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CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor

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ABSTRACT **In *Vibrio cholerae*, the genes encoding cholera toxin (*ctxAB*) are located on a segment of DNA (termed the "core" region) that is flanked by two or more copies of a repeated sequence called RS1. Together these DNA units comprise the CTX genetic element. Evidence presented here suggests that RS1 sequences encode a site-specific recombination system, which allows integration of a suicide plasmid carrying RS1 into an 18-base-pair sequence (attRS1) located on the chromosome of nontoxigenic *V. cholerae* strains. Strains of *V. cholerae* with large deletions removing attRS1 and the entire CTX genetic element no longer undergo site-specific recombination with the RS1 sequence. Additionally, these deletion strains show a defect in intestinal colonization. Recombination experiments localize the gene responsible for enhancing colonization to a portion of the core region of the CTX element. The identified gene encodes a peptide that is highly similar in amino acid sequence to the flexible pilin of *Aeromonas hydrophila*. These results have important implications in the construction of stable, live attenuated cholera vaccines.**

The bacterium *Vibrio cholerae* is the causative agent of cholera, a diarrheal disease that has recently caused an explosive epidemic in Latin America (1). Although this organism has been studied for over 100 years, there still remains a need for an effective cholera vaccine. Recent efforts in developing live attenuated vaccines have focused on the construction of recombinant *V. cholerae* strains lacking the gene (*ctxA*) encoding the active subunit of cholera toxin (2–5). Since recombination with wild-type strains could restore toxigenicity to *ctxA* deletion strains, *recA* mutations have been examined as a means of stabilizing such live vaccine candidates (6, 7).

Naturally occurring strains of *V. cholerae* that carry duplications of the *ctx* genes also have duplications of flanking DNA sequences, whereas nontoxigenic strains lack both *ctx* genes and flanking sequences (2, 8, 9). These observations suggest that the toxin genes are part of a larger genetic element (2, 8, 9). In *V. cholerae* strains of the El Tor biotype, this genetic element has been found to consist of a "core" region, which carries the *ctxAB* operon (10) and the *zot* gene encoding zona occludens toxin (11), flanked by two or more copies of a 2.7-kb repetitive sequence called RS1. Thus, the *ctx* genetic element is structurally analogous to compound transposons such as Tn*9* (12) with RS1 sequences corresponding to IS*1* insertion sequences directly duplicated at the termini. Unlike most transposons, the *ctx* genetic element appears to be located at the same chromosomal site in all El Tor strains (8), suggesting it may display a high degree of insertion site specificity, as do transposons Tn*7* (13) and Tn*554* (14).

In this paper we show that the RS1 sequences associated with the CTX genetic element apparently mediate *recA*-independent site-specific recombination between the element and an 18-bp target site on the *V. cholerae* chromosome called attRS1. In addition to this recombination system, we also show that the CTX element encodes a colonization factor distinct from cholera toxin. We have constructed *V. cholerae* strains that lack attRS1 and the entire CTX genetic element. Such strains no longer recombine with RS1 sequences and are thus promising candidates for the preparation of safer live attenuated cholera vaccines.

METHODS

Bacterial Strains and Genetic Methods. *V. cholerae* and *Escherichia coli* K-12 strains were stored at −70°C in LB medium containing 20% (vol/vol) glycerol (15). *V. cholerae* 2740-80 is a nontoxinogenic El Tor isolate from the Gulf Coast of the United States (16); *V. cholerae* E7946 and P27459 are toxigenic El Tor strains isolated from cholera patients in Bahrain and Bangladesh, respectively (8). *V. cholerae* strains GP1, SM115, and SM44 have been described (10). Standard methods were used for the isolation and manipulation of plasmid DNA (17) and the construction of cosmid libraries (18). Derivatives of plasmid pJM703.1 were maintained in *E. coli* SM10 λpir, which was also used to mobilize pJM703.1 derivatives into *V. cholerae* recipient strains by conjugation (19). Quantitative pGP52 and pGP88 conjugative matings were performed in LB broth by mixing the appropriate *E. coli* SM10 λpir donor strain with streptomycin-resistant *V. cholerae* recipients at a starting density of $\approx 10^8$ cells per ml and a donor-to-recipient ratio of 1:1. After 3 hr at 37°C the culture was diluted in fresh LB broth containing 100 μg of streptomycin per ml (LB-Sm) and grown for 20 generations to select against the *E. coli* donor. The resulting culture was plated on LB-Sm agar containing 50 μg of ampicillin per ml to select transconjugants. The transfer frequency is reported as the ratio of transconjugants to initial donor cells.

