

Novel Type of Specialized Transduction for CTX ϕ or Its Satellite Phage RS1 Mediated by Filamentous Phage VGJ ϕ in *Vibrio cholerae*

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The main virulence factor of *Vibrio cholerae*, the cholera toxin, is encoded by the *ctxAB* operon, which is contained in the genome of the lysogenic filamentous phage CTX ϕ . This phage transmits *ctxAB* genes between *V. cholerae* bacterial populations that express toxin-coregulated pilus (TCP), the CTX ϕ receptor. In investigating new forms of *ctxAB* transmission, we found that *V. cholerae* filamentous phage VGJ ϕ , which uses the mannose-sensitive hemagglutinin (MSHA) pilus as a receptor, transmits CTX ϕ or its satellite phage RS1 by an efficient and highly specific TCP-independent mechanism. This is a novel type of specialized transduction consisting in the site-specific cointegration of VGJ ϕ and CTX ϕ (or RS1) replicative forms to produce a single hybrid molecule, which generates a single-stranded DNA hybrid genome that is packaged into hybrid viral particles designated HybP ϕ (for the VGJ ϕ /CTX ϕ hybrid) and HybRS ϕ (for the VGJ ϕ /RS1 hybrid). The hybrid phages replicate by using the VGJ ϕ replicating functions and use the VGJ ϕ capsid, retaining the ability to infect via MSHA. The hybrid phages infect most tested strains more efficiently than CTX ϕ , even under *in vitro* optimal conditions for TCP expression. Infection and lysogenization with HybP ϕ revert the *V. cholerae* live attenuated vaccine strain 1333 to virulence. Our results reinforce that TCP is not indispensable for the acquisition of CTX ϕ . Thus, we discuss an alternative to the current accepted evolutionary model for the emergence of new toxigenic strains of *V. cholerae* and the importance of our findings for the development of an environmentally safer live attenuated cholera vaccine.

The filamentous phage CTX ϕ contains the *ctxAB* genes encoding cholera toxin (CT), the main virulence factor of the pathogenic gram-negative bacterium *Vibrio cholerae* (49). In toxigenic El Tor and O139 strains of *V. cholerae* CTX ϕ is integrated at the *dif* site in the bacterial genome arrayed in different tandem structures along with the related satellite phage RS1 (11, 39). The genome of RS1 is a short version of the genome of CTX ϕ , which contains genes encoding proteins needed for replication (RstA), integration (RstB), and regulation of gene expression (RstR and RstC) but lacks the genes encoding proteins needed for assembling and secretion of viral particles (Psh, Cep, pIII^{CTX}, Ace, and Zot), as well as CT, which is not necessary for phage morphogenesis (11). Thus, satellite phage RS1 can replicate autonomously but depends on its helper phage CTX ϕ for assembly and secretion and thereby for transmission of RS1 viral particles (11). Conversely, RS1 encodes the antirepressor RstC, which is not present in CTX ϕ (9). This protein promotes transcription of CTX ϕ and RS1 genes by counteracting the activity of the phage repressor RstR (9). Thus, RS1 enhances transmission of both CTX ϕ and itself by means of RstC antirepressor activity (9).

Classical strains of *V. cholerae* contain nonfunctional CTX ϕ prophages, whereas El Tor and O139 strains contain fully active prophages that produce infective CTX ϕ viral particles

(10). CTX ϕ site specifically integrates into the host chromosome by a process dependent on the host recombinases XerC and XerD, which ordinarily catalyze the resolution of chromosome dimers at the *dif* recombination site (25). Other filamentous phages such as f237 of *Vibrio parahaemolyticus*, ϕ Lf of *Xanthomonas campestris*, Xf ϕ 1 of *Xilella fastidiosa*, CUS ϕ -2 of *Yersinia pestis*, and VGJ ϕ of *V. cholerae* seem to exploit the XerCD recombination system to integrate into the chromosome of their hosts, suggesting that lysogenic filamentous phages are more common than initially thought (5, 25, 26).

CTX ϕ infects *V. cholerae* through toxin-coregulated pilus (TCP) (49), a type IV pilus essential for intestinal colonization (47) that is encoded by a gene cluster contained in the *V. cholerae* pathogenicity island (VPI) (33). Although VPI seems to move horizontally between bacterial populations of *V. cholerae*, the transfer mechanism is still controversial. Karaolis et al. presented data suggesting that VPI is the prophage state of another filamentous phage, VPI ϕ , which they thought to use TcpA as major capsid protein (34); however, this hypothesis has raised several unanswered questions (11), and the results from that study could not be reproduced by other researchers (16). Perhaps VPI transmission is mediated by several mechanisms that follow different pathways; for example, O'Shea and Boyd have found that VPI can be mobilized by the generalized transducing phage CP-T1 (43); however, the main mechanism accounting for VPI transmission probably has not been discovered yet. Whatever the mechanism, VPI, carrying the CTX ϕ receptor, also has the ability to move horizontally between bacterial populations, providing CTX ϕ with the advantage of

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amplifying its host range when VPI moves toward new TCP-negative strains.

An evolutionary model for the origin of pathogenic *V. cholerae* has been proposed in which this bacterium first acquired the *tcp* operon and then TCP-producing strains were infected and lysogenized by CTX ϕ (2, 11, 17, 18, 49). However, this model has been impugned by the isolation of several strains of *V. cholerae* (both O1 and non-O1) that lack TCP genes but contain the CTX ϕ prophage (20, 45). It has been suggested that such strains arose by the mentioned model with a subsequent loss of the VPI (17, 18). Another possibility is that such strains have alternative CTX ϕ receptors (2, 3), but filamentous phages that use more than one receptor have not been described. However, CTX ϕ can infect *V. cholerae* in a TCP-independent fashion that requires the TolQ, TolR, and TolA proteins, but the efficiency of infection by this mechanism is quite low and needs direct cell-cell contact (23). Boyd and Waldor demonstrated that a specialized receptor such as TCP is not always essential for acquisition of CTX ϕ , since these authors showed that *V. cholerae* generalized transducing bacteriophage CP-T1 can transfer the whole CTX ϕ genome toward TCP-negative strains of *V. cholerae* (3). However, the presence of TCP-devoid, non-O1, non-O139 strains of *V. cholerae* that contain CTX ϕ prophages cannot be explained by this mechanism, since CP-T1 only infects strains belonging to the O1 serogroup (21).

We recently described a *V. cholerae*-specific filamentous phage, VGJ ϕ , which infects host cells through the mannose-sensitive hemagglutinin (MSHA) pilus and that is able to integrate its genome at the same *attB* chromosomal site as CTX ϕ (5). We describe here a new type of specialized transduction mediated by VGJ ϕ through which CTX ϕ and its satellite phage RS1 are transmitted to new hosts in a TCP-independent fashion. We also discuss the potential implications of this alternative mechanism for the emergence of new toxigenic serotypes of *V. cholerae* and for the development of an environmentally safer live attenuated cholera vaccine.

MATERIALS AND METHODS

Phages, strains, and media. Bacterial strains and phages used in the present study are described in Table 1. Ordinarily, strains were grown in Luria-Bertani (LB) medium at 37°C. Kanamycin was added, when necessary, at 50 μ g/ml. The expression of MshA and TcpA proteins was analyzed in the following media: LB medium (pH 6.5), LB medium (pH 7.0), AKI medium (29), tryptone soy broth (TSB), TSB plus 2.5 g of glucose/liter (TSB_G), Dulbecco modified Eagle medium (DMEM; Sigma), and protein-free hybridoma medium (PFHM; Gibco-BRL).

