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The Hybrid Pre-CTXΦ-RS1 Prophage Genome and Its Regulatory Function in Environmental *Vibrio cholerae* O1 Strains

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The cholera toxin genes of *Vibrio cholerae* are encoded by CTXΦ, a lysogenic bacteriophage. Infection with this phage plays a determinant role in toxigenicity conversion and the emergence of new clones of pathogenic *V. cholerae*. Multiple phage alleles, defined by sequence types of the repressor gene *rstR*, have been found, showing the divergence of phage genomes. Pre-CTXΦ, which is characterized by the absence of toxin genes, is predicted to be the precursor of CTXΦ. We have found a new pre-CTXΦ prophage genome (named pre-CTX^{ZJ}Φ for its novel *rstR* allele) in nontoxigenic *V. cholerae* O1 isolates that were obtained during surveillance of the estuary water of the Zhujiang River. A novel hybrid genome of the helper phage RS1 was identified in an environmental strain carrying pre-CTX^{ZJ}Φ in this study. The chromosomal integration and genomic arrangement of pre-CTX^{ZJ}Φ and RS1 were determined. The RS2 of pre-CTX^{ZJ}Φ was shown to have a function in replication, but it seemed to have lost its ability to integrate. The RstR of pre-CTX^{ZJ}Φ exerted the highest repression of its own *rstA* promoter compared to other RstRs, suggesting *rstR*-specific phage superinfection immunity and potential coinfection with other pre-CTXΦ/CTXΦ alleles. The environmental strain carrying pre-CTX^{ZJ}Φ could still be infected by CTX^{ET}Φ, the most common phage allele in the strains of the seventh cholera pandemic, suggesting that this nontoxigenic clone could potentially undergo toxigenicity conversion by CTXΦ infection and become a new toxigenic clone despite already containing the pre-CTXΦ prophage.

Vibrio cholerae is primarily an inhabitant of estuarine water, particularly in estuary waters (1). More than 200 serogroups of *V. cholerae* have been recognized (2), although only serogroups O1 and O139 have caused epidemics (3). *V. cholerae* has caused seven pandemics historically. The ongoing seventh pandemic is caused by *V. cholerae* O1 biotype El Tor.

It has been proposed that the sixth and seventh pandemic strains evolved from nontoxigenic environmental strains (4). Whole-genome comparisons have shown that horizontal gene transfer plays critical roles in the emergence of toxigenic strains and the divergence of epidemic strains (5, 6). Epidemic strains of *V. cholerae* are characterized by the production of cholera toxin (CT) and toxin-coregulated pilus (TCP) (7). The CT gene cluster *ctxAB* is located in the genome of the lysogenic bacteriophage CTXΦ, which is integrated into the chromosome of toxigenic *V. cholerae* and may be transferred from toxigenic to nontoxigenic strains (8, 9). After induction, CTXΦ can infect *V. cholerae* by using TCP as its receptor and by interacting with its major subunit, TcpA; it then integrates into the chromosome at the *attB* site (10–12). The TCP gene cluster resides in the *Vibrio* pathogenicity island (VPI), an essential virulence gene cluster of *V. cholerae* (13). The acquisition of the VPI gene cluster by horizontal gene transfer is generally thought to be a requirement for conversion from nontoxigenic to pathogenic strains due to the production of the TCP, which mediates infection with CTXΦ (14, 15).

The typical CTXΦ prophage genome is composed of RS2 and the core region (9). The core region contains the genes involved in phage morphogenesis and the *ctxAB* genes. CTX prophages that are devoid of the *ctxAB* genes have also been identified (16, 17) and are named pre-CTXΦ; these prophages are predicted to be precursors of CTXΦ. RS2 is the regulation region of CTXΦ and contains the *rstR*, *rstA*, and *rstB* genes and two intergenic regions, ig-1 and ig-2. RstA and RstB are required for CTXΦ replication and integration, and RstR is a repressor that inhibits the transcription of *rstA* and *rstB* genes (18). Certain *V. cholerae* El Tor strains also carry RS1, which is absent in the classical biotype and integrated upstream and/or downstream of the CTX element. RS1 is similar to RS2 except that it contains an additional gene, *rstC*, and is considered to be a satellite phage of CTXΦ (19, 20). The *rstC* gene, which encodes an anti-repressor of RstR, may promote the propagation and transmission of RS1/CTXΦ (19).