DNA Sequencing and Polymerase Chain Reaction (PCR). DNA sequencing was performed by the Sanger dideoxy chain-termination procedure (20) using either Klenow fragment of DNA polymerase (Amersham) or Sequenase (United States Biochemical) according to the protocols supplied by the manufacturers. The DNA sequence of the attRS1 site was determined from *V. cholerae* 2740-80 chromosomal DNA by PCR sequencing as follows. Two oligonucleotide primers were synthesized on an Applied Biosystems 381A DNA synthesizer. Oligonucleotide UP (5′-CGCAGCAGACGAACTCTATGTC-3′) is complementary to the chromosomal DNA sequence located 101–122 nucleotides from the left end of RS1a (Fig. 1*A*). Oligonucleotide DOWN (5′-CACTTTGGTGCACACAATTGACG-3′) is complementary to the chromosomal DNA sequence located 165–187 nucleotides from the right end of RS1c (Fig. 1*A*). DNA from *V. cholerae* strain 2740-80 was amplified by PCR using a Perkin–Elmer/Cetus thermal DNA cycler and GeneAmp DNA amplification kit according to the procedure of Shyamala and Ames (21).

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Abbreviation: ER, end repeat.

Briefly, 1 μg of 2740-80 DNA was amplified through 30 cycles with oligonucleotide UP at 0.6 M and oligonucleotide DOWN at 0.012 M, thus producing a product in which the strand primed by oligonucleotide UP was in 50-fold excess. Similar reactions were carried out with oligonucleotide DOWN in excess. The DNA sequence was then determined by utilizing one-third of each crude product as DNA template in the standard Sequenase protocol with the limiting oligonucleotide as the primer.

Animal Studies. The competition assay of Freter *et al.* (22) was performed as described with minor modifications (15). The mice were inoculated orally with 5×10^4 colony-forming units of each test strain. The ratio of the two strains was determined 24 hr later by plating homogenates of the intestine on LB agar containing antibiotics to differentiate one strain from the other or by colony hybridization with appropriate DNA probes.

RESULTS

RS Sequences and Site-Specific Recombination. The CTX genetic element is composed of a 4.5-kb core region flanked by copies of directly repeated sequences that were originally named RS1 (8). Due to the divergence between repeated sequences, we refer to the 2.7-kb sequence as RS1 and the 2.4-kb sequence as RS2 and refer to them generically as "RS" sequences (Fig. 1). All RS sequences contain *Bgl* II and *Eco*RV sites; the end closest to the *Bgl* II site corresponds to the left end of the element in each case.

