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Replicating function of the RS1 element associated with *Vibrio cholerae* CTXΦ prophage

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Abstract

The RS1 element associated with *Vibrio cholerae* CTXΦ prophage was cloned from an El Tor biotype *Vibrio cholerae* strain. We used the *recA*$^-$ vaccine strain Peru-15, that lacks the target for RS-mediated site-specific integration, to show that RS1 promotes autonomous replication of a suicide vector. A linker insertion in the *rstR* open reading frame abolished autonomous replication in Peru-15 but not in a strain containing an RS1 in the chromosome. An AT-rich region containing *cis*-acting elements involved in autonomous replication was identified by deletion. This region was sufficient to support autonomous replication in a strain containing an RS1 in the chromosome. DNA sequence analysis of a region present in RS1 and not RS2 revealed the presence of putative binding sites for host proteins involved in plasmid replication. These results indicate that RS1 contains a replicon distinct from RS2 which could be involved in replicative recombination events associated with tandem amplification of the CTX element.

Keywords: *Vibrio cholerae*; Prophage; Toxin; RS1; El Tor biotype

1. Introduction

Vibrio cholerae of serogroups O1 and O139, the causative agents of cholera, secrete a potent enterotoxin, cholera toxin (CT), that causes the severe diarrhea of cholera [1]. The genes encoding cholera toxin A (*ctxA*) and toxin B (*ctxB*) subunits are located in a transposon-like structure initially called a 'virulence cassette' [2] or CTX element [3]. The 'virulence cassette' consists of a core region encompassing genes *cep*, *ace*, *orfU*, *zot*, *ctxA* and *ctxB* (Fig. 1A). The core region is flanked by one or several repeat sequences (RS) of type RS1 or RS2 [2,3]. RS elements have been proposed to mediate amplification of the core region during animal passage [4] and to encode a site-specific recombination system [3,4]. The virulence cassette is the prophage state of the CTXΦ filamentous phage that uses a major colonization factor, the toxin co-regulated pilus (TCP), as receptor [5,6]. The CTXΦ phage genome contains the core region of the virulence cassette and a RS2 [5]. The CTXΦ phage can integrate into an 18-bp sequence (*attRS*) in the chromosome or replicate as a plasmid [3,5]. Recently, it has been demonstrated that the genes required for integration and replica-

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tion of CTXΦ are located in element RS2 [7]. In the replicative state, single stranded CTXΦ DNA is packed into filamentous bacteriophage particles and exported to the culture medium [5,7].

In the present work we show that element RS1 associated with the CTXΦ prophage promotes autonomous replication in *V. cholerae*. The genetic study of RS1-mediated replication indicated that element RS1 encodes a replicon different from RS2.

2. Materials and methods

2.1. Strains and media

Bacterial strains and plasmids are described in Table 1. All strains were grown in LB medium and conserved at −70°C in the same medium supplemented with 20% glycerol. Antibiotics were added, when necessary, at the following concentrations: ampicillin (Amp) (100 μg ml^{-1}); kanamycin (Km) (50 μg ml^{-1}); and polymyxin B (PolB) (100 U ml^{-1}).

2.2. Polymerase chain reaction

The RS1 sequence of *V. cholerae* strain 81 was amplified using oligonucleotide UP (5′-CGCAGCAGACGAACTCTATGTC-3′) complementary to chromosomal DNA sequences located 101–122 nucleotides upstream from the 5′ terminus of RS1a (Fig. 1A) and oligonucleotide DOWN (5′-CACTTTGGTGCACACAATTGACG-3′) complementary to chromosomal DNA sequences located 165–187 nucleotides downstream from the 3′ terminus of RS1b (Fig. 1A) [3]. The reaction mixture consisted of chromosomal DNA, 500 ng; oligonucleotides, 300 nM; dNTPs, 200 μM; Vent DNA polymerase (New England Biolabs), 2 U; in a final volume of 100 μl. Amplification was achieved in 30 cycles of denaturation (1 min, 94°C), annealing (1 min, 56°C) and extension (4 min, 72°C).