Phage methods. Phage particles of VGJ ϕ , CTX-Kn ϕ , RS1-Kn ϕ , HybP-Kn ϕ , or HybRS-Kn ϕ were purified and concentrated as described in (5). For infection assays, phage donor strains were grown until they reached an optical density (OD) at 600 nm of 1.5. One-milliliter portions of the cultures were filtered through a 0.22- μ m-pore-size filter (Sartorius), and 50- μ l portions of the filtrates were plated on solid LB medium to check for sterility. Then, 100 μ l of pure and diluted cell-free culture supernatants of the donor strains were mixed with 20 μ l ($\sim 10^8$ cells) of a fresh culture of the receptor strains grown under optimal conditions for TCP expression. The mixture was incubated 20 min at room temperature to allow infection and plated on solid LB medium supplemented with kanamycin to count kanamycin-resistant (Kn^r) transductants. Alternatively, infected cells were grown in LB broth to purify phage particles or single-stranded DNA (ssDNA) according to Sambrook et al. (46).

DNA methods. Total DNA was prepared according to the method of Ausubel et al. (1). Plasmid DNA was prepared by using the WizardPlus SV System (Promega). DNA restriction and modification enzymes were used according to the manufacturer's instructions (Promega). Southern blot analyses were performed with the following digoxigenin (DIG)-labeled probes: a 643-bp fragment

containing part of *ctxAB* genes amplified by PCR with the primer pairs 5'-ATG ATCATGCAAGAGGAAGACTC-3' and 5'-AGGTGTTCATGTGCATATG C-3' was used as the *ctxAB*-specific probe; the 954-bp *SacI*-*EcoRI* fragment of VGJ ϕ RF, containing open reading frame 81 (ORF81), ORF44, ORF29, and part of ORF493, was used as the VGJ ϕ -specific probe (see the genome sequence of VGJ ϕ ; GenBank no. AY242528); the 564-bp *SacI*-*SphI* fragment containing the *rstC* gene from plasmid pURS1 (4) was used as the *rstC*-specific probe; and, finally, the 2.9-kb *EcoRI*-*PstI* fragment containing the RS1 element from pURS1 (4) was used as the RS-specific probe.

Strand-specific probes were generated by asymmetric PCR with HybP-Kn ϕ RF as the template. Primer CTB1 (5'-GCGATTGAAAGGATGAAGG-3'), hybridizing with the negative strand of CTX ϕ and inside *ctxB*, was used to generate strand-specific HybP-A probe and primer NJ2 (5'-TAGAACGTGTCATTGCA TCG-3'), hybridizing with the negative strand of VGJ ϕ and inside ORF136 (AY242528), was used to generate the complementary strand HybP-B probe. Nucleotides were added at the following final concentrations: 100 μ M dATP, 100 μ M dCTP, 100 μ M dGTP, 65 μ M dTTP, and 35 μ M DIG-dUTP. Amplification reactions were performed with the *Taq* bead hot-start polymerase system (Promega).

Sequences of the novel junctions between CTX ϕ and VGJ ϕ in HybP-Kn ϕ were obtained by sequencing its replicative form (RF) with primers CTB1 (see above) for the junction *att*-CTX/VGJ and NJ4 (5'-CCTGTAGAAATTCGTC TCC-3'), hybridizing inside the intergenic region I of CTX ϕ for the junction *att*-VGJ/CTX. Similarly, the sequence of the novel junctions between VGJ ϕ and RS1 in HybRS-Kn ϕ was obtained by sequencing its RF with the primers NJ5 (5'-CGCTCATCAGGTTCAAACC-3') for the *att*-RS1/VGJ junction and NJ4 for the *att*-VGJ/RS1 junction. Sequencing reactions were performed with the Thermo Sequenase Cy5 dye terminator kit and an ALFexpress DNA sequencer (Amersham Pharmacia Biotech).

Stability of HybP-Kn ϕ in the bacterial host. To evaluate the in vitro stability of HybP-Kn ϕ , *V. cholerae* 1333 infected with this phage [1333(HybP-Kn ϕ)] was inoculated into LB medium and grown without kanamycin selection until late stationary phase. The ratio of infected cells (Kn^r) to the total number of bacteria was determined every 2 h for 24 h. At this time, the integrity of HybP-Kn ϕ RF was studied by restriction analysis of plasmid DNA preparations from 12 independent Kn^r clones.

The in vivo stability of replicating or integrated HybP-Kn ϕ was evaluated in BALB/c suckling mice. Ten mice were orogastrically inoculated with 10^6 cells of 1333(HybP-Kn ϕ) or 1333-HybI2 (an integrant of HybP-Kn ϕ in 1333) and then incubated 24 h at 30°C without their mothers. The mice were then sacrificed; the small intestines were homogenized to recover colonizing vibrios, and the ratio of Kn^r cells to the total number of bacteria was determined.

Quantification of CT production. The ability of 1333(HybP-Kn ϕ) and 1333-HybI2 to produce CT was determined in AKI (29) and LB media by the G_{M1} ganglioside-dependent enzyme linked immunosorbent assay (G_{M1}-ELISA) (24) by using a standard curve of purified CT and the anti-CTA monoclonal antibody (MAb) 1G10G5.

Virulence evaluation of the HybP-Kn ϕ lysogen 1333-HybI2. Toxicity was evaluated by orogastric inoculation of groups of 15 BALB/c suckling mice with 10^6 cells of the strains 1333-HybI2, 1333 (negative control), and C6706 (positive control) diluted in 50 μ l of phosphate-buffered saline. Mice were fasted 4 h before and after inoculation and incubated for 6 days with their mothers. The survival was monitored daily during this lapse.

Protein methods. To determine the protein composition of the hybrid phage capsids, samples of HybP-Kn ϕ (10^8 particles), HybRS-Kn ϕ (10^8 particles), or VGJ ϕ as control (10^9 particles) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (1) with the MAb 2H1H9 specific for the major capsid protein of VGJ ϕ phage.

Expression of MshA and TcpA protein subunits was studied by growing the strains of interest in LB medium (pH 6.5, 30°C), LB medium (pH 7.0, 37°C), TSB (37°C), or TSB_G (37°C) with overnight shaking (240 rpm) and by the AKI procedure (29). For growth in DMEM or PFHM, starting suspensions of 10^6 bacteria/ml in each medium were incubated for 8 h static at 37°C in an atmosphere of 5% CO₂. Whole-cell lysates containing $\sim 2 \times 10^7$ bacteria grown in each condition were fractionated by SDS-PAGE and analyzed by Western blot with the anti-TcpA MAb 10E10E1 and the anti-MshA MAb 2F12F1 (15).

The nucleotide sequences of the novel junctions *att*CTX/VGJ, *att*VGJ/CTX, *att*RS1/VGJ, and *att*VGJ/RS1 have been deposited in the GenBank database under the accession numbers AY268047, AY268048, AY349613, and AY349614, respectively.