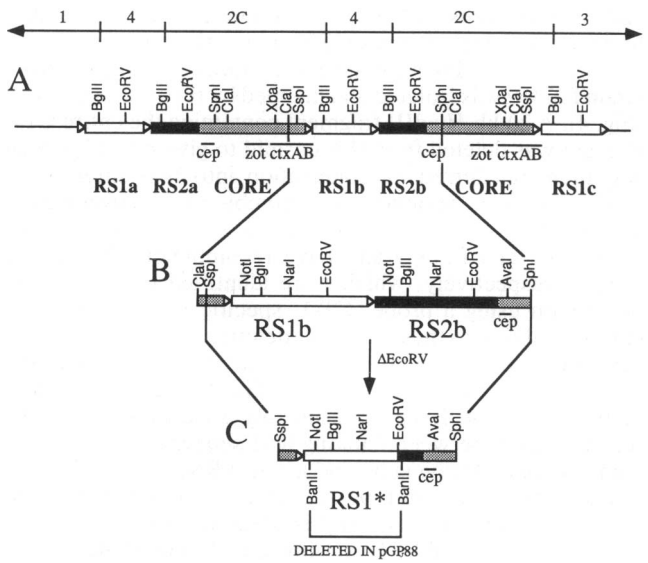

FIG. 1. Restriction map of the E7946 CTX region and construction of plasmid subclones. (*A*) Schematic representation of the duplicated CTX genetic element from El Tor strain E7946. Open boxes, RS1 sequences; filled boxes, RS2; shaded boxes, core sequences. The positions of end repeat (ER) sequences are shown by open arrowheads and *V. cholerae* DNA flanking the *ctx* genetic element is represented by thin lines. The approximate positions of the two copies each of the *ctxAB* operon and the *zot* and *cep* genes are shown by bars below the two core sequences. Above the schematic, a doubleheaded arrow designates *Bgl* II restriction fragments that correspond to those labeled in the Southern blot shown in Fig. 2. (*B*) Restriction map of the insert in E7946 subclone pGP20. A *Cla* I–*Sph* I fragment derived from pGP7 (2) was subcloned into pBR322 to give pGP20. After deletion of the indicated *Eco*RV fragment from pGP20, the *Ssp* I–*Sph* I fragment shown was subcloned into pJM703.1 digested with *Nru* I and *Sph* I, yielding plasmid pGP52, which carries a hybrid RS sequence designated RS1*. (*C*) Restriction map of the insert in pGP52. Deletion of the indicated 1.7-kb *Ban* II fragment gave pGP88, which lacks sequences entirely internal to RS1*.

The RS sequences that flank the core of the CTX genetic element have previously been shown to be involved in the duplication and amplification of the core region (10). We wished to characterize the transposition and recombination properties of RS sequences. After several unsuccessful attempts to obtain evidence for transposition of an RS sequence in *E. coli*, we succeeded in developing an assay for RS-mediated recombinational events in *V. cholerae* strain 2740-80, a nontoxigenic isolate that is apparently clonally related to toxigenic isolates from the United States Gulf Coast (16). Chromosomal DNA from this strain failed to hybridize to *ctx*, RS, or core restriction fragment probes (9), suggesting that this strain lacks the entire CTX genetic element.

To test the ability of RS sequences to mediate recombination with the *V. cholerae* 2740-80 chromosome, we introduced into this strain a conditionally replication-defective plasmid, pGP52, which carries a single copy of a hybrid RS (termed RS1*). RS1* is flanked by core DNA and therefore lacks sequences derived from chromosomal DNA typically found adjacent to the CTX genetic element (Fig. 1). Conjugation was used to mobilize pGP52 from *E. coli* SM10 λpir into *V. cholerae* 2740-80 and ampicillin transconjugants were selected. Southern blot analysis of chromosomal DNA from 14 transconjugants showed that pGP52 had integrated into the chromosomes of these strains to produce two different structures, C and D (Fig. 2). Structure D was found in 12 of the transconjugants and structure C was found in 2. Structure D resulted from integration of a single copy of pGP52, whereas structure C resulted from the integration of two copies of pGP52 in tandem repeat. Southern blot and sequence analysis indicated that pGP52 had inserted into the same chromosomal site at which toxin genes are found in natural isolates of El Tor strains such as E7946 and that the recombination had taken place between this chromosomal site and the core-RS1* left end junction on pGP52 (Figs. 1*C* and 2).

