VEJ ϕ , a novel filamentous phage of *Vibrio cholerae* able to transduce the cholera toxin genes

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A novel filamentous bacteriophage, designated VEJ ϕ , was isolated from strain MO45 of *Vibrio cholerae* of the O139 serogroup. A molecular characterization of the phage was carried out, which included sequencing of its whole genome, study of the genomic structure, identification of the phage receptor, and determination of the function of some of the genes, such as those encoding the major capsid protein and the single-stranded DNA-binding protein. The genome nucleotide sequence of VEJ ϕ , which consists of 6842 bp, revealed that it is organized in modules of functionally related genes in an array that is characteristic of the genus *Inovirus* (filamentous phages). VEJ ϕ is closely related to other previously described filamentous phages of *V. cholerae*, including VGJ ϕ , VSK and fs1. Like these phages, VEJ ϕ uses as a cellular receptor the type IV fimbria called the mannose-sensitive haemagglutinin (MSHA). It was also demonstrated that VEJ ϕ , like phage VGJ ϕ , is able to transmit the genome of phage CTX ϕ , and therefore the genes encoding the cholera toxin (CT), horizontally among populations of *V. cholerae* expressing the MSHA receptor fimbria. This suggests that the variety of phages implicated in the horizontal transmission of the CT genes could be more diverse than formerly thought.

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INTRODUCTION

Filamentous bacteriophages belong to the genus *Inovirus*, members of which infect almost exclusively Gram-negative bacteria. The virions of these phages are slender filaments usually about 6 nm in diameter and 800–2000 nm long, comprising a helical capsid of several thousand copies of the major coat protein (pVIII) surrounding a single-stranded circular DNA genome about of 5–8 kb, encoding around 10 genes (Marvin, 1998). These phages usually use type IV pili as receptors to infect bacterial cells (Marvin, 1998).

Vibrio cholerae, the Gram-negative bacterium causing the acute diarrhoeal disease cholera, hosts several filamentous phages (Campos *et al.*, 2003b; Ehara *et al.*, 1997; Faruque *et al.*, 2005; Jouravleva *et al.*, 1998; Kar *et al.*, 1996; Waldor & Mekalanos, 1996). Some of them are able to transduce portions of alien DNA among bacterial populations, including the transmission of virulence genes mediated by different mechanisms of horizontal gene transfer (Campos *et al.*, 2003a; Davis & Waldor, 2003; Faruque

et al., 2002). In this respect, most significant was the finding that the genes encoding cholera toxin (CT), the main virulence attribute of *V. cholerae*, are contained in the genome of the CTX ϕ phage, which is integrated in the chromosome of toxigenic strains of this bacterium at the chromosomal *dif* site (Huber & Waldor, 2002; Val *et al.*, 2005; Waldor & Mekalanos, 1996). CTX ϕ is also able to replicate directly from the bacterial chromosome and to produce infective phage particles with potential for transducing the CT genes to non-pathogenic environmental strains (Davis & Waldor, 2003; Waldor & Mekalanos, 1996). The receptor of this phage is the toxin co-regulated pilus (TCP), a type IV fimbria essential for colonization of the human intestine by *V. cholerae* (Waldor & Mekalanos, 1996). The genes required for TCP expression and assembly at the bacterial surface are located in the vibrio pathogenicity island (VPI), which has also been horizontally acquired by this pathogen during evolution, although its mechanism of transmission is still controversial (Faruque *et al.*, 2003; Karaolis *et al.*, 1998; Karaolis & Kaper, 1999; Lee, 1999; Rajanna *et al.*, 2003).

More recently, another filamentous phage was described – VGJ ϕ – which is also able to transmit the CT genes by means of a mechanism of specialized transduction, and it uses the type IV pilus mannose-sensitive haemagglutinin (MSHA) as a receptor (Campos *et al.*, 2003a, b). The genomes of VGJ ϕ and CTX ϕ are able to recombine when both phages co-infect the same cell. As a result, a hybrid genome is formed that is packaged into the sheath of VGJ ϕ ,

Abbreviations: CT, cholera toxin; MSHA, mannose-sensitive haemagglutinin; RF, replicative form; TCP, toxin co-regulated pilus.