Within the CTX and pre-CTX prophage genomes, the sequence of the *rstR* gene showed high divergence. The *rstR* genes are preliminarily designated *rstR*^{class}, *rstR*^{ET}, or *rstR*^{calc} based on nucleotide sequence variation from serogroup O1 biotype classical (class) and El Tor (ET) strains and some serogroup O139 strains (e.g., strain O139 Calcutta [calc]) (21, 22). Accordingly, CTXΦs are classified as CTX^{class}Φ, CTX^{ET}Φ, and CTX^{calc}Φ by their *rstR* sequences. Other *rstR* alleles, including *rstR-4** (23), *rstR-4*** (23), *rstR-5* (23), *rstR6* (24, 25), *rstR-232* (26) and *rstR*^{ZJ} (27), are found in nontoxigenic environmental strains of O1 and O139 and non-O1/non-O139 serogroups. RstR may provide immunity to recurrent infection by an identical CTXΦ (21, 22, 28,

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29). This type of CTX immunity is *rstR* allele specific and is determined by the RstR sequence and its binding site in ig-2 (21, 22, 28). Moreover, CTX prophages with different *rstR* sequence types in certain *V. cholerae* strains have been reported (21, 30–32).

It has been strongly suggested that the *ctxAB* genes are acquired subsequently to the development of the pre-CTXΦ (9, 16, 17), whereas the biological significance of pre-CTXΦ and its infection of *V. cholerae* is unclear. The identification of new CTX prophage genomes will provide more evidence for exploring the evolution of the CTXΦ family and lineages of toxigenic *V. cholerae*. During our surveillance of O1/O139 *V. cholerae* in the estuarine water of the Zhujiang River in Guangzhou, China, a higher frequency of strains carrying pre-CTXΦ was found in the nontoxigenic environmental strains (27). Eleven nontoxigenic O1 strains carrying a novel pre-CTX^{ZJ}Φ with unique *rstR*^{ZJ} and *tcpA* genes were isolated in successive months and became the dominant environmental strains carrying pre-CTXΦ in the Zhujiang River (27). In this study, we unraveled the genomic structure of this prophage and the potential immunity of this novel RstR to other CTXΦ alleles. Considering the prevalence of these nontoxigenic strains in the environment and the fact that they encode a TCP that is similar to those encoded by the epidemic strains, we also explored the possibility that these strains could acquire CTX^{ET}Φ and found that they had the potential to become toxigenic.

MATERIALS AND METHODS

Strains and growth conditions. The *V. cholerae* strains and *Escherichia coli* strains used in this study are described in Table 1. Strains were grown in Luria broth (LB) with agitation (200 rpm) at 37°C unless otherwise indicated. When necessary, the culture medium was supplemented with ampicillin (Amp; 100 μg/ml), tetracycline (Tet; 30 μg/ml), chloramphenicol (Cm; 30 μg/ml for *E. coli* or 15 μg/ml for *V. cholerae*), IPTG (isopropyl-β-D-thiogalactopyranoside; 20 μg/ml), or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 20 μg/ml).

PCR amplification, sequencing, and sequence alignment. *V. cholerae* genomic DNA was extracted using a NucleoSpin tissue kit (Macherey-Nagel, USA) according to the instructions of the manufacturer. We amplified the entire pre-CTXΦ genome and its chromosomal integration sites using different combinations of the primers zot1, zot2, ctxAB1, ctxAB2, orfU1, orfU2, rstC1, rstC2, inA, inB, inI, and inJ (see Table S1 in the supplemental material). PCR amplification was performed using *Taq* DNA polymerase or LA *Taq* DNA polymerase (TaKaRa). All amplicons were sequenced using the primers listed in Table S1. BioEdit was used to perform multiple alignments of predicted amino acid sequences. All GenBank accession numbers are listed in Table S2.