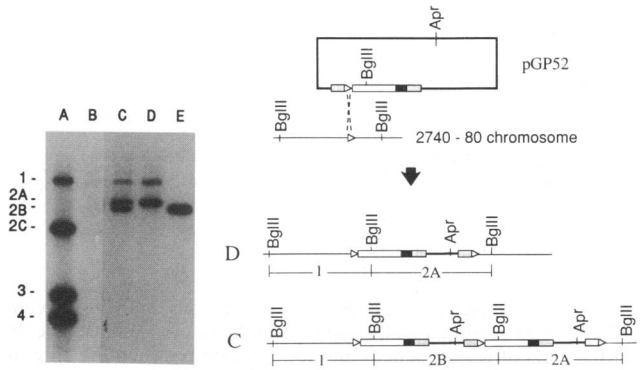

FIG. 2. Southern blot analysis of integration of pGP52 into the 2740-80 chromosome. *E. coli* SM10λpir pGP52 was used to transfer pGP52 to *V. cholerae* strain 2740-80. (*Left*) Autoradiograph of Southern blot analysis of DNA digested with *Bgl* II and hybridized with RS probe (equivalent to fragment 4 in Fig. 1*A*). Lanes: A, E7946; B, 2740-80; C, representative transconjugant with C structure; D, representative transconjugant with D structure; E, pGP52 plasmid DNA. The labels at the left correspond to the fragments labeled in Fig. 1*A* and below. (*Right*) Diagrammatic representation of the structures associated with the integration of pGP52. All of the transconjugants analyzed had integrated pGP52 at a chromosomal site (open arrowhead; attRS1) in which the CTX genetic element is naturally found in toxigenic El Tor strains. Structure D was produced by pGP52 integration taking place via recombination (dashed lines) between the single ER sequence present at the core-RS1* left end junction (represented by the open arrowhead; see also Fig. 1*C*). Structure D is similar to structure C except that two copies of pGP52 inserted tandemly. The fragments designated below each structure correspond to the labeled bands in the Southern blot analysis shown above. Ap^r denotes vector sequences.

The site on the *V. cholerae* 2740-80 chromosome into which pGP52 inserted was sequenced by amplifying this region from chromosomal DNA by PCR using oligonucleotide primers complementary to chromosomal sequences known to flank the element. As shown in Fig. 3 *Top*, the *V. cholerae* 2740-80 chromosomal DNA sequence determined overlaps with sequence of the left end of the CTX element and therefore the left end of RS1a. Comparison of this overlap shows that the 18-bp chromosomal sequence CCTTAGTGCGTATTATGT defines the integration target site. We have called this 18-bp chromosomal sequence attRS1 and called the identical 18-bp or similar 17-bp sequences located at the ends of RS1 "ERs" to distinguish them from attRS1 (Fig. 3 *Top*). Extensive sequence and hybridization analysis has shown that ER sequences exist at the junction between chromosomal DNA and the CTX element in all *V. cholerae* strains thus far examined and that ER sequences also mark the junction between tandem copies of RS1 and RS2 (Fig. 1). However, as shown in Fig. 3 *Middle*, the junction between the right end of RS2 and the core does not contain an ER sequence. Thus, pGP52 contains only one ER sequence and it is through this site that recombination with the chromosomal attRS1 occurs (Figs. 1 and 2).