TABLE 1. *V. cholerae* strains and phages used in this study and susceptibility of *V. cholerae* strains to phages VGJ-Kn ϕ , HybP-Kn ϕ , and CTX-Kn ϕ

Strain ^a or phage	Description and remarks	Source, yr or reference ^c	Susceptibility to phage ^b :		
			VGJ-Kn ϕ	HybP-Kn ϕ	CTX-Kn ϕ
Strains					
569B	O1, classical, Inaba	Calcutta, India, 1945	+++	+++	+++
O395	O1, classical, Ogawa	Calcutta, India, 1964	+++	+++	++
1395	O1, classical, Inaba	CIEI	+++	+++	++
CA385	O1, classical, Ogawa	Calcutta, India, 1953	-	-	+
CA401	O1, classical, Inaba	Calcutta, India, 1953	+++	+++	+
CA-EM1	CA401, Δ <i>mshA</i>	CNIC	-	-	+
NIH35A3	O1, classical, Inaba	India, 1941	++	++	++
C6706	O1, El Tor, Inaba	Peru, 1992	+++	+++	-
KHT46	C6706 Δ <i>mshA1</i>	48	-	-	-
KHT52	C6706 Δ <i>tcpA10</i>	48	+++	+++	-
1333	C6706 Δ (CTX ϕ prophage), <i>hap::celA</i> , remnant RS1	CNIC	+++	+++	-
C7258	O1, El Tor, Ogawa: prophage structure: CTX ϕ /RS1	Peru, 1991	+++	+++	-
C72K7	C7258, CTX ϕ /RS1::Kn ^r (inserted in <i>NotI</i> site)	CNIC	NP	NP	NP
N16961	O1, El Tor, Inaba, prophage structure: CTX ϕ /RS1	Bangladesh, 1975	++	++	-
N16K38	N16961, RS1/CTX ϕ ::Kn ^r (inserted in <i>NotI</i> site)/RS1	CNIC	NP	NP	NP
Peru-15	C6709 Δ (CTX ϕ /RS1 prophages, <i>attRS1</i>), <i>rec4::ctxB</i>	35	+++	+++	+++
E7946	O1, El Tor, Ogawa	Bahrain, 1978	+++	+++	-
Lima	O1, El Tor, Inaba	Lima, Peru, 1991	+++	+++	-
3083	O1, El Tor, Ogawa	Viet Nam, 1964	+++	+++	-
CRC262	O139	Bangladesh, 2000	+++	+++	-
CRC266	O139	Bangladesh, 2000	+++	+++	-
Phages					
VGJ ϕ	Filamentous phage of <i>V. cholerae</i>	5	NA	NA	NA
VGJ-Kn ϕ	VGJ ϕ carrying a Kn ^r gene inserted into its unique <i>XbaI</i> site	5	NA	NA	NA
CTX-Kn ϕ	CTX ϕ carrying a Kn ^r gene inserted into its unique <i>NotI</i> site (produced by strain C72K7)	CNIC	NA	NA	NA
RS1-Kn ϕ	RS1 carrying a Kn ^r gene inserted into its unique <i>NotI</i> site (produced by strain N16K38)	CNIC	NA	NA	NA
HybP-Kn ϕ	Recombinant hybrid phage produced by site-specific recombination of CTX-Kn ϕ and VGJ ϕ RFs	This study	NA	NA	NA
HybRS-Kn ϕ	Recombinant hybrid phage produced by site-specific recombination of RS1-Kn ϕ and VGJ ϕ RFs	This study	NA	NA	NA

^a Receptor strains were grown under optimal conditions for expression of TCP, which was checked in parallel by Western blotting.

^b 569B was used as an indicator strain to adjust the titer of each phage suspension to 10⁶ Kn^r transducing particles (TP)/ml, and then 100 μ l of the adjusted suspension (10⁵ Kn^r TP) was used to infect 10⁸ cells of the receptor strains (multiplicity of infection of 10⁻³). + + +, Appearance of >10⁴ Kn^r transductants of the infected strain; + +, appearance of 10² to 10⁴ Kn^r transductants; +, appearance of <10² Kn^r transductants; -, no Kn^r CFU detected. NP, not performed; NA, not applicable. These results are from three independent experiments.

^c CIEI, Centro de Investigación de Enfermedades Infecciosas, Cuernavaca, Mexico; CNIC, Centro Nacional de Investigaciones Científicas, Havana, Cuba.

RESULTS

CTX ϕ transmission mediated by VGJ ϕ . The filamentous phage VGJ ϕ , recently isolated from the O139 *V. cholerae* strain SG25-1, enters the cell using as receptor the MSHA (5), a type IV pilus that also confers hemagglutinating capacity to cholera vibrios (22). VGJ ϕ integrates at the same chromosomal *attB* site as CTX ϕ , and the 17-bp functional core within the *attP* sites of both phages are almost identical (5). Thus, we hypothesized that since both phage RFs integrate at the same *attB* site, they could potentially integrate into each other by their respective *attP* sites if they coexisted in the same cell and that, if such a cointegrate were formed, it could be transduced into a new host cell and disseminated thereafter.

To test this hypothesis, 569B was infected with VGJ ϕ , CTX-Kn ϕ , or coinfecting with both phages. The coinfecting cells were grown under kanamycin selection to warrant the permanency of CTX-Kn ϕ , whereas VGJ ϕ was self-maintained because it is a very prolific phage and its receptor, MSHA, is constitutively expressed in 569B (see below). Infection assays were done with

cell-free culture supernatants of the classical biotype strain 569B infected with VGJ ϕ or CTX-Kn ϕ or coinfecting with both phages and by using as receptor strains the TCP mutant KHT52 (48), the isogenic MSHA mutant KHT46 (48), their parental strain C6706, the vaccine strain Peru-15, and the classical biotype strain 569B; the last three strains express both TCP and MSHA (Table 2). As expected, 569B infected with CTX-Kn ϕ transmitted this phage only to TCP-expressing strains not restricted by phage immunity (only to 569B and Peru-15 receptors in Table 2). Although KHT46 and C6706 strains expressed TCP, they were resistant to CTX ϕ because they contain CTX ϕ and RS1 prophages expressing RstR^{El Tor} repressor, which confers immunity to CTX ϕ ^{El Tor} infection (36). 569B infected with VGJ ϕ was able to transmit this phage to all strains, except the MSHA mutant KHT46, but this event of transfer never provided Kn^r to the transductants (Table 2). However, 569B coinfecting with CTX-Kn ϕ and VGJ ϕ transduced the Kn^r gene to all MSHA-expressing strains (KHT52, C6706, Peru-15, and 569B in Table 2). Thus, the Kn^r gene

TABLE 2. Transduction of CTX-Knϕ and RS1-Knϕ to different *V. cholerae* strains

Donor strain	No. of Kn ^r transductants of the receptor strains ^a :				
	KHT52 (<i>ΔcpA10</i>)	KHT46 (<i>ΔmshA1</i>)	C6706 (MSHA ⁺ TCP ⁺)	Peru-15 (MSHA ⁺ TCP ⁺)	569B (MSHA ⁺ TCP ⁺)
569B (CTX-Knϕ)	ND	ND ^b	ND ^b	9.85 × 10 ⁴	2.45 × 10 ⁵
569B (VGJϕ)	ND ^c	ND	ND ^c	ND ^c	ND ^c
569B (CTXKnϕ/VGJϕ)	1.22 × 10 ⁵	ND ^b	1.08 × 10 ⁵	8.36 × 10 ⁴	2.07 × 10 ⁵
569B (RS1-Knϕ)	ND	ND ^b	ND ^b	1.83 × 10 ⁵	2.15 × 10 ⁵
569B (RS1-Knϕ/VGJϕ)	5.04 × 10 ⁴	ND ^b	1.02 × 10 ⁵	2.35 × 10 ⁵	2.68 × 10 ⁵
C72K7	ND	ND ^b	ND ^b	8.90 × 10 ³	9.15 × 10 ³
C72K7 (VGJϕ)	5 ^d	ND ^b	21 ^d	8.90 × 10 ³	6.02 × 10 ⁴
N16K38	ND	ND ^b	ND ^b	7.85 × 10 ²	7.64 × 10 ²
N16K38 (VGJϕ)	3 ^d	ND ^b	9 ^d	1.06 × 10 ³	8.04 × 10 ²

^a That is, the number of Kn^r transductants appearing after exposure of 10⁸ receptor cells to 100 μl of cell-free culture supernatants of the donor. All numbers are average from at least three independent experiments. Receptor strains were grown under optimal conditions for TCP expression. ND, no Kn^r transductants detected.