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forming a hybrid phage particle designated HybP ϕ , which is able to infect other vibrio cells via the MSHA receptor (Campos *et al.*, 2003a). Thus, CTX ϕ and therefore the CT genes it carries are transduced by VGJ ϕ through a mechanism independent of the TCP receptor (Campos *et al.*, 2003a). Hybrid phage HypP ϕ could potentially infect vibrios that do not express TCP; once inside the cells phage HypP ϕ is capable of integrating into the bacterial chromosome, guaranteeing the vertical perpetuation of the acquired CT genes (Campos *et al.*, 2003a).

Studies on the horizontal gene transfer mechanisms of CT genes and other virulence-related genes have been of great importance in understanding the evolution of the virulent strains of *V. cholerae*. This knowledge has allowed researchers to gain new perspectives on the possible epidemiological trends that cholera disease could follow in the future and it has had a direct influence on the strategies currently applied in the design of new vaccines based on live attenuated strains of *V. cholerae*, in the sense of trying to avoid the reacquisition of virulence genes by

these strains in the environment (Campos *et al.*, 2003a; Kimsey & Waldor, 1998).

In this study we characterize phage VEJ ϕ , a new filamentous phage hosted by *V. cholerae* that is a close relative of VGJ ϕ , and we explore its ability to transduce the CT genes among bacterial populations of this pathogen.

METHODS

Strains, plasmids, phages and culture media. Strains, plasmids and phages used in this study are described in Table 1. Bacterial strains were grown in Luria–Bertani medium (LB). It was normally adjusted to pH 7.0 or to pH 6.5 for expression of the TCP in classical strains of *V. cholerae*. Ampicillin (100 $\mu\text{g ml}^{-1}$) or kanamycin (50 $\mu\text{g ml}^{-1}$) were added when required.

Isolation of phage VEJ ϕ . Strain MO45 was grown in 5 ml LB in a 25 ml Erlenmeyer flask for 6 h with shaking (240 r.p.m.) at 37 °C. One millilitre of this culture was serially filtered through 0.45 μm and 0.22 μm pore-size filters (Sartorius). An aliquot (50 μl) of the filtered

Table 1. Strains, plasmids and phages used in this study

Strain, plasmid, phage or primer	Description or sequence	Origin or reference
<i>E. coli</i>		Promega
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac proAB)</i>	
<i>V. cholerae</i>		
569B	O1, Classical, Inaba	Calcutta, India, 1945
C6706	O1, El Tor, Inaba	Peru, 1991
KHT46	C6706 Δ <i>mshA1</i>	Thelin & Taylor (1996)
KHT52	C6706 Δ <i>tcpA10</i>	Thelin & Taylor (1996)
MO45	O139	Madras, India, 1993
Plasmids		
pIJ2921	General-purpose vector	Mendez & Chater (1987)
p4	pIJ2921 derivative containing a 435 bp <i>HincII</i> fragment from the RF of VEJ ϕ , inserted at <i>SmaI</i> site of vector	This study
p5	pIJ2921 derivative containing a 1039 bp <i>HincII</i> fragment from the RF of VEJ ϕ , inserted at <i>SmaI</i> site of vector	This study
p11	pIJ2921 derivative containing a 1787 bp <i>HincII</i> fragment from the RF of VEJ ϕ , inserted at <i>SmaI</i> site of vector	This study
Phages		
CTX-Kn ϕ	CTX ϕ containing a Kn ^r gene inserted into the unique <i>NotI</i> site of the phage genome	Campos <i>et al.</i> (2003a)
VEJ ϕ	Filamentous phage of <i>V. cholerae</i>	This study
Primers		
EJ1	5'-GGAAGAAGCAACGATTGTCTG-3'	This study
EJ2	5'-CCGTATTGAGCAAACCTCTC-3'	This study
EJ3	5'-CTATGAGCAAGAAAGCGAGG-3'	This study
EJ4	5'-CTCGACTCAATTCTGACAAAC-3'	This study
EJ5	5'-CATTCGCTTCGATTCATTG-3'	This study
EJ6	5'-ACTCATAGAACCAATGGATG-3'	This study
EJ7	5'-GCTTAGTTCGTCTCGACTTG-3'	This study
EJ8	5'-GTAATGGCTAACATCACTGG-3'	This study
EJ9	5'-CATCGGTGATACCAGAGAAG-3'	This study
EJ10	5'-GTGATCATCAATACTCTTGC-3'	This study
NJ-4	5'-CCTGTAGAAATTCGCTCTCC-3'	Campos <i>et al.</i> (2003a)
CTB1	5'-GCGATTGAAAGGATGAAGG-3'	Campos <i>et al.</i> (2003a)