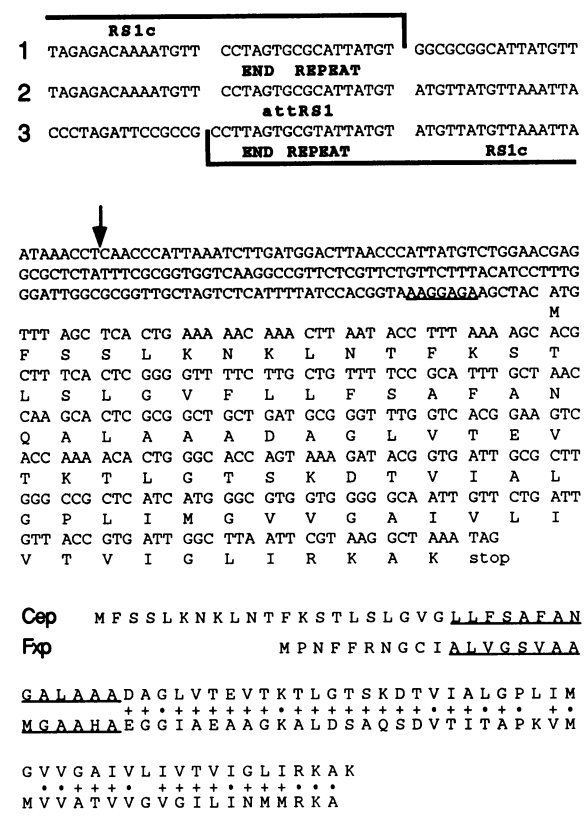

FIG. 3. Sequence analysis of RS junctions and *V. cholerae* 2740-80 chromosomal DNA. (*Top*) Alignment of sequences located at the ends of RS sequences and the junction between the chromosome. No. 1, sequence of upstream chromosomal DNA at the junction where it meets RS1a; no. 2, sequence of 2740-80 chromosomal DNA corresponding to the integration site of pGP52; no. 3, sequence of the junction where RS1c meets downstream chromosomal sequence. The overlap between these three sequences defines ERs located at the ends of RS sequences and attRS1 located on the *V. cholerae* 2740-80 chromosome. (*Middle*) Sequence of DNA defining the RS2b–core region junction. To the left of the indicated arrow the RS2b sequence was identical to sequences derived from other RS sequences (e.g., RS1c; not shown). To the right of this arrow the sequence diverges, indicating that this is the junction between RS2b and the left side of the core region. Downstream of this junction begins an open reading frame that is associated with a ribosome binding site (bar) that encodes the precursor to the peptide designated Cep. (*Bottom*) Deduced amino acid sequence of the Cep peptide and comparison to Fxp. Hydrophobic amino acid residues preceding the proposed processing sites are indicated by underlining. Subsequent lines of amino acid sequence show the mature Fxp peptide (23) aligned with the proposed processed form of the Cep peptide. Identical residues are noted by dots, whereas chemically similar amino acids are indicated by plus signs.

Quantitative measurements of integration frequency were made for pGP52 using our conjugative transfer/integration assay. We used as a control plasmid pGP88, a derivative of pGP52 that carries a 1.7-kb deletion within RS1* but retains the core–RS1 junction and the ER site through which recombination occurs. As shown in Table 1, pGP52 can integrate in the 2740-80 attRS1 site at a frequency at least 10^3-fold greater than pGP88. This result suggests that integration is not simply due to *recA*-dependent homologous recombination between the attRS1 sequence on the *V. cholerae* 2740-80 chromosome and the ER sequence on pGP52 but that it requires factors at least partially encoded by the *Ban* II fragment of RS1*. This conclusion was further supported by mobilizing pGP52 and pGP88 into a *recA*$^-$ derivative of 2740-80. Integration of pGP52 was decreased 10-fold but still occurred at a frequency that was at least 100-fold higher than pGP88 (Table 1). These data suggest that RS1 encodes a site-specific recombination system that can mediate *recA*-independent recombination between the attRS1 site and ER sequences.

Deletion of attRS1 and Its Effect on RS1-Mediated Recombination. To determine whether the chromosomal attRS1 site was the only target site for RS1-mediated recombination, we constructed strains that were deleted for all copies of attRS1 and ER sequences and tested these mutants for their ability to act as recipients in the pGP52 integration assay.

A cosmid (pGP60) carrying the cloned *ctx* region from strain P27459 was isolated and mapped by restriction analysis (Fig. 4). A 20-kb *Hin*dIII fragment containing the entire CTX element was deleted from this cosmid to give pAR62, which was then transferred by conjugation into two different *V. cholerae* strains. Spontaneous recombination between plasmid pAR62 and the chromosome of the two strains (E7947-Sm and P27459-Sm) gave recombinants Bah-2 and Bang-2, respectively, which were identified by colony hybridization using a probe, CT-1, specific for the *ctx* genes (10). In addition to these "attRS1 deletion mutants," we also separately obtained recombinants that had deleted only the core region of the CTX element. The "core deletion mutants" Bah-1 and Bang-1 presumably arise by spontaneous recombination between flanking RS1 sequences, thus deleting the core and leaving behind a single RS1 (ref. 10; Fig. 4). The structures of both types of deletion mutants were confirmed by Southern blot analysis (data not shown).

