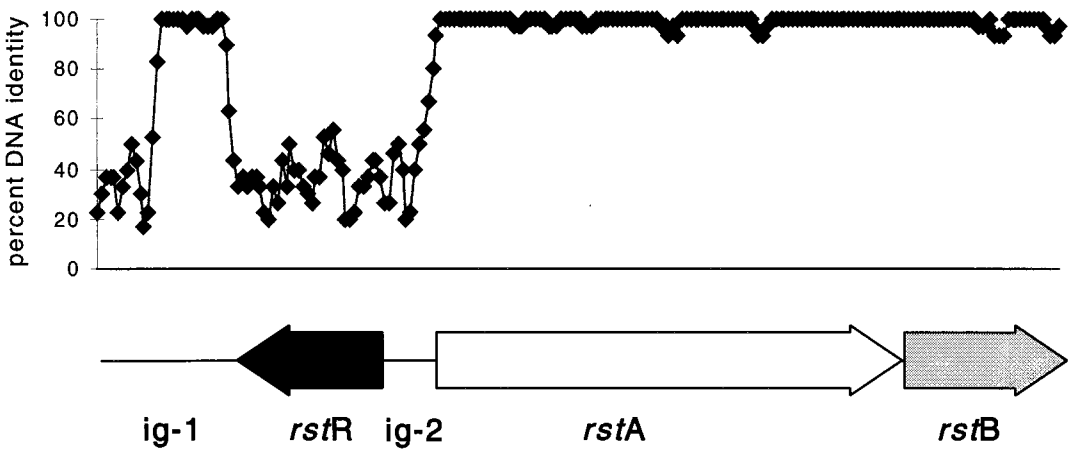

FIG. 2. Comparison of the nucleotide sequences of the El Tor and classical RS2 regions. DNA sequences were aligned by using GENEWORKS 2.5.1. Percent identity scoring was accomplished by moving a 30-bp window along the alignment in 10-bp increments.

the number of transductants of O395(pHK1) with CTX-Knϕ compared with the number of transductants of O395(pHK1) after growth in the absence of arabinose. Arabinose did not alter the transducibility of O395 harboring the pBAD33 vector without an insert. These findings indicate that El Tor RstR is sufficient to render classical *V. cholerae* immune from transduction with El Tor-derived CTXϕ. The converse experiment, testing whether the classical RstR can render an El Tor strain immune to infection with classical CTXϕ, is not possible at this time because to date we have not been able to induce CTXϕ virion production from classical lysogens. This may suggest that the classical CTX prophage is defective.

Divergence of the Classical and El Tor CTXϕ Repressors. To begin to address the molecular basis for the lack of immunity of classical lysogens to El Tor CTXϕ infection, we sequenced the RS2 region of the classical CTXϕ by using subclones (17) of the chromosomal copies of the classical CTX prophage. Previous studies of the RS2 region of the El Tor CTXϕ genome have revealed that this region encodes products required for phage DNA replication and integration (*rstAB*) as well as a repressor, *rstR*, which is transcribed divergently from all the other CTXϕ genes and which represses transcription of *rstA* (9) (Fig. 2). DNA sequence analysis revealed that like the El Tor CTXϕ RS2 region, classical CTXϕ RS2 contains three ORFs, designated *rstR*^{CL}, *rstA*^{CL}, and *rstB*^{CL}, and two intergenic regions (ig-1^{CL} and ig-2^{CL}) (Fig. 2). Also, like the El Tor RS2 region, an intergenic region (ig-2^{CL}) separates the divergently transcribed *rstR*^{CL} and *rstA*^{CL}. The nucleotide sequences of *rstA*^{CL} and *rstB*^{CL} were 94% identical to their El Tor homologues, and the predicted amino acid sequences of these proteins are 99% identical. In striking contrast to this, the nucleotide sequences of the *rstR* and ig-2 sequences were highly divergent in the two biotypes (Fig. 2). Despite the nucleotide and predicted amino acid sequence divergence of the classical and El Tor *rstR* genes (RstR and RstR^{CL} are 24% identical and 32% similar), the classical RstR^{CL}, like the El Tor RstR (9), is similar to other bacteriophage repressor proteins.