^b Although the TCP is intact in KHT46 and C6706, these strains are not efficiently infected by CTX-Knϕ or RS1-Knϕ due to phage immunity provided by RstR^{EI} T_{OR} repressor present in these strains. Notice that Peru-15 and 569B lack immunity to CTX-Knϕ or RS1-Knϕ because Peru-15 does not contain any copy of the RstR repressor gene and 569B expresses RstR^{CI} repressor, which belongs to a phage immunity group (classical) different from the immunity group of the CTX-Knϕ used in this study (El Tor).

^c No Kn^r transductants were detected, but RFs were isolated from the same recipient strains grown without Kanamycin selection.

^d These colonies were determined to be transductants of HybP-Knϕ by Southern blot. A total of 10⁸ cells of the receptor strains plated in LBK did not produce any Kn^r spontaneous mutant.

originally carried by CTX-Knϕ was transduced to the TCP mutant KHT52 with great efficiency, acquiring the pattern of transmission of VGJϕ. Since KHT52 cannot produce TCP (the receptor of CTX-Knϕ), but normally assembles MSHA pilus (the receptor of VGJϕ), the appearance of KHT52 Kn^r transductants could only be explained by some kind of interaction between VGJϕ and CTX-Knϕ.

Plasmid DNA preparations from 24 Kn^r transductants of KHT52 from three independent experiments digested with *Eco*RI, which cuts once into VGJϕ RF but does not cut into CTX-Knϕ RF, produced two bands of ca. 15.7 and 7.5 kb, respectively (not shown). Analysis by Southern blot with two DIG-labeled probes specific for VGJϕ and CTXϕ showed that the smaller 7.5-kb band hybridized only with the VGJϕ-specific probe while the larger 15.7-kb band hybridized with both the VGJϕ- and the CTXϕ-specific probes (not shown). These results indicated the simultaneous presence of two plasmids: the VGJϕ RF of 7.5 kb and a larger plasmid whose size (15.7 kb) was compatible with a cointegrate structure comprising both VGJϕ and CTX-Knϕ RFs fused into a single recombinant molecule, thus supporting our initial hypothesis. We designated the larger new plasmid pHybP-Knϕ (from hybrid phage). Electroporation of these plasmid DNA preparations (containing both plasmids) into KHT46 allowed us to propagate and purify clones containing pHybP-Knϕ alone, giving final confirmation of its existence (VGJϕ was washed out due to lack of its receptor, MSHA, in this strain).

Restriction analysis of pHybP-Knϕ produced bands characteristic of CTXϕ or VGJϕ and new bands corresponding to the novel junctions between both phage DNAs (Fig. 1AI). In addition, pHybP-Knϕ hybridized with VGJϕ- and CTXϕ-specific probes in Southern blots (Fig. 1AII and III), indicating that this molecule contained sequences from both phages. These results were absolutely compatible with the theoretic cointegrate structure of pHybP-Knϕ derived from our initial hypothesis in which the RFs of VGJϕ and CTX-Knϕ recombined site specifically through their respective *attP* sites (Fig. 1B). Finally, DNA sequencing of the novel junctions of pHybP-Knϕ con-

firmed our hypothesis and showed that the genome of both phages were opposite in the hybrid RF (Fig. 1C).

KHT46 carrying pHybP-Knϕ transmitted this hybrid molecule to several MSHA-expressing *V. cholerae* strains, indicating that pHybP-Knϕ was generating an ssDNA genome that was being packaged and exported in a new phage particle designated HybP-Knϕ. KHT46 produced ca. 10⁷ HybP-Knϕ particles/ml/OD unit of culture when the titers were measured by using 569B as receptor strain. In general, the titers of HybP-Knϕ ranged from 10⁶ to 10⁸ phage particles/ml/OD unit of cultures, depending on the donor and receptor strain used to measure phage titer (not shown). This value was intermediate between titers of CTXϕ (10⁵ to 10⁶) and VGJϕ (10¹⁰ to 10¹¹) when measured under the same conditions.

RS1 transmission mediated by VGJϕ. The RF of the satellite phage RS1 contains an *attP* site nearly identical to that of CTXϕ; we therefore evaluated whether VGJϕ could also transmit the satellite phage RS1 according to the same procedure described above to assess CTXϕ transmission. Thus, RS1-Knϕ was used in place of CTX-Knϕ to coinfect strain 569B, together with VGJϕ (Table 2). As occurred with CTX-Knϕ, RS1-Knϕ was efficiently transmitted to the TCP mutant KHT52 (Table 2), suggesting that a hybrid phage was also formed by recombination between RS1 and VGJϕ RFs. Here again, the analysis of several KHT52 transductants by restriction and Southern blotting (not shown) confirmed that the RFs of RS1-Knϕ and VGJϕ had recombined site specifically to form a hybrid RF designated pHybRS-Knϕ, which generated an ssDNA hybrid genome that was packaged and exported in a phage particle designated HybRS-Knϕ. The analysis of one representative clone of pHybRS-Knϕ purified from KHT46 (as described above for pHybP-Knϕ) is shown in Fig. 1D. Sequencing of the novel junctions between RS1 and VGJϕ DNAs (Fig. 1F) confirmed the structure of HybRS-Knϕ RF and that RS1 and VGJϕ DNAs were opposite in the hybrid molecule (Fig. 1E).

VGJϕ-dependent transmission of CTXϕ and RS1 from their lysogenic state. Since the initial source of HybP-Knϕ