As shown in Table 1, strains Bah-1 and Bang-1 each functioned as recipients of pGP52 in our quantitative conjugative transfer/integration assay. Southern analysis of several representative transconjugants showed that pGP52 had integrated into the ER sequence located at the left end of the

Table 1. Transfer of pGP52 and pGP88 from *E. coli* into *V. cholerae* recipient strains

Donor	Recipient	attRS1	Transfer frequency
Sm10λpir pGP52	2740-80	Yes	1.8×10^{-5}
Sm10λpir pGP88	2740-80	Yes	$<2.5 \times 10^{-8}$
Sm10λpir pGP52	2740-80 *recA*	Yes	2.0×10^{-6}
Sm10λpir pGP88	2740-80 *recA*	Yes	$<2.5 \times 10^{-8}$
Sm10λpir pGP52	Bah-1	Yes	1.2×10^{-5}
Sm10λpir pGP52	Bah-2	No	$<2.5 \times 10^{-8}$
Sm10λpir pGP52	Bang-1	Yes	2.0×10^{-5}
Sm10λpir pGP52	Bang-2	No	$<2.5 \times 10^{-8}$

Transfer frequency is expressed as the number of ampicillin-resistant transconjugates per donor cell.

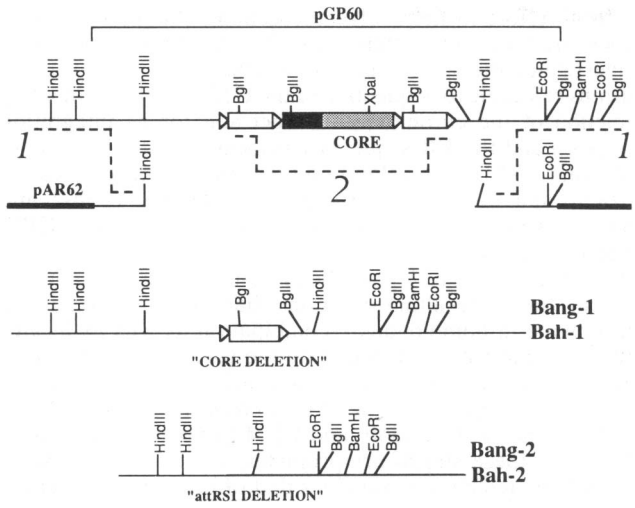

FIG. 4. Construction of *V. cholerae* core and attRS1 deletion strains. At the top is shown the restriction map of the P27459 chromosome in the region of the CTX genetic element (with RS1, RS2, and core sequences designated by open, filled, and shaded boxes, respectively). The structure of the E7946 chromosome is similar except for a tandem duplication of the CTX element as shown in Fig. 1. Copies of ER repeats are indicated by open arrowheads. The bracket above the map indicates a fragment from this region cloned in plasmid pGP60. As shown below the map, an internal *Hin*dIII fragment of pGP60 was deleted to give pAR62. Recombination between plasmid pAR62 and flanking chromosomal sequences (dashed line labeled 2) gave rise to attRS1 deletion strains Bang-2 and Bah-2. Recombination between flanking chromosomal RS1 sequences (dashed lines labeled 1) gave rise to core deletion strains Bang-1 and Bah-1.

RS1 element remaining on the chromosome of each of these core deletion strains. In contrast, strains Bah-2 and Bang-2 were completely defective as pGP52 recipients in this assay (Table 1). These results suggest that the chromosomal attRS1 and ER sequences are essential participants in the site-specific recombination reaction that integrates pGP52 and presumably the CTX genetic element into the chromosome.

The CTX Element Encodes a Colonization Factor. The availability of isogenic strains differing only in the presence or absence of the entire CTX element provided an opportunity to test the effect of this element on the intestinal colonization properties of *V. cholerae*. The relative ability of these strains to colonize the intestines of suckling mice was assessed by a competition assay in which mixtures of Ctx deletion mutants and their isogenic Ctx$^+$ parents were used to infect mice (15, 22).