2.3. Plasmid constructions and genetic techniques

The RS1 fragment amplified from strain 81 was cloned into the *Sma*I site of pUC19 to create plasmid pURS1. Plasmid pURSB6 was constructed by digestion of pURS1 with *Bgl*II, filling the overhanging strands with Klenow enzyme and insertion of a *Bam*HI octameric linker. Plasmids pGPRS16 and pGPRS61 (Fig. 1B) were constructed by subcloning *Kpn*I-*Sph*I fragments, containing the wild-type and

Table 1
Strains and plasmids

Strain	Description and remarks	References
E. coli		
MM294	*SupE44 hsdR endA1 pro thi,* host for general purpose cloning vectors	[18]
SY327λpir	Δ*(lac pro) argE(Am) rif nalA recA56 (λpirR6K)*, host for suicide vectors	[8]
SM10λpir	*thi thr leu tonA lacY supE recA::RP4-2Tc::Mu (λpirR6K)*, Km^r, host for suicide vectors with transfer functions integrated in chromosome	[8]
V. cholerae		
C7258	Wild type, O1, El Tor, Ogawa, Perú 1991	[9]
81	Δ*(cep orfU ace zot ctxA ctxB)* mutant from C7258	[9]
Peru-15	Δ*(cep orfU ace zot ctxA ctxB* RS) *recA::ctxB* mutant from C6709	[13]
Plasmids		
pUJΔEV	Amp^r, *Xba*I-*Eco*RV fragment containing part of *ctxA* , *ctxB* and the RS1 region 5′ to the *Eco*RV site cloned in pUC19	[9]
pURS1	Amp^r; RS1 fragment amplified from 81 cloned in *Sma*I site of pUC19	This study
pURSB6	Amp^r; *Bam*HI linker insertion in blunted *Bgl*II site of pURS1	This study
pGP704	Amp^r; suicide vector containing *ori* R6K and mobRP4 (mobilization) genes	[8]
pGPRS16	Amp^r; *Kpn*I-*Sph*I fragment from pURS1 containing RS1 sequence, cloned in pGP704	This study
pGPRS61	Amp^r; *Kpn*I-*Sph*I fragment from pURB6 cloned in pGP704	This study
pGPRS5	Amp^r; RS1 deleted of sequences downstream of the *Eco*RV site, cloned in pGP704	This study
pGPRS8	Amp^r; RS1 deleted of sequences upstream of the *Eco*RV site, cloned in pGP704	This study

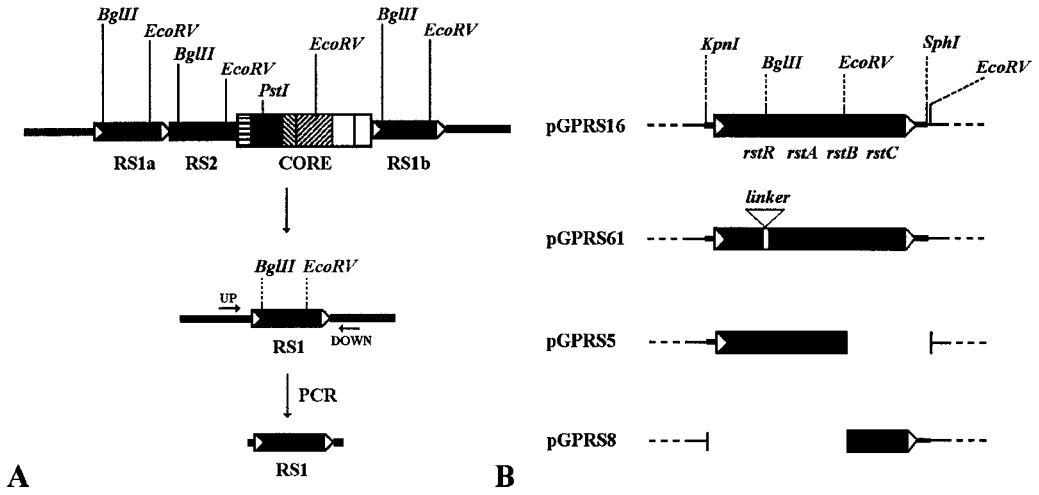

Fig. 1. A: Structure of CTXΦ prophage in wild type *V. cholerae* and amplification of the RS element from the core deleted recombinant 81. The following features are displayed: solid boxes, RS elements; non-filled arrow heads, ER sites; horizontal-lined box, *cep*; shaded box, *orfU*; right-slanted box, *ace*; left-slanted box, *zot*; non-filled boxes, cholera toxin A (*ctxA*) and B (*ctxB*) subunits. PCR primers UP and DOWN are indicated with arrows. B: Structure of suicide vectors containing the whole RS1 element, a linker insertion mutant and deletions. The RS1 fragments cloned in each vector are aligned so that empty regions indicate deletions. A solid line represents *V. cholerae* flanking chromosomal DNA. The interrupted line indicates pGP704 sequences.