Construction of plasmids. Plasmid pACYC184-*rstR*^{ZJ} containing the *rstR*^{ZJ} gene and its promoter was constructed by amplifying the *rstR*^{ZJ} coding and promoter regions using primers rstRZJ1 and rstRZJ2 (see Table S1 in the supplemental material) and genomic DNA from strain VC06-18 (Table 1). Next, the PCR product was cloned into TA cloning vector pMD18T (TaKaRa) and sequenced. An EcoRI fragment containing the insertion was then ligated to EcoRI-digested and -dephosphorylated pACYC184 to generate pACYC184-*rstR*^{ZJ}. The plasmids pACYC184-*rstR*^{class}, pACYC184-*rstR*^{ET}, pACYC184-*rstR*^{calc}, pACYC184-*rstR6*, and pACYC184-*rstR-232*, which contain the *rstR*^{class}, *rstR*^{ET}, *rstR*^{calc}, *rstR6*, and *rstR-232* genes and their promoters, respectively, were constructed using the same strategy. To construct pRS591-*rstA*^{ZJ}, the *rstA*^{ZJ} promoter region and the *Renilla* luciferase reporter gene (*rluc*) were amplified using primers rstAZJP1/rstAZJP2 (see Table S1) and genomic DNA from strain VC06-18 and using primers RlucZJ1/Rluc2 (see Table S1) and plasmid pRL-null, respectively. Next, the overlapping fragment containing the *rstA*^{ZJ} promoter and *rluc* reporter was amplified with primers rstAZJP1/Rluc2. To construct pRS591-*rluc*, the *rluc* reporter gene was amplified with primers Rluc1/Rluc2 (see Table S1). Following subcloning of these PCR products into pMD18T and sequencing, EcoRI fragments containing the insertion were ligated into EcoRI-digested and -dephosphorylated pRS591 to generate pRS591-*rstA*^{ZJ} and pRS591-*rluc*, respectively. Plasmids pKTN701-RS2^{ZJ} and pKTN701-RS2^{ET} were constructed by amplifying the region of partial *zot*-RS2-*psh*-*cep*-partial *gIII*^{CTX} with primers RS-F and RS-R (see Table S1) and genomic DNA from strain VC06-18 or strain 86015 (Table 1). Next, the PCR products were cloned into pMD18T

TABLE 1 Characterization of strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source and/or reference(s)
V. cholerae strains		
VC06-18	O1, pre-CTX^{ZJ}Φ, Δ*ctxAB*, *tcpA*$^+$	Water (27)
VC06-41	O1, Δ*zot*, Δ*ctxAB*, *tcpA*$^+$	Water
1119	O1, classical, Inaba, ΔCTX^{ET}Φ, CTX^{class}Φ$^+$, *tcpA*$^+$	33
N16961	O1, El Tor, CTX^{ET}Φ, *ctxAB*$^+$, *tcpA*$^+$	Patient
86015	O1, El Tor, CTX^{ET}Φ, *ctxAB*$^+$, *tcpA*$^+$	Water
N-Φc	N16961 *ctxAB*::*cat*	33
SCE264	O42, RS1$^+$, ΔCTXΦ, Δ*ctxAB*, *tcpA*$^+$	Water (23)
MS6	O1, El Tor, CTX^{ET}Φ, *ctxAB*$^+$, *tcpA*$^+$	Patient (34, 35)
234-93	O141	Patient (3)
E. coli strains		
JM109	*recA1 supE44 endA1 hsdR17 thi*Δ(*lac-proAB*)F′[*traD36 proAB*$^+$ *lacI*^{q}*Z*ΔM15]	TaKaRa
SM10λpir	*supE recA*::RP4-2-Tc::Mu λpir; Km^r	Our laboratory
Plasmids		
pRL-null	*rluc* reporter gene	Promega
pACYC184	*ori*p15A, Cm^r, Tc^r	NEB
pRS591	*ori*ColE1, Ap^r, Tc^r	36
pACYC184-*rstR*^{ZJ}	pACYC184 containing *rstR*^{ZJ}, Tc^r	This study
pACYC184-*rstR*^{class}	pACYC184 containing *rstR*^{class}, Tc^r	This study
pACYC184-*rstR*^{ET}	pACYC184 containing *rstR*^{ET}, Tc^r	This study
pACYC184-*rstR*^{calc}	pACYC184 containing *rstR*^{calc}, Tc^r	This study
pACYC184-*rstR6*	pACYC184 containing *rstR6*, Tc^r	This study
pACYC184-*rstR*-232	pACYC184 containing *rstR*-232, Tc^r	This study
pRS591-*rstA*^{ZJ}	pRS591 containing *rstA*^{ZJ} promoter and *rluc*, Ap^r, Tc^r	This study
pRS591-*rluc*	pRS591 containing *rluc* without promoter, Ap^r, Tc^r	This study
pKTN701	Suicide plasmid vector, *mobRP4 oriR6K*, Cm^r	37
pKTN701-RS2^{ZJ}	pKTN701 containing RS2 of pre-CTX^{ZJ}Φ, Cm^r	This study
pKTN701-RS2^{ET}	pKTN701 containing RS2 of CTX^{ET}Φ, Cm^r	This study

[a] Ap, ampicillin; Cm, chloramphenicol; Tc; tetracycline.