supernatant was plated on solid LB to check for sterility. Another aliquot (20 µl) was mixed with 20 µl of a culture of 569B (grown under the same conditions as MO45) and incubated for 20 min at room temperature. The mixture was then inoculated into 100 ml LB in a 500 ml Erlenmeyer flask and grown at 37 °C with shaking (240 r.p.m.) overnight. The culture was centrifuged (8000 g, 20 min), and the supernatant was filtered through a 0.45 µm pore-size filter. Phage particles in the filtrate were precipitated by addition of NaCl and polyethylene glycol to final concentrations of 3 and 5% (w/v), respectively. The mixture was incubated on ice for 30 min and centrifuged at 12 000 g for 20 min. The supernatant was discarded, and the phage-containing pellet was resuspended in 1 ml PBS.

DNA isolation and manipulation. Plasmid DNA was prepared by using the Wizard plus SV system (Promega), and genomic ssDNA from phage particles was prepared as described by Sambrook *et al.* (1989). DNA restriction and modification enzymes were used according to the manufacturer's instructions (Promega). The DNA was electrophoresed on 0.8% (w/v) agarose gels and was visualized with ethidium bromide (1 µg ml⁻¹) or with acridine orange (1 µg ml⁻¹), which fluoresces green with double-stranded DNA and orange with ssDNA (McMaster & Carmichael, 1977). Sequencing reactions were ordered from Macrogen. The whole sequence of the VEJφ genome was assembled by combining data obtained from sequencing of cloned fragments of the replicative form (RF) in the pIJ2921 vector with modified universal and reverse primers and from direct sequencing of the purified genomic ssDNA of the phage (plus strand) or the RF with primers EJ1–EJ10 (Table 1).

Nucleotide and protein sequence analysis and alignment. Sequences were assembled and analysed by using Vector NTI advance 10 (Invitrogen), which was also used for alignment analyses. The BLAST program was used to search for homologous DNA or protein sequences in databases.

Electron microscopy. Purified phage particles were negatively stained with 4% (w/v) uranyl acetate and mounted on freshly prepared Formvar grids, which were examined with a JEOL JEM-2000EX transmission electron microscope.

Infection assay. VEJφ or CTX-Knφ donor strains were grown in LB to an OD₆₀₀ of 1 measured in a BioPhotometer (Eppendorf). One millilitre of culture was filtered through a 0.22 µm pore-size filter (Sartorius). Aliquots (100 µl) of pure or serially diluted cell-free supernatant of the donor were mixed with 20 µl (~10⁸ cells) of a culture of the recipient strain grown in LB for 6 h at 37 °C and 240 r.p.m. The mixture was incubated for 20 min to allow infection and then plated onto a soft LB agar (0.4%) overlay in the case of VEJφ or onto solid LB plates supplemented with kanamycin (LBKn) in the case of CTX-Knφ. The number of opaque plaques or Kn^r c.f.u. was determined at 20 h. Alternatively, the infection mixture was seeded directly into LB broth if necessary.

Protein methods. Preparations of phage proteins were analysed by SDS-PAGE. For amino-terminal sequencing, the proteins were first separated by SDS-PAGE, then electro-transferred to a PVDF membrane. After transfer the membrane was stained with Coomassie blue (0.1% in 50% methanol) for 5 min and subsequently destained with 50% methanol until the protein bands were clearly visible (5–10 min). The membrane was washed twice for 5 min with distilled water and the bands of interest were cut from the membrane with a new scalpel. The piece of membrane with each band was used for amino-terminal protein sequencing by the Edman degradation method using an Applied Biosystems Procise automatic sequencer.

Nucleotide sequence accession numbers. The nucleotide sequence of VEJφ has been deposited in the GenBank database under accession number FJ904927.

RESULTS AND DISCUSSION

Isolation of the new filamentous phage VEJφ

We noticed the presence of an extrachromosomal genetic element in total DNA preparations of *V. cholerae* O139 strain MO45. We investigated if this episome corresponded to the genome of a phage. Cell-free culture supernatants of MO45 were used to infect the phage-free indicator strain 569B, which acquired the same episome, as checked by restriction analysis (data not shown). The transmission of the episome from MO45 to 569B without direct cell–cell contact indicated the presence of a phage in the supernatants of MO45. Restriction analyses showed that this episome was a circular, double-stranded DNA molecule with an approximate size of 7 kb (data not shown). The restriction map generated was different from those previously described for *V. cholerae* phages, so we named this new phage VEJφ.