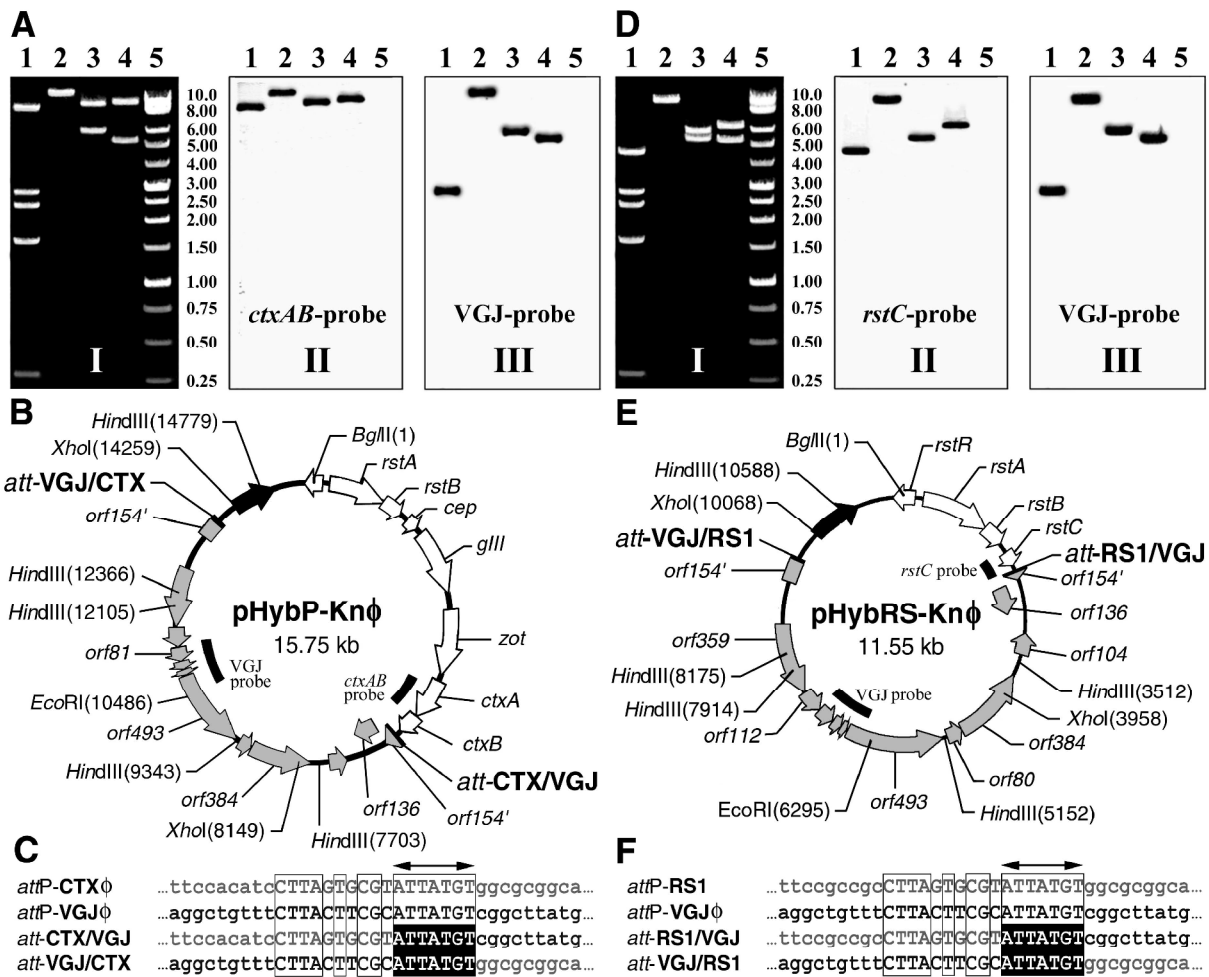


FIG. 1. Analysis of HybP-Kn ϕ and HybRS-Kn ϕ RF structure. (A) Restriction and Southern analysis of HybP-Kn ϕ RF. In subpanel I, DNA samples of HybP-Kn ϕ RF digested with *Hind*III (lane 1), *Eco*RI (lane 2), *Xho*I (lane 3), *Eco*RI-*Bgl*II (lane 4), and appropriate molecular size markers (lane 5) were electrophoresed in 0.8% agarose gels. In subpanels II and III are shown Southern blot analyses with the *ctxAB*- and VGJ ϕ -specific probes represented in panel B. The lanes are the same as in subpanel I, except for lane 5, which was loaded with VGJ ϕ RF DNA in subpanel II or with CTX-Kn ϕ RF DNA in subpanel III as negative controls. (B) Structure of pHybP-Kn ϕ . The genes of CTX ϕ are represented in white, the genes of VGJ ϕ are represented in gray, and the Kn r gene is represented in black. The enzymes and probes used to determine pHybP-Kn ϕ structure by restriction and Southern in panel A are shown. The novel junctions between phage DNAs, *att*-VGJ/CTX and *att*-CTX/VGJ, are also represented. Notice that the VGJ ϕ and CTX ϕ genomes are opposite. (C) Nucleotide sequences of *attP* sites of CTX ϕ and VGJ ϕ before site-specific recombination and sequences of the novel junctions after recombination, as determined by DNA sequencing. The core sequences of homology are shown in uppercase, with identities boxed. DNA sequences of CTX ϕ or VGJ ϕ origin are shown in gray or black, respectively, or in white over black for sequences of undetermined origin at the moment of recombination (5). A double-headed arrow delimits the region where the cut and rejoining of the recombination partners took place. (D, E, and F) The same analyses as in panels A, B, and C, respectively, but for HybRS-Kn ϕ . In DIII (lane 5) the negative control loaded was RS1-Kn ϕ RF DNA.

(and HybRS-Kn ϕ) was 569B coinfecting with both CTX-Kn ϕ (or RS1-Kn ϕ) and VGJ ϕ , where both phages were replicating, we studied HybP-Kn ϕ and HybRS-Kn ϕ production from El Tor strains with resident Kn r -labeled CTX ϕ or RS1 prophages after infection with VGJ ϕ . Thus, we used strains C72K7 and N16K38, which normally produce CTX-Kn ϕ and RS1-Kn ϕ particles, respectively, from the resident prophages (Fig. 2). When these strains were infected with VGJ ϕ , they detectably produced HybP-Kn ϕ or HybRS-Kn ϕ , albeit with significantly reduced titers (Table 2). However, once the few HybP-Kn ϕ or HybRS-Kn ϕ particles produced by these strains infected another receptor like KHT52, they could replicate and produce

large amounts of hybrid phage particles ($\sim 10^7$ particles/ml/OD unit) from the new host.

DNA and protein composition of HybP-Kn ϕ and HybRS-Kn ϕ particles. Genomic ssDNA preparations of HybP-Kn ϕ particles obtained from KHT46(HybP-Kn ϕ) could only be sequenced with oligonucleotides hybridizing with the positive DNA strand of VGJ ϕ or the negative DNA strand of CTX ϕ ; also, this genomic DNA hybridized only with the HybP-A probe, which is specific for the positive strand of VGJ ϕ and the negative strand of CTX ϕ but not with the reverse probe HybP-B (Fig. 3A to C). These results were identical for HybRS-Kn ϕ (not shown), clearly indicating that the replica-

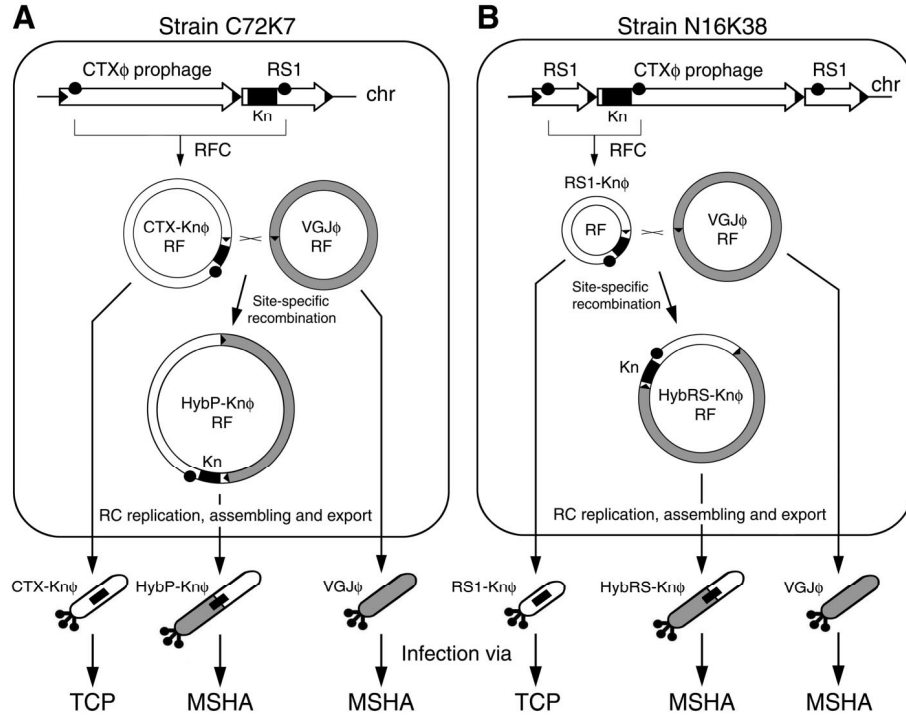


FIG. 2. Schematic representation of hybrid phages HybP-Kn ϕ and HybRS-Kn ϕ formation in *V. cholerae* El Tor strains. (A) Production of the hybrid phage HybP-Kn ϕ in the El Tor strain C72K7 infected with VGJ ϕ . Extrachromosomal CTX ϕ RF is generated by replication from the chromosome (RFC) by a process dependent on the presence of replication origins (dots) in tandem (40), and then CTX ϕ and VGJ ϕ RFs recombined site specifically through their respective *attP* sites (solid arrowheads) to produce HybP-Kn ϕ RF, which generates hybrid phage HybP-Kn ϕ particles. Only hybrid particles can transduce the Kn^r trait (solid box) by using the MSHA receptor. (B) Production of the hybrid phage HybRS-Kn ϕ in the El Tor strain N16K38 infected with VGJ ϕ by the same mechanism described in panel A for HybP-Kn ϕ . Again, only HybRS-Kn ϕ hybrid particles transduce the Kn^r trait through the MSHA.