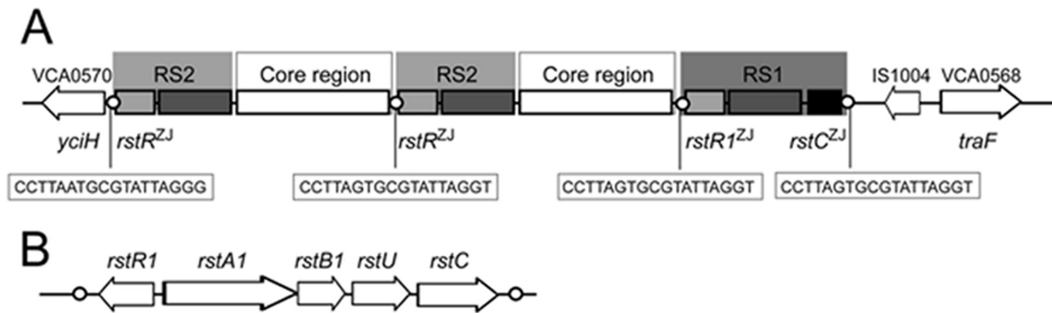

FIG 1 Genetic organization of pre-CTX^{ZJ}Φ (A) and RS1 (B) in the small chromosome of strain VC06-18 (not drawn to scale). Circles represent integration site *attB* of pre-CTXΦ, and the sequences are shown in the rectangles.

and sequenced. The SalI/EcoRI fragments containing the insertion were then ligated to SalI/EcoRI-digested pKTN701 to generate pKTN701-RS2^{ZJ} and pKTN701-RS2^{ET}.

Quantitative real-time PCR and calculation of the *zot* gene copy numbers. Quantitative real-time PCR was performed using primers zotF1/zotR1 (see Table S1 in the supplemental material) targeting the *zot* gene and ThyA1/ThyA2 (see Table S1) targeting the thymidylate synthase (*thyA*) gene, fluorescent dye SYBR green I (TaKaRa), and a LightCycler 2.0 (Roche Diagnostics, USA) system. Melting curve analysis was performed for each reaction to confirm the specificity of the PCR. The number of PCR threshold cycles (*CT*) required for the fluorescent intensities to exceed a threshold just above the background level was calculated for each reaction. The $2^{-\Delta\Delta CT}$ method was used to calculate the copy number of the *zot* gene as previously described (38, 39). The average copy number and 95% confidence interval (CI) of the *zot* gene were calculated for six experiments by the use of SPSS 10.0.

***Renilla* luciferase luminescence assay.** JM109-derived strains containing different combinations of pACYC184 and pRS591 derivatives were incubated at 37°C with shaking. The optical density at 595 nm (OD_{595}) of triplicate bacterial suspensions was measured every 30 min. Simultaneously, 95-μl culture samples were mixed with 5 μl of 1.2 mM ViviRen live cell substrates (Promega, USA) in triplicate and incubated for 10 min at room temperature. Luminescence was then measured using a luminometer (Tecan). The combination of pACYC184 and pRS591-*rstA* served as a positive control, while the combination of pACYC184 and pRS591-*rluc* without the promoter served as a negative control. To calculate the repression multiple of the novel *rstA* promoter mediated by RstR, as a positive control, the luminescence of the test strain at the same OD points was calculated by constructing the bivariate scatter plots with luminescence-OD values. The repression multiple of every RstR was obtained by dividing the luminescence of the positive control by the corresponding luminescence of the test. The repression curve of the *rstA* promoter was constructed with the OD value on the *x* axis and the repression multiple along the *y* axis.