VEJφ phage particles were purified from culture supernatants of infected 569B and their genetic material was isolated. It was resistant to hydrolysis with several restriction enzymes and RNase, but was susceptible to mung-bean nuclease degradation (data not shown), and it fluoresced orange when it was electrophoresed in the presence of acridine orange (data not shown), indicating that the nucleic acid extracted from phage particles was ssDNA.

Phage suspensions of VEJφ were able to produce opaque plaques when spread onto a lawn of the sensitive strain 569B grown on soft agar, which allowed the titration of the phage. The mean titre from three independent experiments was 7.4×10^{10} p.f.u. (ml culture $\times$ OD unit)⁻¹, indicating that it was a very prolific phage, comparable in this respect to VGJφ and M13. The ability of VEJφ to form opaque plaques, taken together with the size and the single-stranded nature of its genome, suggested this was a filamentous phage. This hypothesis was confirmed by visualization of the phage particles by electron microscopy (not shown), as well as by the DNA sequence similarity of the VEJφ genome with those of other filamentous phages stored in DNA databases.

Genomic organization

The VEJφ DNA sequence is similar to sequences of other previously described phages, such as VSK (93.1%), fs1 (89.9%) and VGJφ (84.2%). The whole genome of VEJφ consisted of 6842 nucleotides and had a G + C content of 42.95 mol%, which is slightly lower than the G + C content of its *V. cholerae* host (47.7% for chromosome I and 46.9% for chromosome II). The sequence of VEJφ deposited in the GenBank database (FJ904927) corresponds to the positive DNA strand of the phage, i.e. the same strand that is packaged in the phage capsid. This was confirmed because only oligonucleotides hybridizing with this DNA strand exhibited sequence reactions when phage genomic ssDNA was used as the template.

The VEJ ϕ genome has 11 putative ORFs organized in four modular structures: the replication module, the structural module, the assembly module and the regulation module (Fig. 1). The replication module contains ORF361 and ORF112 (Fig. 1). ORF361 is similar to rolling circle replication proteins of other filamentous phages (VGJ ϕ , 95.8%; KSF, 76.2%; CTX ϕ , 34.1% similarity), while ORF112 is identical to the same ORF of VGJ ϕ and is similar in size and position to ssDNA-binding proteins of other inoviruses. Indeed we demonstrated that the protein product of ORF112 functions as the ssDNA-binding protein of VEJ ϕ and VGJ ϕ , by retardation assays, which showed that it binds specifically to the phage genomic ssDNA (data not shown; while this paper was under revision, the purification and characterization of the ORF112 product was published by Falero *et al.*, 2009). Thus, we propose to rename the ORF112 gene of VEJ ϕ and VGJ ϕ as gV^{VEJ ϕ} and gV^{VGJ ϕ} and their respective proteins as pV^{VEJ ϕ} and pV^{VGJ ϕ} , to follow the denomination of canonical phages of the *Inovirus* genus as the fd group.

The structural module comprises ORF81, ORF44, ORF29, ORF493 and ORF104 (Fig. 1), whose protein products are similar in size, genomic position and/or sequence similarity to putative or proved capsid proteins of other inoviruses, suggesting that their protein products should accomplish the same function in VEJ ϕ . A supporting example is ORF44, whose product was identified as the major capsid protein of VEJ ϕ and VGJ ϕ in this work by amino-terminal sequencing of these peptides. The predicted amino acid sequence of ORF44 from VEJ ϕ differs in only one residue at position 23 from the same ORF of VGJ ϕ , where a serine residue was substituted by an alanine. Whole particles of VEJ ϕ and VGJ ϕ were electrophoresed in SDS-PAGE (Fig. 2) and the major capsid proteins were used for sequencing by Edman degradation as described in Methods. The first

ten amino acid residues of ORF44 (MADIFGAIDF) from both phages were identified. Consequently, we propose to rename the ORF44 genes of VEJ ϕ and VGJ ϕ as gVIII^{VEJ ϕ} and gVIII^{VGJ ϕ} , and the major capsid proteins as pVIII^{VEJ ϕ} and pVIII^{VGJ ϕ} , to follow the denomination of canonical phages of the *Inovirus* genus.