tion machinery of VGJ ϕ prevailed by far over that of CTX ϕ during replication of the hybrid phages.

HybP-Kn ϕ and HybRS-Kn ϕ particles profiled by SDS-PAGE to study their protein contents revealed a protein profile identical to that of VGJ ϕ (5). The major capsid protein of VGJ ϕ was evident in silver-stained gels (not shown) when HybP-Kn ϕ or HybRS-Kn ϕ particles (10⁸) were electrophoresed, and its identity was confirmed by reaction with a MAbs specific for this protein (Fig. 3D), indicating that the hybrid phage capsid is mostly (if not totally) composed by the same proteins of VGJ ϕ capsid.

Stability of HybP-Kn ϕ . The time course stability and structural integrity of HybP-Kn ϕ studied during in vitro growth of 1333(HybP-Kn ϕ), in the absence of kanamycin selection, demonstrated that HybP-Kn ϕ preserved the same recombinant structure and remained present in about one-third of the bacterial population until the late stationary phase (24 h) (not shown).

Colonization of 1333(HybP-Kn ϕ) in the suckling mice intestines also supported the permanency of HybP-Kn ϕ in a similar fraction of the bacterial population at 24 h of colonization but, in contrast to the in vitro condition, the in vivo conditions promoted site-specific integration of pHybP-Kn ϕ into an as-yet-unidentified chromosomal site different from the integration site of VGJ ϕ , as revealed by the unique Southern blot banding pattern in a significant number of clones tested (see the analysis of one representative clone in Fig. 4). One of these

in vivo isolated Kn^r clones, 1333-HybI2, extensively cultured in vitro and in vivo showed that all vibrios in the bacterial population retained the Kn^r trait. Southern blot analysis of 10 clones isolated from both culture conditions revealed the same original prophage structure of 1333-HybI2 (not shown).

Therefore, the replication and packaging processes used by HybP-Kn ϕ (which are the same used by VGJ ϕ) preserved the structure of the hybrid recombinant genome during cell growth and sustained the inheritance of the hybrid phage by a significant fraction of the cell population in vitro. In vivo, the inheritance of the recombinant phage was guaranteed by the site-specific integration of HybP-Kn ϕ into the bacterial chromosome also in a significant fraction (about one-third) of the bacterial population. Once HybP-Kn ϕ integrated it was stably maintained in the bacterial chromosome.

Expression of CT genes transduced by HybP-Kn ϕ . 1333(HybP-Kn ϕ) and 1333-HybI2 cultured in AKI medium produced 80 to 90 ng of CT/ml as measured by G_{M1}-ELISA. This amount is similar to the CT levels produced by its toxigenic progenitor C6706 in similar condition but contrasted with the null production of CT by the attenuated vaccine strain 1333. Thus, *ctxAB* genes are actively expressing CT from the replicating and lysogenic state of HybP-Kn ϕ in the AKI condition. Curiously, when CT production was measured in LB medium, 1333(HybP-Kn ϕ) produced 60 to 70 ng of CT/ml; however, 1333-HybI2, as well as the control parental strain C6706, did not produce CT under this condition. Thus, CT

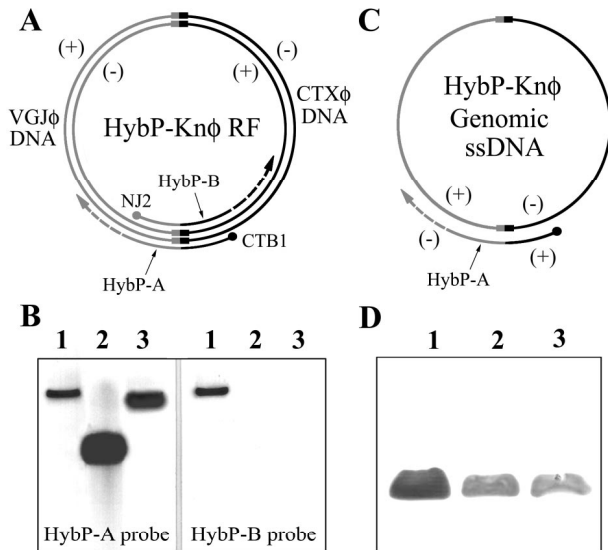


FIG. 3. Analysis of DNA and protein composition of HybP-Kn ϕ . (A) Schematic representation of the construction of HybP-A and HybP-B strand-specific probes by asymmetric PCR with HybP-Kn ϕ RF as a template and the primers CTB1 and NJ2 (represented by dots). VGJ ϕ and CTX ϕ DNAs are represented in gray and black, respectively, connected by their respective *attP* sites. (B) Southern blot analysis of genomic DNA from HybP-Kn ϕ particles with strand-specific probes. Lanes: 1, HybP-Kn ϕ RF linearized with *Sph*I; 2, genomic DNA (positive strand) of VGJ ϕ as a control; 3, genomic ssDNA of phage HybP-Kn ϕ . As expected, both strand-specific probes hybridized with the RF of HybP-Kn ϕ , from where they were generated, but only HybP-A probe hybridized with the ssDNA genome extracted from VGJ ϕ or HybP-Kn ϕ particles. (C) Structure of the ssDNA genome of HybP-Kn ϕ deduced from the Southern results, showing that it is composed of the positive strand of VGJ ϕ and the negative strand of CTX ϕ . (D) Western blot analysis with MAb 2H1H9 specific for the major capsid protein of VGJ ϕ . Lanes: 1, VGJ ϕ (10^9 particles); 2, HybP-Kn ϕ (10^8 particles); 3, HybRS-Kn ϕ (10^8 particles).

expression from HybP-Kn ϕ behaved in a different manner when HybP-Kn ϕ DNA was in the replicative or integrated state showing the same pattern of CT expression from CTX ϕ (38). These results suggest that *ctxAB* genes are not under the control of the ToxR regulon when HybP-Kn ϕ is in the replicative state but that they are regulated by this regulatory system when HybP-Kn ϕ is integrated.

HybP-Kn ϕ mediates virulence conversion. As expected, attenuated strain 1333 was not lethal at all to suckling mice; however, strain 1333-HybI2 inoculated orogastrically into suckling mice at a dose of 10^6 CFU reproduced the toxigenic behavior of the pathogenic parental C6706 in the kinetics of mouse killing (Fig. 5). At day 6 no mice survived inoculation with 1333-HybI2 or C6706 (Fig. 5), indicating that infection and lysogenization with HybP-Kn ϕ have full potential for virulence reversion of attenuated vaccine strains such as 1333.