frame-shifted RS1 from pURS1 and pURSB6, respectively, in pGP704 [8]. Plasmid pGPRS5 (Fig. 1B) was constructed by deletion of an *Eco*RV fragment from pGPRS16. Plasmid pGPRS8 (Fig. 1B) was constructed by subcloning an *Eco*RV-*Sph*I fragment from pURS1, containing the RS1 3′ terminus, in suicide vector pGP704. All derivatives of vector pGP704 were transformed into *E. coli* SY327λpir [8] and the correct clones purified and transferred to *E. coli* SM10λpir [8] for mobilization to *V. cholerae*. Construction of plasmid pUJΔEV is described elsewhere [9]. This plasmid contained the DNA sequence located within the conserved *Xba*I site in *ctxA* and the *Eco*RV site in the downstream RS element cloned in pUC19 [9]. Chromosomal DNA of *V. cholerae* exconjugants was prepared as previously described [10] and characterized by Southern blot hybridization analysis using a DIG-DNA labeling and detection kit from Boehringer Mannheim. *E. coli* was transformed by electroporation [11] and plasmid DNA purified by alkaline extraction [12]. DNA restriction and modification enzymes were used according to manufacturers' instructions (Boehringer Mannheim and New England Biolabs).

2.4. DNA sequencing

DNA sequencing was performed using the T7 Sequenase 7-deaza-dGTP sequencing kit (Amersham, Life Science). Restriction fragments of the amplicon contained in pURS1 were subcloned in pUC19 to facilitate DNA sequencing from internal restriction sites (*Bgl*II, *Eco*RV, *Sac*I) and pUC19 junctions using the universal direct and reverse sequencing primers. DNA sequencing was further extended from these sites using synthethic sequencing primers based on determined sequences.

2.5. Filter mating experiments

The appropriate *E. coli* SM10λpir donor strain was mixed with 10^9 cells of receptor strains 81 [8] or Peru-15 [13] at a donor-to-recipient ratio of 2:1. The mixture was concentrated on the surface of a membrane filter and incubated 4 h on LB agar at 37°C. The cells on the filters were harvested in LB medium, standardized by dilution to an optical density (600 nm) of 0.01, and aliquots plated on LB-Amp-PolB (transconjugants count) and LB-PolB (recipient count). The transfer frequency is reported as transconjugants per recipient.

3. Results and discussion

3.1. Cloning of Vibrio cholerae RS1 element

Vibrio cholerae 81 was used to clone the RS1 element. This strain was derived from El Tor biotype C7258 by insertion of a suicide vector encoding Amp^r in the core region of the CTX element and subsequent selection for Amp^s recombinants [9]. In strain C7258 plasmid pUJΔEV hybridized with two 17.0-kb and 7.5-kb *Pst*I fragments (Fig. 2A, lane 2) indicating the existence of repeated sequences located upstream and downstream from the *Pst*I site (Fig. 1A). In contrast, pUJΔEV hybridized to a single 20-kb *Pst*I fragment in strain 81 (Fig. 2A, lane 3). This result suggests that deletion of the core region in 81 was due to recombination between RS elements (Fig. 1A). This conclusion was ascertained by amplification of the putative RS element remaining in the chromosome of strain 81. The 3.0-kb amplified product (Fig. 2B, lane 2) was cut by *Ban*II, *Bgl*II, *Not*I and *Eco*RV that recognize conserved sites in RS1. The amplicon hybridized to the same *Pst*I fragments as pUJΔEV in Southern blots (data not shown). The identity of the amplified fragment was confirmed by DNA sequencing. Nucleotides 146–398 of the amplification product overlapped nucleotides 1768–2020 and 1–252 of sequences X00171 and U83795, respectively, reported by Mekalanos et al. [7,14]. This region includes the upstream 18-bp end repeat (ER) identical to *attRS* reported in [3]. The nucleotide sequence of the insert cloned in plasmid pGPRS8 (Fig. 4) corresponding to the 3′ end the amplification product overlapped nucleotides 1860–2724 of the RS1 sequence (U83795) reported in [7].