As we have predicted in previous work (Campos *et al.*, 2003b), no leader peptide is present in the ORF44 product. Other filamentous phages such as Pf3 and C2 have major capsid proteins without leader peptides (Kiefer *et al.*, 1997; Kostrikis *et al.*, 1995), which are inserted into the bacterial inner membrane independent of the Sec translocase machinery, driven by hydrophobic forces assisted by YidC protein (Kiefer & Kuhn, 1999; Serek *et al.*, 2004). Since the ORF44 product lacks a leader peptide, we predict that a similar mechanism mediates the insertion of this protein into the inner membrane of *V. cholerae*.

The assembly module of VEJ ϕ only contains ORF411 (Fig. 1), whose protein product is similar to the assembly protein of other filamentous phages such as VGJ ϕ , ϕ Lf and CTX ϕ (82, 52 and 40% similarity, respectively). It is noticeable that ORF411 has accumulated more mutations compared with its equivalent ORF384 of VGJ ϕ (76% similarity at the DNA level and 81.8% at the protein level). The significance of the evolutionary divergence of these ORFs is not known, but we speculate that it could be related to a better fitness of the phages for being hosted by different bacterial strains to evade mechanisms of resistance at the level of phage assembly. Immediately 3' of ORF411 there is an intergenic region where major differences between VEJ ϕ and VGJ ϕ are present. In this region VGJ ϕ contains an insertion of 774 bp with an ORF (ORF104) that is not present in VEJ ϕ (Fig. 1) and whose function is unknown. It seems that this is a more flexible

Fig. 1. Modular structure of the genome of VEJ ϕ compared to those of phages VGJ ϕ of *V. cholerae* and M13 of *E. coli*. ORFs or genes are shown with arrows pointing in the direction of transcription. The number of amino acid residues encoded is shown in the name of each ORF, or below each gene in M13. The replication module is represented in black, the structural module in white, and the assembly and secretion module in dark grey. The ORFs belonging to the regulation module are hatched. The ORF104 of VGJ ϕ , whose function is unknown, is represented in light grey. Genome sizes are shown on the right.

Fig. 2. Major capsid proteins of VEJ ϕ and VGJ ϕ visualized by SDS-PAGE. Purified viral particles of VEJ ϕ and VGJ ϕ were applied in the outer lanes, where the bands corresponding to the major capsid proteins are observed at ~4 kDa. Protein molecular mass markers (M) were applied in the middle lane and the size of each marker is shown to the right.

region of the genome, which can accommodate major changes without affecting the biological cycle of the phage and therefore favouring evolutionary fitness in the long term.

No gene homologous to *gIV* of the Ff phage group of *Escherichia coli*, which encodes the secretin pIV needed for extrusion of the viral particles (Marciano *et al.*, 1999), is present in VEJ ϕ . Therefore, VEJ ϕ may rely upon a host protein for phage export, like CTX ϕ phage, which uses EpsD secretin of the type II secretion system (Davis *et al.*, 2000).

The regulatory module of VEJ ϕ contains ORF136 and ORF98, which are encoded in the opposite direction with respect to the rest of the phage genes (Fig. 1). The product of ORF136 is 99.3 % similar to the homonym hypothetical repressor of VGJ ϕ , 46.1 % similar to repressor 1 of VSK and 35.1 % to a *lacI* family repressor of *Thermotoga maritima*. The ORF98 product is 46.1 % similar to repressor 2 of VSK and is almost identical to the amino-terminal region of the ORF154-encoded hypothetical repressor of VGJ ϕ .

VEJ ϕ uses the MSHA fimbria as a receptor

Due to the similarity of VEJ ϕ with VGJ ϕ , which uses the MSHA fimbria as a receptor, it was very probable that VEJ ϕ also uses this fimbria as a receptor. Therefore, we used the same procedure that was previously described to determine the VGJ ϕ receptor (Campos *et al.*, 2003b). For this, we used the isogenic strains of *V. cholerae* KHT46 and KHT52 (Table 1) mutated at genes *mshA* or *tcpA*, coding for MSHA and TCP main pilin, respectively. Like VGJ ϕ phage, VEJ ϕ was able to infect only the strains with an intact MSHA fimbria and unable to infect the *mshA* mutant KHT46, clearly indicating that VEJ ϕ uses MSHA as a receptor.