HybP-Kn ϕ host range. The host range of HybP-Kn ϕ was the same of VGJ ϕ (5) and was unaffected by the expression or not of TCP, indicating that HybP-Kn ϕ entry into host cells depended only on the MSHA (Table 1). In addition, HybP-Kn ϕ infected most strains tested more efficiently than CTX ϕ , even under optimal conditions for TCP expression (Table 1).

MshA versus TcpA expression. To compare the in vitro relative expression of HybP-Kn ϕ and CTX ϕ receptors, we

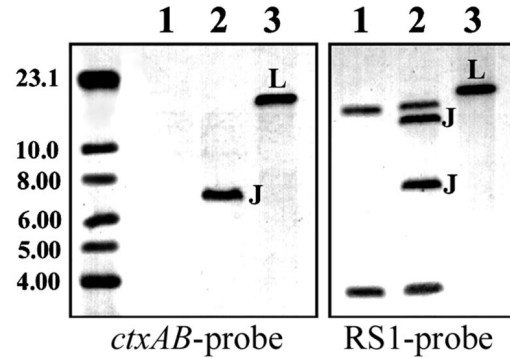


FIG. 4. Site-specific integration of HybP-Kn ϕ into a chromosomal site different from the *attRS1*. Southern blot analysis of total DNAs of 1333 (lane 1), 1333-HybI2 (lane 2), and HybP-Kn ϕ RF DNA (lane 3) digested with *Bgl*II and hybridized with *ctxAB* (left)- and RS1 (right)-specific probes. The “J” denotes bands containing the novel junctions of HybP-Kn ϕ with the chromosome, and “L” denotes the linearized RF. Note that in the right panel the RS1-specific bands, belonging to the RS1 prophage contained in 1333’s chromosome (lane 1) remain identical in 1333-HybI2 after integration of HybP-Kn ϕ (lane 2), indicating that integration occurred by a different locus.

analyzed the expression of their major pilus subunits, MshA and TcpA, in classical, El Tor, and O139 strains under different culture conditions by Western blotting. Although in vitro TcpA expression was detected only in restricted conditions and not in all strains, MshA was expressed at similar levels in all strains and conditions tested (Table 3). These results indicated that MSHA is very likely a more ubiquitously expressed receptor than TCP.

DISCUSSION

The present study describes a novel type of specialized transduction for transmission of CTX ϕ or its satellite phage RS1 between *V. cholerae* strains, which is mediated by the filamentous phage VGJ ϕ . Our results strongly suggest that this mechanism consists in the site-specific recombination of the RF of

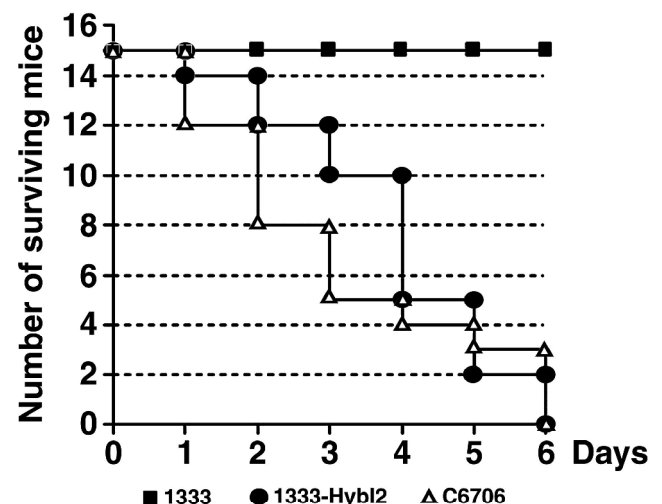


FIG. 5. Time course survival of suckling mice inoculated with 1333, 1333-HybI2, or their parental strain C6706.

TABLE 3. Comparative expression of TcpA and MshA protein subunits under different growth conditions

Protein subunit and condition ^a	Expression in strains ^b													
	O1, classical						O1, El Tor						O139	
	569B	O395	CA401	CA385	NIH35A3	1395	C6706	C7258	N16961	E7946	3083	Lima	CRC262	CRC266
MshA														
LB (pH 7, 37°C)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LB (pH 6.5, 30°C)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AKI	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TSB or TSB _G	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DMEM	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PFHM	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TcpA														
LB (pH 7, 37°C)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LB (pH 6.5, 30°C)	+	+	-	-	-	+	-	-	-	-	-	-	-	-
AKI	+	-	-	-	-	-	+	+	+	+	+	+	+	+
TSB or TSB _G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DMEM	+	NP	NP	NP	NP	NP	-	-	-	-	-	-	-	-
PFHM	+	NP	NP	NP	NP	NP	+	+	+	+	+	+	+	+

^a LB, LB medium.

^b +, Clearly visible band by Western blotting; -, no band detected. NP, not performed.

VGJφ and CTXφ (or RS1) to form a single-hybrid molecule in a donor *V. cholerae* cell. This hybrid RF generates an ssDNA genome that is packaged and exported in hybrid phage particles designated HybPφ (for VGJφ/CTXφ hybrid) or HybRSφ (for VGJφ/RS1 hybrid). HybPφ and HybRSφ are recombinant and stable phages whose genomes consist of the positive strand of VGJφ and the negative strand of CTXφ or RS1, respectively, linked by their respective *attP* sites. Although it is possible that the phages first integrate in tandem in the bacterial chromosome and then excise to form the hybrid phage, this mechanism is very unlikely because (i) integration of VGJφ has not been detected in vitro, a condition in which the hybrid phages are obtained, and (ii) strains in which VGJφ and CTXφ/RS1 are integrated in tandem (5) do not produce hybrid phage particles (data not shown). In addition, the hybrid phages are efficiently produced by classical strains in which VGJφ and CTXφ (or RS1) are replicating in the same host cell (Table 2). However, the hybrid phages are produced at very low levels by El Tor strains of *V. cholerae* (in which CTXφ and RS1 are integrated) when these strains are infected with VGJφ (Table 2), a finding which suggests that the extrachromosomal RFs of VGJφ and CTXφ (or RS1) are needed for production of the hybrid phages by site-specific recombination.

This mechanism of specialized transduction constitutes a novel TCP-independent pathway for CTXφ or RS1 transmission and dissemination. The processes of replication and assembly of HybPφ and HybRSφ, as well as their capsid, are essentially those of VGJφ; thus, HybPφ and HybRSφ also use MSHA as receptor. The MSHA is probably a more available phage receptor than TCP since expression of MshA protein is not restricted to specific growth conditions, as TcpA is, and the MSHA has been found also in non-O1 non-O139 strains of *V. cholerae* (14). Thus, a hybrid phage such as HybPφ could have advantage over CTXφ for transmitting the CT genes under certain conditions, such as those found in the environment. Interestingly, HybPφ infected all El Tor and O139 strains tested in the present study more efficiently than CTXφ, even under optimal conditions for TCP expression (Table 1); there-

fore, phage HybPφ (containing a CTXφ^{El Tor} genome) circumvents phage immunity (36), supporting the idea that the replication machinery of VGJφ is leading replication of HybPφ. Although the differences between the efficiency of infection of classical strains with HybPφ and CTXφ are less pronounced because these strains are not immune to CTXφ^{El Tor} (36), these strains were also infected more efficiently by HybPφ than by CTX-Knφ (Table 1), which likely indicates that HybPφ transduces CT genes more efficiently than CTXφ, the ordinary vehicle of these genes, in all conditions tested.