As shown in Table 2, the competitive index of attRS1 deletion strains Bah-2 and Bang-2 indicated that these strains were approximately 5- to 13-fold reduced in colonization relative to isogenic strains containing the CTX element but lacking the *ctx* genes (strains SM115 and SM44, respectively). A similar intestinal colonization defect was noted for core deletion mutants Bah-1 and Bang-1, which showed a 14- to 16-fold reduction in colonization relative to their isogenic core-positive strains SM155 and SM44 (Table 1). Transduction of the CTX element back into Bah-1 and Bang-2 using bacteriophage CP-TI (24) gave strains GP16 and GP17, which again colonized 13- to 21-fold better than Bah-1 and Bang-1. These results suggest that a gene product associated with the core of the CTX genetic element stimulates intestinal colonization. Strains SM44, SM115, GP17, and GP16 all carry a deletion-insertion in the *ctxAB* operon (10), thus ruling out the possibility that the core-associated colonization factor is cholera toxin.

The DNA sequence analysis that defined the right end of RS2 and the left end of the core region indicated the presence of an open reading frame beginning with a typical ribosome binding site 153 bp within the core region (Fig. 3 *Middle*). The protein encoded by this sequence contains 82 amino acids and has what appears to be a typical hydrophobic signal sequence at its amino terminus. Removal of the signal sequence would yield a 47-residue peptide (Fig. 3 *Bottom*). We have isolated a Tn*phoA* fusion to this gene that gives rise to a fusion protein with alkaline phosphatase activity, indicating that the gene does encode a secreted product (25). The deduced amino acid sequence of the gene's processed product shows 91% similarity to the processed product of the *fxp* gene of *Aeromonas hydrophila* (ref. 26; Fig. 3 *Middle*). The 48-amino acid residue Fxp protein is the major subunit of a flexible pilus thought to be involved in adherence and colonization of the enteric pathogen *A. hydrophila* (23). These results suggest that a gene encoding a pilin-like subunit, which we now designate *cep* (*c*ore *e*ncoded *p*ilin), may be responsible for the enhanced intestinal colonization associated with the core region of the CTX genetic element.

Table 2. Intestinal colonization assay of attRS1 and core deletion derivatives of *V. cholerae*

Strain 1		Strain 2		Competitive index* (range)
Name	Relative genotype	Name	Relative genotype	
E7946 derivative†				
GP1	ΔctxABN4	SM115	ΔctxABN4 Kan^r	1.0 (0.1–1.2)
SM115	ΔctxABN4 Kan^r	Bah-2	attRS1Δ	13 (8.7–16)
SM115	ΔctxABN4 Kan^r	Bah-1	core Δ	16 (22–38)
GP16	ΔctxABN4 Kan^r	Bah-1	core Δ	13 (10–27)
JM1840	core Δ (pGP52)	Bah-1	core Δ	47 (14–77)
P27459 derivative†				
SM44	ΔctxABN4 Kan^r	Bang-2	attRS1Δ	5.1 (3.2–7.6)
SM44	ΔctxABN4 Kan^r	Bang-1	core Δ	14 (6.7–39)
GP17	ΔctxABN4 Kan^r	Bang-1	core Δ	21 (6.1–15)
GP688	core Δ (pGP52)	Bang-1	core Δ	11 (4.3–17)

*Change in the ratio of strain 1 relative to strain 2 after a mixed intestinal infection in 3- to 5-day-old suckling mice (15, 22). Values reported are the average of those determined in four to six mice with the range of values indicated in parentheses.

†Strains SM44 and SM115 carry an internal deletion in the *ctx* locus marked by an insertion that encodes kanamycin resistance (Kan^r) (10). Strain GP1 is a derivative of strain SM115 that carries the same *ctx* deletion but lacks the insertion encoding Kan^r. Strains GP16 and GP17 are CP-T1 transductants of Bah-1 and Bang-1 that have reacquired the ΔctxABN4 Kan^r allele from SM44 or SM115, respectively. Strain JM1840 and GP688 are derivatives of Bah-1 and Bang-1, respectively, that have a single copy of pGP52 integrated into the chromosome.