The integration site of VEJ ϕ

Some filamentous phages of *V. cholerae* are able to integrate into the bacterial genome (Campos *et al.*, 2003b; Kar *et al.*, 1996; Waldor & Mekalanos, 1996). In the case of CTX ϕ it has been proven that integration is catalysed by recombinases XerCD (Val *et al.*, 2005), which use as a substrate the genomic ssDNA of the phage for recombination with the bacterial chromosomal *difI* site (Val *et al.*, 2005). VGJ ϕ is also able to integrate into the *difI* site, as well as to form a cointegrate with the genome of CTX ϕ (Campos *et al.*, 2003a, b). It is highly probable that XerCD also catalyse recombination reactions in these cases. Based on these antecedents we identified the sites similar to *difI* in VEJ ϕ and other integrative phages, which were possible substrates for XerCD catalysis. Taking the *difI* site of *V. cholerae* as a template, we found candidate sites on the negative strand of phages VEJ ϕ , VGJ ϕ and VSK, which were found to be almost identical and which differed by only a few bases from the *difI* site (Fig. 3).

The putative XerC-binding sites differ in two bases from *difI*, while the putative XerD-binding sites are identical in every phage and differ in two bases from *difI*. The central regions consist of seven nucleotides in place of six in *difI*, and these are identical in the 5' portion, where every one contains the TGT triplet (Fig. 3). However, the 3' extremes of the central regions differ significantly from *difI*. The XerC- and XerD-binding sites, as well as the central region, are identical in VGJ ϕ and VEJ ϕ ; therefore VEJ ϕ is probably able to integrate into the bacterial chromosome as does VGJ ϕ , although this has not been tested yet.

	XerC	XerD
<i>difI</i> (<i>V. cholerae</i>)	AGTGCGCATTA TGTATG-	TTATGTTAAAT
XerCD-VEJ ϕ	ACTTCGCATTA TGTCGGC	TTATGGTAAAA
XerCD-VGJ ϕ	ACTTCGCATTA TGTCGGC	TTATGGTAAAA
XerCD-VSK	ACTTCGCAGTA TGTCGGC	TTATGGTAAAA

Fig. 3. Sequence alignment of the *difI* site of *V. cholerae* with similar sites encoded on the negative DNA strand of VEJ ϕ , VGJ ϕ and VSK. Identical bases for each position are highlighted in grey. The probable binding sites for the recombinases XerC and XerD are shown.

VEJ ϕ transduces CTX ϕ

The finding of binding sites for XerCD recombinases in VEJ ϕ identical to those of VGJ ϕ and similar to the *dif* site of chromosome I of *V. cholerae* strongly suggested that VEJ ϕ could transduce CTX ϕ and hence the CT genes by forming a cointegrate between their genomes, as does VGJ ϕ (Campos *et al.*, 2003a). To determine whether VEJ ϕ was able to transduce CTX ϕ we used a variant of CTX ϕ labelled with a kanamycin-resistant gene (CTX-Kn ϕ), so that transduction events could be followed by the transmission of the resistant phenotype between bacterial strains. *V. cholerae* strain 569B was infected with CTX-Kn ϕ or VEJ ϕ , or co-infected with both phages. These three variants were used as donor strains of phage particles to infect strains KHT46 and KHT52 (isogenic mutants lacking MSHA and TCP pili, respectively), as well as 569B itself (Table 2). As expected, 569B infected with CTX-Kn ϕ transmitted the kanamycin-resistant trait to 569B, which expresses the CTX ϕ receptor TCP, but was unable to infect KHT52, the TCP-defective mutant (Table 2). Although KHT46 expresses TCP, this strain was not infected because it contains the CTX ϕ prophage of the El Tor group that confers phage immunity (Kimsey & Waldor, 1998). However, 569B co-infected with CTX ϕ and VEJ ϕ was able to transfer the kanamycin-resistant trait to virgin 569B and to KHT52 (Table 2). Since KHT52 does not express TCP, and its isogenic MSHA-negative relative, KHT46, was unable to be infected by VEJ ϕ , the transfer of the kanamycin trait without direct cell–cell contact likely proceeds through the MSHA fimbria by a phage containing the CTX-Kn ϕ genome or a part of it containing the Kn^r gene. Donor strain 569B infected with VEJ ϕ alone, used as negative control, was unable to transfer the kanamycin-resistant trait to any of the recipient strains (Table 2).