Repression Mediated by the RstR Alleles Is Biotype-Specific. To address the specificity of repression mediated by the classical and El Tor *rstR* genes, *lacZ* transcriptional fusions to the classical and El Tor *rstA* alleles (pHK101 and pHK102, respectively) were constructed. Also, plasmids containing the classical and El Tor *rstR* genes under the transcriptional control of an arabinose-inducible promoter (12) were constructed. Combinations of plasmids containing either of the two biotype *rstA*::*lacZ* reporters and either of the two biotype-inducible repressors were introduced into an *E. coli* Δ *lacZ* strain [CC118 (21)]. After growth in the presence of the inducer arabinose, El Tor RstR repressed expression of the El Tor *rstA-lacZ* fusion nearly 200-fold but had no repressive effects on expression of the classical *rstA-lacZ* fusion (Table 1). Similarly, RstR^{CL} repressed expression of the classical *rstA-lacZ* fusion nearly 80-fold but had no repressive effect on expression of the El Tor *rstA-lacZ* fusion. That is, RstR-mediated repression of *rstA* expression is biotype-specific. Expression of the *rstA-lacZ* reporters also was repressed in a biotype-specific manner after these reporters were introduced into El Tor (E7946) or classical (O395) lysogens (Table 1). This indicates that the repressors are expressed and active in lysogens of their respective biotypes. Our data suggest that classical lysogens lack immunity to El Tor CTXϕ infection because the classical RstR^{CL} is unable to repress El Tor *rstA* expression.

The molecular bases of phage immunity have been well studied in the temperate, double-stranded lambdoid phages. Among this large group of bacteriophages it has been

Table 1. Specificity of CTXϕ RstR repressors for *rst*A promoters

	RstR repressor*				*V. cholerae* CTXϕ lysogen†	
	Classical (pHK2)		El Tor (pHK1)			
Reporter	−Arabinose	+Arabinose	−Arabinose	+Arabinose	Classical	El Tor
Classical *rst*A-*lac*Z (pHK 101)	527	7	688	933	5	74
El Tor *rst*A-*lac*Z (pHK102)	157	167	255	1.2	260	1

β-Galactosidase units are reported as nmol of *o*-nitrophenyl β-D-galactoside hydrolyzed per min per OD_{600}. Assays were performed in triplicate, and the average value is presented.

*The arabinose-inducible classical (pHK2) or El Tor (pHK1) *rst*R plasmids were introduced along with an *rst*A-*lac*Z reporter plasmid into *Escherichia coli* strain CC118 (21). Cultures were grown in L broth (25) for 16 hr in the presence or absence of 0.05% arabinose, and the β-galactosidase activity was determined (25).

†The classical and El Tor *rst*A-*lac*Z reporters were introduced into *lac*Z$^-$ derivatives of classical (0395) and El Tor (E7946) *V. cholerae* CTXϕ lysogens, and the activity of β-galactosidase was determined (25).

Table 2. Protection of live-attenuated *V. cholerae* vaccine strains from intraintestinal CTXϕ transduction by *rstR*

Recipient strain*	CFU in intestinal homogenates†	
	Total no. of recipients	Transductants
BAH-2(pHK3)	3.7×10^5	0
BAH-2(pBR322)	5.6×10^5	5.3×10^4
O395-N1(pHK4)	2.2×10^6	0
O395-N1(pACYC184)	1.9×10^6	8.1×10^4

**V. cholerae* CTXϕ donor strain LAC-1(pCTX-Kn) was mixed with each of the different recipient strains and gastrointestinally inoculated into suckling mice.