***In vitro* and *in vivo* CTX^{ET}Φ infection assays.** Cm-resistant CTX^{ET}Φ (CTXΦc) was isolated from strain N-Φc as described previously (33, 40). For *in vitro* infection assays, *V. cholerae* strains were incubated without shaking in colonization factor broth (0.15% yeast extract, 1% casein hydrolysate, 50 ppm $MgSO_4$, 5 ppm $MnCl_2$, pH 6.8) at 30°C for 18 to 24 h, as described previously (33, 40). Control strain 1119, which is commonly used to validate the infectivity of CTXΦ (33, 40), and test strain VC06-18 were mixed with CTXΦc in triplicate and incubated without shaking at 30°C for 3 h, and the cells were then spread onto plates containing Cm. The *in vivo* CTXΦc infection was conducted as described previously (33). Briefly, as parallel controls, two New Zealand White rabbits (2 to 2.5 kg of body weight) were fasted for 24 h prior to surgery. Rabbits were anesthetized with ether. The small intestines were separated under sterile conditions and tied into 4-to-5-cm-long loops separated by 2 cm of interloops. Each loop was inoculated with the mixture of recipient *V. cholerae* and donor CTXΦc particles in triplicate. The *V. cholerae* strain and CTXΦc particles alone served as negative controls. The intestines were returned to the peritoneal cavity. The rabbits were sacrificed 16 to 18 h later. The small intestine was dissected, and each loop was washed with 1 ml of 0.9% saline solution. The number of CTXΦ-infected cells and the total number of cells were obtained by plating the ileal loop fluid onto LB agar plates containing Cm and onto antibiotic-free LB agar plates. Each colony was confirmed with antisera diagnostic for *V. cholerae*. Both experiments were performed three times.

Nucleotide sequence accession number. Sequence data for V06-18 have been deposited in GenBank under accession number KP768424.

RESULTS AND DISCUSSION

Zhujiang estuarine strain VC06-18 carries a novel hybrid RS1. We identified a pre-CTX^{ZJ}Φ genome from 11 *V. cholerae* O1 nontoxigenic strains isolated during our Zhujiang estuarine water surveillance (27). The pre-CTX^{ZJ}Φ carries a hybrid RS2 (*rstR*^{ZJ} *rstA*^{232} *rstB*^{232}). The satellite phage RS1, which encodes an additional *rstC* gene compared to RS2, is also found in some *V. cholerae* strains (27). Here, we confirmed the presence of RS1 in the 11 strains carrying pre-CTX^{ZJ}Φ by PCR and by sequencing *rstC* with the primers rstC1 and rstC2 (see Table S1 in the supplemental material) and the regions between pre-CTX ^{ZJ}Φ and its integration site in the chromosome with combinations of primers rstRZJ1/rstRZJ2/zot1/zot2 targeting the *rstR*^{ZJ} and *zot* genes and InI/InJ/inA/inB targeting the integration site. Only strain VC06-18 has the RS1 region (data not shown), although the 11 strains have the same *rstR*^{ZJ}, *rstA*^{232}, and *rstB*^{232} genes in the RS2 region (data not shown). Subsequently, we explored the organization of this RS1. Combinations of primers located in the *rstR*^{ZJ}, *rstC*, and *zot* genes (see Table S1) were used to amplify RS1 from strain VC06-18. A 4.3-kb fragment was amplified with primers rstC1 and zot1 (see Table S1) and sequenced. Based on the open reading frame (ORF) prediction and comparison, this RS1 was composed of ORFs *rstR1*, *rstA1*, *rstB1*, *rstU*, and *rstC* (Fig. 1B), and RstR1, RstA1, and RstB1 showed 21.4%, 11.6%, and 9.7% amino acid identity with RstR, RstA, and RstB of RS2 in its host VC06-18 strain (see Fig. S1, S2, and S3 in the supplemental material), respectively. We found that the amino acid sequence of RstC was highly conserved in all searched *V. cholerae* strains (see Fig. S4). RstA1 and RstB1 of VC06-18 RS1 showed 94.2% and 100% amino acid identity, respectively, with those of *V. cholerae* O1 El Tor strain MS6, which was isolated from Thailand-Myanmar in 2008 and carries CTX^{ET}Φ (34, 35), and 72.5% and 64.5% amino acid identity with those of O42 strain SCE264, which contains RS1 but

lacks the CTX element (23) (see Fig. S1 and S2). In addition, no nucleotide or amino acid sequences similar to sequences of the *rstA* and *rstB* genes of VC06-18 were found by BLASTN or BLASTP in the whole-genome shotgun sequencing results seen with toxigenic *V. cholerae* O141 strain 234-93 (isolated in India in 1998), for which annotated *rstA* and *rstB* genes are unavailable (41). RstR1 of VC06-18 RS1 has 95.3% amino acid identity with a hypothetical helix-turn-helix family protein of strain 234-93, but it showed 20.4% identity with RstR1 of MS6 and also very little identity with other RstR and RstR1 sequences (see Fig. S3). In addition, a putative 117-amino-acid (aa) ORF located between *rstB1* and *rstC* showed 100% nucleotide identity with the gene encoding fumarate reductase subunit D, which resides in the region between *rstB1* and *rstC* of MS6 (data not shown). However, this is only a predicted ORF, and there is no other information about its function, but we have provisionally designated this ORF *rstU* (Fig. 1B). These analyses indicated that *rstR1* and the *rstA1*, *rstB1*, and *rstU* genes of RS1 in strain VC06-18 derived from different *V. cholerae* strains, and even different serogroups, and formed a new RS1 variant with a conserved *rstC* gene.