Due to the similarity between VEJ ϕ and VGJ ϕ , we expected that VEJ ϕ uses the same mechanism of CTX ϕ transduction used by VGJ ϕ (Campos *et al.*, 2003a), i.e. the formation of a hybrid phage genome between CTX ϕ and VEJ ϕ genomes. To study the mechanism of CTX ϕ transduction mediated by VEJ ϕ , we made plasmid DNA preparations from 12 of the Kn^r transductants of KHT52 from three independent experiments. The DNAs were digested with *Nco*I, which cuts once into VEJ ϕ RF but does not cut into CTX-Kn ϕ

RF; this produced two bands, of ~15.0 and 6.8 kb, respectively (not shown). These results indicated the simultaneous presence of two plasmids: the VEJ ϕ RF of 6.8 kb and a larger plasmid, whose size (15.0 kb) was compatible with a cointegrate structure comprising both VEJ ϕ and CTX-Kn ϕ RFs fused into a single recombinant molecule, thus supporting our hypothesis. We designated the larger plasmid pHybP-VEJ/CTX ϕ (from hybrid phage). Electroporation of these plasmid DNA preparations (containing both plasmids) into KHT46 allowed us to propagate and purify clones containing pHybP-VEJ/CTX ϕ alone under Kn selection (VEJ ϕ was washed out due to lack of its receptor, MSHA, in this strain). Finally, DNA sequencing of the novel junctions of pHybP-VEJ/CTX ϕ showed that they were produced by recombination of the XerCD sites of both phages' genomes (Fig. 4). This confirmed our hypothesis of the hybrid genome and showed that the genomes of both phages were oriented in opposite directions in the hybrid RF (Fig. 4).

Conclusions

Seven filamentous bacteriophages hosted by *V. cholerae* have been described so far: CTX ϕ , fs1, fs2, VSK, 493, VGJ ϕ and KSF-1 (Campos *et al.*, 2003b; Ehara *et al.*, 1997; Faruque *et al.*, 2005; Jouravleva *et al.*, 1998; Kar *et al.*, 1996; Nakasone *et al.*, 1998; Waldor & Mekalanos, 1996). Some of them have been implicated in the horizontal transfer of genetic material between strains of *V. cholerae*. The discovery that what was formerly called the CTX element is in fact the integrated genome of the filamentous phage CTX ϕ (Waldor & Mekalanos, 1996) has profoundly modified the perspective with which scientists regard *V. cholerae*. It implies the existence of a horizontal transfer mechanism of the CT genes, which potentially pose the risk of virulence conversion of non-toxigenic environmental strains of *V. cholerae*. However, transduction of CT genes mediated by CTX ϕ depends on the expression of the phage receptor TCP by *V. cholerae*. An alternative mechanism of transmission of the CT genes, not dependent on TCP, is the one mediated by VGJ ϕ , through which these genes could be acquired by non-toxigenic strains not sensitive to CTX ϕ infection (Campos *et al.*, 2003a). This mechanism depends on the formation of a hybrid genome between

Table 2. Evaluation of CTX ϕ transduction to different strains mediated by VEJ ϕ

Donor strain*	Recipient strain†		
	KHT46 (MSHA [−])	KHT52 (TCP [−])	569B
569B (CTX-Kn ϕ)	ND	ND	4.1×10^6
569B (CTX-Kn ϕ /VEJ ϕ)	ND	5.5×10^4	4.2×10^5
569B (VEJ ϕ)	ND	ND	ND

*Cell-free culture supernatants of these strains were used for the infection assays.

†Recipient strains were grown under optimal conditions for the expression of the TCP receptor. The mean number of Kn^r transductants of each recipient strain, from three independent assays, is shown. ND, Not detected.

Fig. 4. Structure of the hybrid genome formed by the recombination of VEJφ with CTX-Knφ. (A) Map of the hybrid plasmid. Genes of CTXφ are shown in white, while genes of VEJφ are in grey. The Kn^r gene is shown in black. The novel junctions at the boundaries of both genomes are also shown. (B) Nucleotide sequences of the boundaries of recombination sites of CTXφ and VEJφ before site-specific recombination and sequences of the novel junctions after recombination, as determined by DNA sequencing. DNA sequences of CTXφ or VEJφ origin are shown in grey or black, respectively. The XerC- and XerD-binding sites are indicated and the XerC-cutting site is represented with an arrowhead.

those of VGJφ and CTXφ; thus a hybrid phage forms that uses MSHA as receptor (Campos *et al.*, 2003a). MSHA is a fimbria more ubiquitously expressed among vibrios, increasing the risk of virulence conversion through this phage receptor.