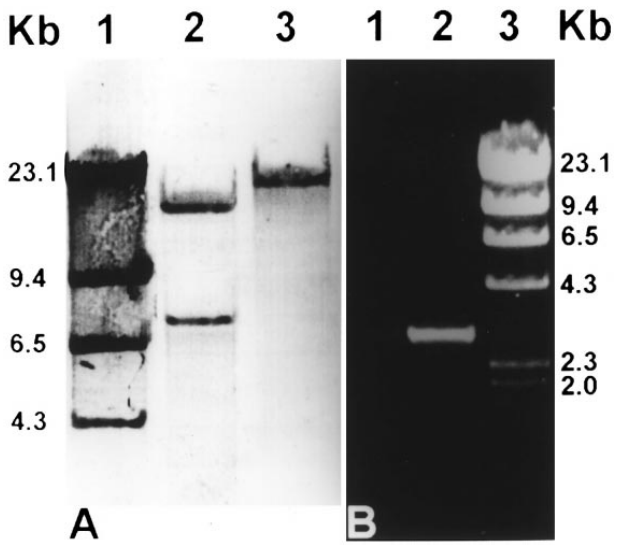

Fig. 2. A: Southern blot of DNA from strains C7258 and 81 hybridized with pUJΔEV. Lane 1: λ*Hin*dIII molecular weight marker; lane 2: DNA from strain C7258 digested with *Pst*I; lane 3: DNA from strain 81 digested with *Pst*I. B: Agarose gel electrophoresis of PCR amplification products. Lane 1: Peru-15 DNA template; lane 2: strain 81 DNA template; lane 3: λ*Hin*dIII molecular weight marker.

3.2. Transfer of a suicide vector containing RS1 fragments to Vibrio cholerae

We selected the vaccine strain Peru-15 to study the replicative function of RS1 because it is *recA*$^-$ and lacks the chromosomal RS element encoding the *attRS*-mediated site-specific recombination system [13] (Fig. 2B, lane 1). The RS1 element amplified from strain 81 was cloned in suicide vector pGP704 to obtain pGPRS16 (Fig. 1B). Suicide vector pGP704 and its derivatives contain the R6K origin of replication (*ori*) that requires the π protein encoded by the *pir* gene [8]. The π protein is supplied *in trans* in *E. coli* hosts by prophage λpir [8]. Since *V. cholerae* does not contain the *pir* gene, pGP704 based vectors can only transform this bacterium by homologous recombination with the bacterial chromosome to form an Amp^r co-integrate. We have observed that this vector can integrate to unspecified chromosomal sites with a low frequency (Table 2; Fig. 3, lanes 7, 10, 13). Transfer frequency is increased by insertion of homologous sequences or sequences promoting autonomous replication. Previous researchers reported higher transfer frequencies [3] than the ones reported in this study for strain 81 (Table 2). These differences can be explained by the use of different strains and a distinct mating procedure. In our studies the mating mixture was plated directly while in the previous work [3] it was diluted in fresh medium and grown for 20 generations prior to plating.

The RS1 insert in pGPRS16 increased the transfer frequency to Peru-15 1000-fold in comparison to pGP704 (Table 2). Total DNA from transconjugants was digested with an enzyme cutting once in the plasmid (*Sph*I). Appearance of a single 6.7-kb band (probe: pGP704), of size identical to linear plasmid,