Two copies of the pre-CTX^{ZJ}Φ integrated in a tandem manner in the small chromosome of VC06-18. CTXΦ/pre-CTXΦ integrates between the toxin-linked cryptic (TLC) element and RTX toxin loci in the large chromosome of epidemic strains or between the *traF* (VCA0570) and *yciH* (VCA0568) loci in the small chromosome of classical strains and in certain clinical El Tor strains (20, 42–45). To identify the integration site of pre-CTX^{ZJ}Φ in strain VC06-18, PCR and sequencing were performed using primers InI/InJ and inA/inB (see Table S1 in the supplemental material), which span the *attB* sites in the small and large chromosomes of *V. cholerae*, respectively. An empty *attB* site was detected in the large chromosome but was absent from the small chromosome, implying that the pre-CTX^{ZJ}Φ integrated into the small chromosome of strain VC06-18.

The CTXΦ/pre-CTXΦ element may be present in more than one copy in the *V. cholerae* chromosomes (46). We determined the copy number of the pre-CTX^{ZJ}Φ element in strain VC06-18 with quantitative real-time PCR using the *zot* core region gene of CTXΦ/pre-CTXΦ and the *thyA* housekeeping gene of *V. cholerae* as the indicators. A single copy of *thyA* exists in *V. cholerae* based on the analysis of seven complete genomes (NC_016445, NC_016944, NC_017270, NC_012578, NC_012668, NC_002505, and NC_012582) and 159 shotgun whole genomes (data not shown). El Tor strain N16961, which contains one copy of the *zot* gene in its chromosome, was used as a control. The data were analyzed as described in Materials and Methods. The average copy number of the *zot* gene of strain VC06-18 was calculated to be 2.1 (95% confidence interval, 1.91 to 2.17); therefore, VC06-18 has two copies of *zot* and two copies of core regions of pre-CTX^{ZJ}Φ.

Subsequently, the RS1, core, and RS2 regions of the pre-CTX^{ZJ}Φ genome and the junction regions between the chromosome and the pre-CTXΦ genome/RS1 were amplified with combinations of primers located in these regions (see Table S1 in the supplemental material), and all amplicons were sequenced. By sequence comparison and assembly, we obtained the following information regarding the arrangement of pre-CTX^{ZJ}Φ-RS1 in the chromosome of VC06-18. (i) The pre-CTX^{ZJ}Φ-RS1 genome was integrated into the intergenic region between VCA0570 and VCA0568 in the small chromosome, with two tandem copies in the arrangement (Fig. 1A). (ii) The RS2 region is composed of *rstR*^{ZJ}, *rstA*^{232}, and *rstB*^{232}, as described in our previous report (27), and its core region contains the *psh*, *cep*, *gIII*^{CTX}, *ace*, and *zot* genes and is thus quite similar to the core region of CTX^{ET}Φ. (iii) The region downstream of RS1 is adjacent to the second copy of pre-CTX^{ZJ}Φ. We drew the physical map of pre-CTX^{ZJ}Φ-RS1 with the pattern (*rstR*^{ZJ}-*rstA*^{232}-*rstB*^{232}-core region without *ctxAB*)$_2$-*rstR1-rstA1-rstB1-rstU-rstC* in strain V06-18 (Fig. 1), which can be simplified as (RS2-core)$_2$-RS1.