CTXφ transmission mediated by a VGJφ-like phage is a plausible explanation for the occasional emergence of non-O1, non-O139 TCP-devoid toxigenic strains of *V. cholerae*. It is highly probable that other environmentally circulating *V. cholerae* filamentous phages can potentially transduce CTXφ or RS1 by a mechanism similar to the one described here. As a supporting example, phage fs-2 contains a 715-bp fragment that is highly homologous (97% identity) to part of RS1 satellite phage, flanked at one side by an *attRS1* (27), strongly suggesting that fs-2 and RS1 recombined once upon their evolutionary history. In addition, it has been recently found that RS1 is transduced by the filamentous phage KSFφ by an unknown TCP-independent mechanism (19) that perhaps is the same mechanism described in the present study. More recently, we isolated another related filamentous phage, designated VEJφ, from the *V. cholerae* O139 strain MO45, which is also able to transmit CTXφ by the mechanism described here mediated by VGJφ (results not shown). Phages such as VGJφ and VEJφ seem to be relatively abundant in strains of the O139 serogroup of *V. cholerae*, since other related filamentous phages have been isolated from strains of this serogroup, such as VSK, fs1, and 493 (30, 31, 32, 42).

Consequently, in Fig. 6 we propose an evolutionary model that could explain the origin of new virulent strains from non-toxigenic environmental strains of *V. cholerae* or from another related bacterial species. Acquisition of CTXφ phage by a nontoxigenic *V. cholerae* strain does not automatically create a new pathogen since other factors besides CT are needed for

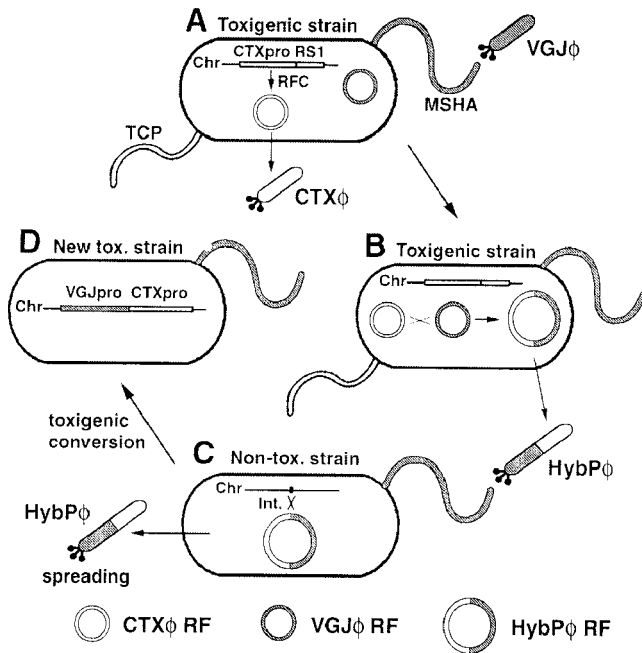


FIG. 6. Speculative model for the emerging of new pathogens from *V. cholerae* (non-O1, non-O139), or another bacterium species, mediated by a phage such as VGJ ϕ . (A) A VGJ ϕ -like phage infects a toxigenic strain of *V. cholerae* through MSHA pilus. (B) Incoming RF of this phage site specifically recombines with CTX ϕ RF, producing a hybrid genome, which is packaged and exported in hybrid phage particles such as HybP ϕ . (C and D) Hybrid particles eventually encountered, infected, and integrated their hybrid genome into the chromosome of an MSHA⁺ nontoxic strain of *V. cholerae* or into another related host species expressing an MSHA homologous pilus (C) to produce a new genetically stable toxigenic strain (D). The nontoxic *V. cholerae* receptor could be a VPI⁺ strain, expressing MSHA but not TCP in the environment conditions, such as live attenuated vaccine strains. Although not represented, the inverse situation in which CTX ϕ infects *V. cholerae* with a resident phage such as VGJ ϕ would also originate a hybrid phage such as HybP ϕ . CTX ϕ phage, its RF, and its receptor (TCP) are represented in white; VGJ ϕ phage, its RF, and its receptor (MSHA) are represented in gray; and HybP ϕ phage and its RF are represented in gray and white. Chr, bacterial chromosome; RFC, replication from the chromosome; Int., integration; CTXpro, CTX ϕ prophage; VGJpro, VGJ ϕ prophage.

manifestation of the full virulence phenotype (12, 13, 37). Among these factors are TCP, which allows virulent strains to colonize the human small intestine (47), and the lipopolysaccharide, since only two serogroups of *V. cholerae* (O1 and O139) are known to cause epidemic cholera (7, 28). Therefore, for a phage such as HybP ϕ to convert a nontoxic *V. cholerae* strain into a full pathogen it is necessary that the phage infect a host cell with adequate virulence factors. One possibility is that these hybrid phages infect *V. cholerae* strains (non-O1, non-O139) which do not contain CT encoding genes but which contain VPI and thereby the TCP gene cluster and *toxT* gene, whose product is needed to activate TCP and CT expression. Although, they are not the rule, such strains have been isolated, including some with new variant alleles of *toxT* and *tcpA* (6, 20, 41). If such strains additionally have the MSHA gene cluster, they could express this pilus in a broader number of conditions than TCP, giving advantage to a phage

such as HybP ϕ over CTX ϕ to infect them. Also, the possibility that a phage such as HybP ϕ infects *V. cholerae* strains with other unknown virulence attributes, such as a colonization factor different from TCP cannot be absolutely excluded. Furthermore, another possibility for the emergence of new pathogens is that HybP ϕ -like phages infect other bacterial species expressing MSHA homologous pili where the hybrid phage could integrate and express CT. The homology between the putative adsorption proteins of phages VGJ ϕ and Vf33, which infects the enteropathogen *Vibrio parahaemolyticus*, suggests that this bacterium species expresses a pilus homologous to MSHA; in fact, VGJ ϕ seems to be more related to Vf33 than to other *V. cholerae* phages such as CTX ϕ or fs2 (5).

More tangible is the fact that live attenuated vaccine strains, which contain MSHA and TCP gene clusters, can potentially reacquire *ctxAB* genes in the environment by infection with a phage such as HybP ϕ . Infection with HybP ϕ mediates virulence conversion of the *V. cholerae* vaccine attenuated strain 1333, in which this phage is able to integrate into the chromosome during in vivo growth in the small intestines of suckling mice, stabilizing the inheritance of CT genes. In contrast to VGJ ϕ and CTX ϕ , which integrate preferentially at the right flanking end repeat of RS1 (*attRS1*) (5, 8), HybP ϕ integrates at an as-yet-unidentified chromosomal site of 1333 (Fig. 4), indicating that the recombinant nature of HybP ϕ shifts integration specificity. Therefore, previously conceived mutations, such as the deletion of the *attRS1* site (44), directed to avoid stable reversion to virulence of live attenuated vaccine strains, could be ineffective to prevent integration of HybP ϕ -like phages. Thus, it would be desirable that live attenuated vaccine strains do not express MSHA to diminish the risk of virulence reversion in the environment after the release of these strains during massive vaccination campaigns. In addition, it would be desirable that vaccine strains lack any VGJ ϕ -related phage (replicating or lysogenized) to avoid dissemination of CTX ϕ in case it is reacquired.

The present study emphasizes the importance and versatility of horizontal gene transfer mechanisms for the evolution of bacterial pathogens. To our knowledge, this is the first report of the transmission of one filamentous phage genome by another filamentous phage, which constitutes an example of how mobile genetic elements can subtly interact to expand the pathways for gene transfer. This finding should lead to future studies about the interaction between filamentous phages in particular and between mobile genetic elements in general for horizontal gene transfer in bacteria.

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