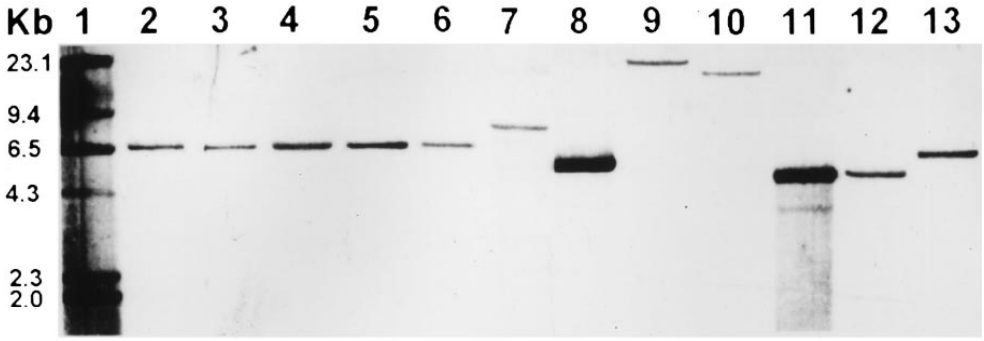

Fig. 3. Southern hybridization patterns of *V. cholerae* exconjugants. Lane 1: λ*Hin*dIII molecular weight marker; lane 2: pGPRS16 digested with *Sph*I; lane 3: DNA from 81-pGPRS16 digested with *Sph*I; lane 4: DNA from Peru-15-pGPRS16 digested with *Sph*I; lane 5: pGPRS61 digested with *Sph*I; lane 6: DNA from 81-pGPRS61 digested with *Sph*I; lane 7: DNA from Peru-15-pGPRS61digested with *Sph*I; lane 8: pGPRS5 digested with *Xba*I; lane 9: DNA from 81-pGPRS5 digested with *Xba*I; lane 10: DNA from Peru-15-pGPRS5 digested with *Xba*I; lane 11: pGPRS8 digested with *Sph*I; lane 12: DNA from 81-pGPRS8 digested with *Sph*I; lane 13: DNA from Peru-15-pGPRS8 digested with *Sph*I. The banding pattern in each lane is representative of at least five independent exconjugants.

was observed in all transconjugants analyzed indicating autonomous replication (Fig. 3, lane 4). The same plasmid was transferred to strain 81 with a frequency two orders of magnitude higher than pGP704 (Table 2). In all transconjugants analyzed by Southern blot hybridization, plasmid pGPRS16 was autonomously replicating (Fig. 3, lane 3). Plasmid DNA isolated from these transconjugants by alkaline extraction transformed *E. coli* SY327 to Amp^r (data not shown). These observations suggest that element RS1 contains an *ori* that functions in *V. cholerae* to promote autonomous replication of pGP704.

Plasmid pGPRS61 contains a linker insertion in the unique *Bgl*II site of RS1. DNA sequencing confirmed that the linker insertion interrupted an ORF identified as *rstR* [5,15]. This insertion reduced 1000-fold the transfer frequency to Peru-15 (Table 2). Southern blot hybridization of transconjugants showed that plasmid pGPRS61 integrated in Peru-15 at different chromosomal sites (Fig. 3, lane 7). The above result suggests that the drop in transfer frequency resulted from the loss of vector capacity for autonomous replication. The insertion mutation in plasmid pGPRS61 did not affect transfer to strain 81. In this case, all transconjugants analyzed by Southern blot hybridization contained autonomously replicating pGPRS61 (Fig. 3, lane 6). We suggest that strain 81 produces a factor that complements, alleviates or bypasses the mutation created in pGPRS61. A plausible candidate factor could be a protein required *in trans* for autonomous replication provided by the RS1 element in the chromosome. In element RS2, RstR has been proposed to repress transcription of *rstA2* which in turn encodes a protein required for replication of CTXΦ [7]. The repressor role of RstR was evidenced by its capacity to diminish expression of an *rstA*2-*lacZ* fusion in *E. coli* [7]. The negative replication phenotype of pGPRS61 in Peru-15 suggests a different role for RstR in RS1-mediated replication. The products of *rstR* and/or *rstA*1 could posses dual regulatory functions as reported for RepA in plasmid pSC101 [15].

Table 2
Transfer of suicide vectors with cloned RS1 sequences to *V. cholerae*

Plasmid	Transfer frequency to[a]
Peru-15	
pGP704	5.3×10^{-7}
pGPRS16	4.7×10^{-4}
pGPRS61	4.0×10^{-7}
pGPRS5	6.1×10^{-7}
pGPRS8	9.0×10^{-7}
81	
pGP704	3.0×10^{-7}
pGPRS16	5.8×10^{-5}
pGPRS61	6.9×10^{-5}
pGPRS5	3.0×10^{-6}
pGPRS8	2.3×10^{-5}

[a]Transfer frequency is expressed as the number of Amp-resistant transconjugants per total count of recipient vibrios at the end of mating.