RS2 of pre-CTX^{ZJ}Φ has replication but no integration function. The *rstA* and *rstB* genes in the RS2 region are responsible for the replication and integration of CTXΦ (18). To study the replication and integration functions of the RS2 region of pre-CTX^{ZJ}Φ, we constructed plasmids pKTN701-RS2^{ZJ} (containing the RS2 region of pre-CTX^{ZJ}Φ) and pKTN701-RS2^{ET} (containing the RS2 region of CTX^{ET}Φ) as a control. Among the environmental *V. cholerae* strains isolated from the estuary waters of the Zhujiang River (27), strain VC06-41 (Table 1) has no CTX or pre-CTX element, has empty CTXΦ integration sites on both chromosomes, and carries the same *tcpA* gene as strain VC06-18 (detected by PCR and sequencing; data not shown). This strain was isolated from the same estuarine site as V06-18. Therefore, VC06-41 was chosen to be the donor strain for this experiment. Plasmids pKTN701-RS2^{ZJ} and pKTN701-RS2^{ET} were introduced into strain VC06-41 by conjugation. Sixty-five colonies of Cm^r transconjugants were picked randomly and confirmed to contain pKTN701-RS2^{ZJ} by PCR with primers RS1/RS2 and plasmid extraction. However, no pKTN701-RS2^{ZJ} was found to integrate in the large and/or small chromosomes of VC06-41 by PCR with primers inI/inJ and inA/inB and by sequencing. In contrast, chromosomal integration of pKTN701-RS2^{ET} in the transconjugants was confirmed. Among 65 randomly selected transconjugants, 16, 40, and 9 demonstrated pKTN701-RS2^{ET} integration in the large and/or small chromosomes. This result demonstrated that RS2 of pre-CTX^{ZJ}Φ can mediate replication but not integration. It seems that the integration function of RS2 in pre-CTX^{ZJ}Φ is lost. Whether *rstB* in this pre-CTX^{ZJ}Φ is dysfunctional or the integration is recipient dependent still needs to be defined. In comparison to the results seen with CTX^{ET}Φ, infection of the recipient *V. cholerae* strain with pre-CTX^{ZJ}Φ is less stable because the pre-CTX^{ZJ}Φ remains in plasmid form rather than undergoing chromosomal integration.

Repression of the *rstA* promoter in pre-CTX^{ZJ}Φ has an *rstR* allele specificity. In CTXΦ, repression of *rstAB* transcription by RstR is biotype specific (22, 28), which confers immunity to CTXΦ superinfection. In this study, we investigated whether the expression of *rstAB* of VC06-18 could be repressed by other RstR alleles. pRS591-*rstA*^{ZJ}, which contains an *rluc* transcriptional fusion under the control of the *rstA* promoter of pre-CTX^{ZJ}Φ, was constructed as the reporter plasmid. Six pACYC184 plasmid derivatives containing *rstR* genes (*rstR*^{class}, *rstR*^{ET}, *rstR*^{calc}, *rstR6*, *rstR-232*, and *rstR*^{ZJ}) and their promoters were constructed. Each of these six plasmids was combined with pRS591-*rstA*^{ZJ} and introduced into *E. coli* JM109. Luminescence activity was measured, and the curves of luminescence activity relative to the OD for each plasmid combination showed that the activity of the pACYC184-*rstR*^{ZJ}/pRS591-*rstA*^{ZJ} combination was lower than that of all other combinations tested except for the negative-control pACYC184/pRS591-*rluc* combination (Fig. 2A). RstR^{ZJ} repressed the expression of *rluc* in pRS591-*rstA*^{ZJ} by nearly 3.5-fold, but the other five

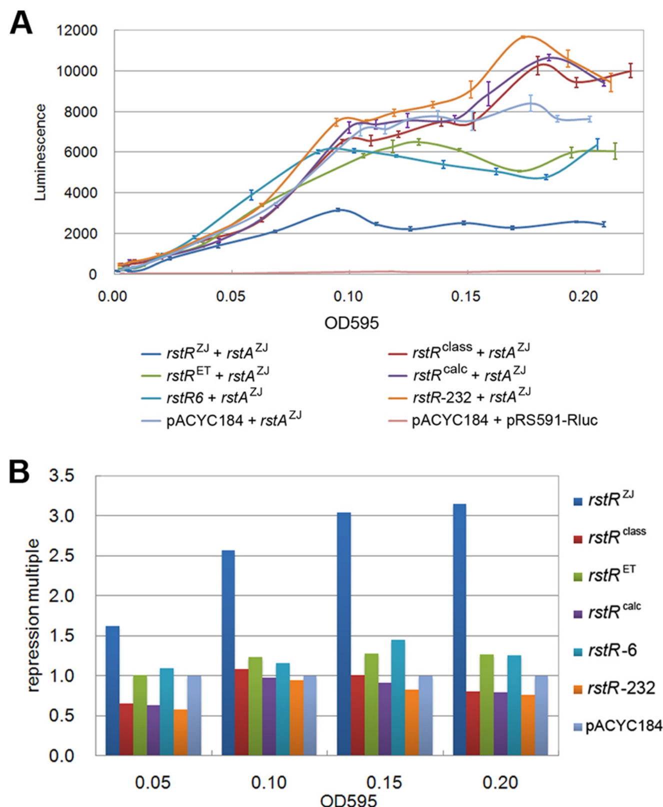

FIG 2 Specific repression of the *rstA*^{ZJ} promoter by RstR^{ZJ}. (A) Luminescence values generated under the control of the *rstA*^{ZJ} promoter combined with different *rstR*s during culture with different OD_{595} values. (B) Repression presented with alternation of repression multiples under conditions of different OD_{595} values. Each value represents the mean of the results from six independent experiments. Error bars denote the standard deviation.