†Intestinal homogenates were plated on selective media to enumerate the total numbers of colony-forming units (CFU) of each recipient strain and the numbers of kanamycin-resistant CFU (tranductants) of each recipient strain after 20 hr of intraintestinal growth. There were at least eight mice in each group, and the median number of CFU in each group is presented.

demonstrated that divergence of the sequences of the repressor, cI, and its operators establishes the molecular basis for the finding that λ lysogens are not immune to lytic infection by closely related lambdoid phages such as 434 (11). This lack of immunity among very closely related lambdoid phages is referred to as ''heteroimmunity'' (11). Our observations strongly suggest that the divergence of the classical and El Tor *rstR* genes and their operators in ig-2 establishes a heteroimmunity-like phenomenon among CTXϕ. Because filamentous CTXϕs and lambdoid phages are distinct classes of viruses, it is remarkable that in both cases repressors mediate phage immunity and divergence of repressors and their cognate operators accounts for heteroimmunity. This may suggest that the evolutionary history of CTXϕ included acquisition of the *rstR*-ig-2 immunity region by horizontal gene transfer.

Use of *rstR* to Protect Live-Attenuated *V. cholerae* Vaccine Strains from CTXϕ Infection. The live-attenuated *V. cholerae* vaccine strains derived from both biotypes are transducible with the El Tor-derived CTXϕ (7) and therefore are capable of reversion to toxinogenicity. The finding that *rstR* and ig-2 function as an immunity region for CTXϕ suggested that El Tor RstR could be used to protect El Tor-derived live vaccine strains, which contain a deletion of the entire CTXϕ (5, 6), as well as classical-derived vaccine strains, which contain a deletion of *ctxA* (4), from infection with the El Tor CTXϕ. To test this possibility, plasmids containing El Tor *rstR* (pHK3 or pHK4) or the same plasmids without inserts, pBR322 and pACYC184, respectively, were introduced into the El Tor vaccine strain Bah-2 (10), a CTXϕ^- attRS$^-$ strain, and into classical vaccine strain O395-N1 (22), a *ctxA*$^-$ strain. Then, the numbers of intestinally derived CTX-Knϕ transductants of these vaccine strains were determined by using the *in vivo* transduction assay as described above.

No detectable intestinally derived Kn^R transductants of Bah-2 (pHK3) or O395-N1 (pHK4), which contain plasmid-expressed El Tor *rstR*, were recovered in intestinal homogenates (Table 2). In contrast, nearly 1 in 10 Bah-2 (pBR322) and 1 in 20 O395-N1 (pACYC184) were transduced with CTX-Knϕ within the intestine (Table 2). This intestinal transduction assay can detect transduction frequencies as low as 1 in 100,000. Thus, introduction of the El Tor *rstR* into live-attenuated *V. cholerae* vaccine strains provides a means to ensure that these vaccines will be immune to CTXϕ infection and thereby should significantly lower the possibility of vaccine reversion to toxinogenicity.

Other strategies to prevent CTXϕ-mediated reversion of live vaccine strains can be envisioned. For example, deletion of the CTXϕ receptor, TCP, is another approach. However, strains harboring *tcpA* deletions do not colonize the intestine and do not induce a protective immune response (23). Therefore, unless TCP pili, which are not functional CTXϕ receptors but which enable *V. cholerae* colonization, can be constructed, this approach may not be successful. Expression of *rstR* in *V. cholerae* vaccine strains has potential limitations as well. For example, analogous to other temperate phages, *vir* mutants of CTXϕ may arise. Similarly, if there are many *ctxAB*$^+$ CTX phages with different immunity regions present in the world, then this strategy will not be useful. To begin to address this possibility, we compared the nucleotide sequences of *rstR*-ig-2 derived from El Tor clinical isolates from different continents over the past 25 years. The nucleotide sequences were identical in all five strains studied. This finding supports the clonality of the seventh pandemic of cholera (24). In addition, the nucleotide sequence of *rstR*-ig-2 in MO45, a *V. cholerae* O139 serogroup strain, was identical to the El Tor sequence. The conservation of the sequence of the immunity region of CTXϕ in these El Tor strains suggests that the existence of many different CTXϕ immunity regions is improbable. Thus, introduction of a stably maintained *rstR* into the existing *V. cholerae* vaccine candidates should improve their biosafety. Heterologous expression of viral regulatory genes may have applications in the development of other live bacterial vaccines and potentially in the design of human antiviral gene therapy as well.

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