To test this possibility, we asked whether the *cep* gene alone could complement the colonization defect displayed by core deletion mutants. As shown in Fig. 1, the fragment cloned in plasmid pGP52 carries, in addition to RS1* sequences, a small portion of the core region that includes the *cep* gene. Thus, we integrated pGP52 into core deletion mutants Bah-1 and Bang-1 to produce isogenic transconjugants JM1840 and GP688, respectively, each carrying a single copy of the *cep* gene. Competition assays between core deletion mutants Bah-1, Bang-1, and their pGP52 derivatives were performed in infant mice. As shown in Table 2, the derivatives carrying pGP52 were able to colonize 11- to 47-fold better than their respective core deletion parental strains. These data suggest that the *cep* gene encodes the colonization factor associated with the core region of the CTX genetic element.

DISCUSSION

The natural conversion of toxigenic strains to toxigenicity via acquisition of genetic elements is a common theme in several bacterial species (27). In the case of diphtheria, at least two outbreaks have been shown to occur as a result of the conversion of geographically resident nontoxigenic strains to toxigenicity through the acquisition of a phage carrying the diphtheria toxin gene (28). The structure of the CTX element of *V. cholerae* is consistent with the idea that the *ctx* genes are also located on a mobile genetic element. Furthermore, the GC content and codon usage of the *ctx* genes are not consistent with a *V. cholerae* origin (29), suggesting that an as yet unidentified environmental organism may harbor and donate the CTX genetic element to nontoxinogenic strains of *V. cholerae*.

We believe the integration of plasmid pGP52 into the chromosome of *V. cholerae* 2740-80 serves as a valid model for understanding the transposition of the CTX genetic element. The conserved chromosomal location of the CTX genetic element in El Tor strains is consistent with the site-specific recombination we observed (8). We demonstrated here that the CTX element (tagged with a kanamycin resistance cassette) could be transferred into core deletion strains by transduction with phage CP-T1 (24). *V. cholerae* also possesses conjugative plasmids that can mobilize chromosomal and plasmid DNA at high efficiency (30). Thus, transfer of the CTX genetic element to nontoxigenic strains of *V. cholerae* can be envisioned to occur by various different mechanisms.

These results have important implications for the development of safe, live attenuated cholera vaccines. Current vaccine prototypes are attenuated typically by deletion of the *ctxA* gene encoding the active A subunit of cholera toxin. Since live vaccines will be tested and used in cholera endemic areas, it is highly likely that vaccine strains will come in contact with DNA-transfer-proficient, toxigenic *V. cholerae* strains in the environment or in the intestine of a vaccinee. The studies detailed above demonstrate that if this occurred, then the vaccine strain could regain wild-type cholera toxin genes along with any other virulence genes present on the CTX genetic element by a transposition event. In this regard, we have demonstrated here that the core region of the CTX element enhances colonization by a cholera toxin-independent mechanism. The gene responsible for this effect appears to be *cep*, which apparently encodes a peptide that is highly similar to the major subunit of a flexible pilus of *A. hydrophilia*. The *cep* gene may therefore encode a pilus-like structure that enhances colonization. The colonization-enhancing properties of the core region and cholera toxin (31) might be expected to drive the acquisition of the CTX element by nontoxigenic strains (naturally occurring and vaccine constructs) as well as promote the observed duplication and amplification of this element in toxigenic strains (8).

In an effort to help solve the genetic stability problems associated with live attenuated cholera vaccines, we have investigated the possibility of rendering *V. cholerae* vaccine strains refractile to acquisition of the CTX genetic element by deletion of the chromosomal target site for its integration (attRS1) and the ER sequences associated with the ends of RS1 and RS2 repeats. We found that the strains deleted for attRS1 and the entire CTX genetic element (Bah-2 and Bang-2) were completely deficient as recipients in the pGP52 integration assay (Table 1). Derivatives of these strains that are defective in *recA* have been constructed (unpublished observations), resulting in strains that lack the capacity to participate in homologous recombination as well as illegitimate recombination mediated by the CTX genetic element. Also, we have recently constructed analogous vaccine strains from a 1991 Peruvian isolate of *V. cholerae* (A.W., G.D.N.P., C. L. Gardel, J. C. Sadoff, and J.J.M., unpublished results). Together, these strains are promising candidates for use as live attenuated cholera vaccines that offer an unprecedented level of recombinational stability and safety.

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