alleles of RstR did not clearly repress the expression of *rluc* (Fig. 2B). This result indicated that RstR^{ZJ} strongly represses only the transcription of its own *rstAB* and that other RstRs with different sequence types have a quite diminished capacity to inhibit the transcription of RstA^{ZJ}. It can be deduced that pre-CTX^{ZJ}Φ or CTX^{ZJ}Φ can still infect the *V. cholerae* strains carrying a pre-CTX/CTX element with other sequence types of *rstR*.

CTX^{ET}Φ converts pre-CTX^{ZJ}Φ lysogen VC06-18 into a toxigenic strain. Infection and lysogenic conversion by CTXΦ may result in the emergence of new toxigenic strains or clones, and the reversion of live-attenuated *V. cholerae* vaccine strains to toxigenicity is a possibility (22). Lysogenic strains with pre-CTX^{ZJ}Φ, such as VC06-18, were more frequently isolated during our environmental surveillance. Whether such strains could be converted to toxigenicity by CTXΦ should be considered. The seventh-pandemic El Tor strains and toxigenic O139 strains carried CTX^{ET}Φ, the most common prophage of the current epidemic strains; therefore, we experimentally tested whether VC06-18 could be infected by this phage. In the *in vitro* experiments, strain VC06-18 was not found to be infected by CTXΦc, which is consistent with other El Tor strains (33), while the average infection rate (average number of CTXΦ-infected cells/100 total cells) of control strain 1119 was 3.09×10^{-5} with a 95% confidence interval (CI) of 1.09×10^{-4} to 8.75×10^{-6}. When these strains were tested in rabbit ileal loops, CTXΦc could infect VC06-18 with an average infection rate of 2.6×10^{-7} (95% CI, 2.56×10^{-6} to 7.29×10^{-8}) and strain 1119 with a rate 4.68×10^{-4} (95% CI, 2.56×10^{-4} to 5.84×10^{-5}), showing that VC06-18 could still be infected by El Tor type CTXΦ *in vivo* even though it carries the prophage pre-CTX^{ZJ}Φ. These results may also suggest that RstR of pre-CTX^{ZJ}Φ does not effectively inhibit the transcription of *rstA* and *rstB* of CTX^{ET}Φ and permits the infection of the latter. Strains carrying pre-CTX^{ZJ}Φ are predominant in estuarine water (27); therefore, it could be predicted that, during the coexistence of this type of strain and toxigenic El Tor (or O139) strains in a specific microenvironment such as a biofilm on plankton or in human intestines, these strains could obtain CTX^{ET}Φ released from the toxigenic strains and could become toxigenic, thus generating a new pathogenic or epidemic clone.

In conclusion, we describe a pre-CTX^{ZJ}Φ genome with a novel hybrid RS1. Divergence of CTXΦ/pre-CTXΦ genomes showed dynamic genomic recombination in the evolution of the CTXΦ family. Because phage immunity to CTXΦ superinfection mediated by RstR is limited by its sequence, pre-CTXΦ alleles can play roles in the complexity of the pre-CTXΦ family through coinfection and subsequent genomic recombination among the different alleles, cause the emergence of new CTXΦ alleles, and further increase the complexity of toxigenic *V. cholerae* clones. Pre-CTX^{ZJ}Φ is not rare, because environmental strains carrying this prophage were isolated from different surveillance months in the Zhujiang estuary. The strain carrying pre-CTX^{ZJ}Φ has no resistance to infection by CTX^{ET}Φ. Therefore, toxigenic conversion of these strains should be surveyed, although they have already integrated the pre-CTXΦ allele. In addition, based on the unique or uncommon prophage arrays of CTXΦ and pre-CTXΦ and their *rstR* sequences, these structures or sequence types may also be used as epidemiological genetic markers to trace the spread of specific *V. cholerae* clones.

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