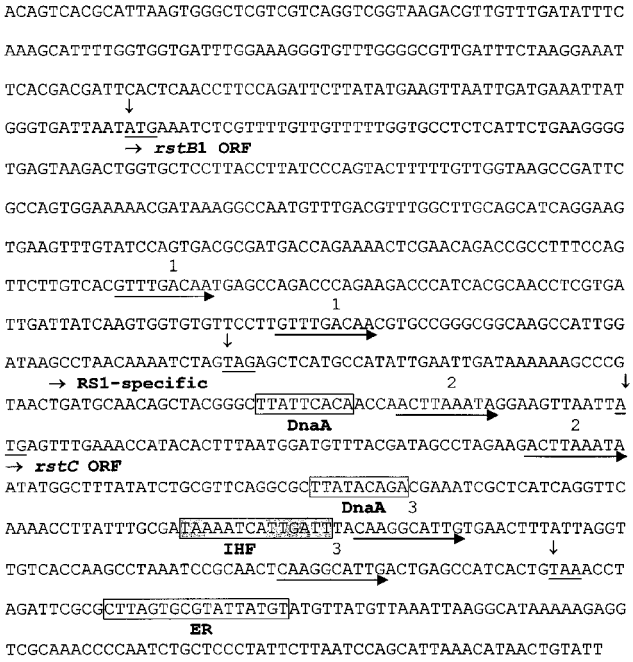

Fig. 4. DNA sequence of the RS1 fragment cloned in plasmid pGPRS8. Direct repeats are indicated with arrows. These repeats occur in three clusters which are numbered. The start and stop codons of ORFs *rstB*1 and *rstC* are underlined and marked with a vertical arrow.

In plasmid pGPRS5 the region downstream from the *Eco*RV site in RS1 was deleted (Fig. 1B). This deletion reduced the transfer frequency to Peru-15 1000-fold (Table 2). As in pGPRS61, hybridization analysis of total DNA purified from transconjugants indicated that the plasmid integrated into the chromosome (Fig. 3, lane 10). The deletion in pGPRS5 diminished to a lesser extent transfer to strain 81 (Table 2). All exconjugants of strain 81 examined showed the same 21.4-kb *Xba*I band indicating that pGPRS5 integrated into the same chromosomal site (Fig. 3, lane 9). This result indicates that, as in Peru-15, plasmid pGPRS5 cannot replicate in strain 81 but retains the capacity to integrate by homologous or site specific recombination in this host. Thus, the deletion in pGPRS5 removed RS1 sequences required for autonomous replication that are not complemented by the RS1 in the chromosome. DNA sequencing (Fig. 4) showed that this deletion removed the *rstB*1 and *rstC* ORF. The homologous *rstB2* ORF and *rstC* are not required for RS2-promoted replication [7]. Therefore, our results strongly suggest that RS1 encodes a distinct replicon not involved in phage replication.

Plasmid pGPRS8 contains the putative *cis*-acting elements, deleted in pGPRS5, subcloned in pGP704. This vector was mobilized to strain 81, with a frequency similar to that of pGPRS16. Southern blot hybridization studies (Fig. 3A, lane 12) revealed that pGPRS8 was autonomously replicating in this strain. The transfer frequency of this plasmid to Peru-15 was as low as pGP704 (Table 2).

Previous studies failed to detect autonomous replication of suicide vector pGP704 containing cloned RS2/RS1 sequences in *attRS* strains [3,7]. In the present study plasmids pGPRS16, pGPRS61 and pGPRS8 autonomously replicated and could be recovered from strain 81 that contains a chromosomal RS1.

3.3. DNA sequence analysis of the RS1 fragment cloned in pGPRS8

Plasmid replication control in Gram-negative bacteria can be RNA-regulated (ColE1, IncFII), iteron-regulated (pSC101, R6K, F, P1 etc.) or follow in essential details the rolling circle pattern of the single stranded coliphages [16]. The DNA sequence of the RS1 insert cloned in pGPRS8 is shown in Fig. 4. This region is particularly A-T rich (60% A+T). Analysis of the nucleotide sequence revealed the presence of three clusters of two direct repeats each. The first two clusters had 9-bp repeats while the third contained 10-bp repeats. Direct repeats within each cluster were regularly spaced by 50–71 base pairs. The spacing within clusters varied from 100 to 125 nucleotides. This structure is reminiscent of the iteron-regulated replication origins [15,16]. Additionally, several consensus sequences for binding of host proteins were found. These consensus sequences include two DnaA boxes adjacent to a putative integration host factor (IHF) binding site [17]. IHF is known to mediate integrative recombination of phage λ and to enhance replication of f1 filamentous phage [17]. All these elements are located in a region of RS1 not contained in RS2 and upstream from the 18-bp end repeat. Since the RS2 is sufficient for integration and replication of CTXΦ [15], the function of this region is not clear. The important differences between RS1- and RS2-medi-

ated replication of a suicide vector indicate that RS1 encodes an alternative mode of replication. A possible role for this region in the choleragenic vibrio could be to mediate replicative recombination events associated with tandem amplification of the CTX element in the chromosome [4].

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