In this work we characterized VEJφ, a new filamentous phage also capable of transmitting the CT genes among strains of *V. cholerae* expressing the MSHA receptor by the same mechanism as used by VGJφ. This constitutes another example of gene transfer dependent on phages in *V. cholerae*, in particular for the transmission of the CT genes, the main virulence factor of this pathogen. The greater variety of phages that use MSHA as a receptor compared with those that use TCP confirms that the former is a pilus much more accessible to phages in the environment. Other described phages, such as VSK, could also potentially transduce the CT genes, since we found that VSK contains

integration sites similar to the *dif* site (Fig. 3). Thus, the diversity of phages capable of transducing genetic material in *V. cholerae* could be greater than formerly thought. The study of horizontal gene transfer in this bacterium is of great importance for understanding the future evolution of this pathogen and for the design of safer live attenuated vaccines.

REFERENCES

- Campos, J., Martinez, E., Marrero, K., Silva, Y., Rodriguez, B. L., Suzarte, E., Ledon, T. & Fando, R. (2003a). Novel type of specialized transduction for CTXφ or its satellite phage RS1 mediated by filamentous phage VGJφ in *Vibrio cholerae*. *J Bacteriol* **185**, 7231–7240.
- Campos, J., Martinez, E., Suzarte, E., Rodriguez, B. L., Marrero, K., Silva, Y., Ledon, T., del Sol, R. & Fando, R. (2003b). VGJφ, a novel filamentous phage of *Vibrio cholerae*, integrates into the same chromosomal site as CTXφ. *J Bacteriol* **185**, 5685–5696.
- Davis, B. M. & Waldor, M. K. (2003). Filamentous phages linked to virulence of *Vibrio cholerae*. *Curr Opin Microbiol* **6**, 35–42.
- Davis, B. M., Lawson, E. H., Sandkvist, M., Ali, A., Sozhamannan, S. & Waldor, M. K. (2000). Convergence of the secretory pathways for cholera toxin and the filamentous phage, CTXφ. *Science* **288**, 333–335.
- Ehara, M., Shimodori, S., Kojima, F., Ichinose, Y., Hirayama, T., Albert, M. J., Supawat, K., Honma, Y., Iwanaga, M. & Amako, K. (1997). Characterization of filamentous phages of *Vibrio cholerae* O139 and O1. *FEMS Microbiol Lett* **154**, 293–301.
- Falero, A., Caballero, A., Ferrán, B., Izquierdo, Y., Fando, R. & Campos, J. (2009). DNA binding proteins of the filamentous phages CTXφ and VGJφ of *Vibrio cholerae*. *J Bacteriol* **191**, 5873–5876.
- Faruque, S. M., Asadulghani, Kamruzzaman, M., Nandi, R. K., Ghosh, A. N., Nair, G. B., Mekalanos, J. J. & Sack, D. A. (2002). RS1 element of *Vibrio cholerae* can propagate horizontally as a filamentous phage exploiting the morphogenesis genes of CTXφ. *Infect Immun* **70**, 163–170.
- Faruque, S. M., Zhu, J., Asadulghani, Kamruzzaman, M. & Mekalanos, J. J. (2003). Examination of diverse toxin-coregulated pilus-positive *Vibrio cholerae* strains fails to demonstrate evidence for *Vibrio* pathogenicity island phage. *Infect Immun* **71**, 2993–2999.
- Faruque, S. M., Bin Naser, I., Fujihara, K., Diraphat, P., Chowdhury, N., Kamruzzaman, M., Qadri, F., Yamasaki, S., Ghosh, A. N. & Mekalanos, J. J. (2005). Genomic sequence and receptor for the *Vibrio cholerae* phage KSF-1φ: evolutionary divergence among filamentous vibriophages mediating lateral gene transfer. *J Bacteriol* **187**, 4095–4103.
- Huber, K. E. & Waldor, M. K. (2002). Filamentous phage integration requires the host recombinases XerC and XerD. *Nature* **417**, 656–659.
- Jouravleva, E. A., McDonald, G. A., Garon, C. F., Boesman-Finkelstein, M. & Finkelstein, R. A. (1998). Characterization and possible functions of a new filamentous bacteriophage from *Vibrio cholerae* O139. *Microbiology* **144**, 315–324.
- Kar, S., Ghosh, R. K., Ghosh, A. N. & Ghosh, A. (1996). Integration of the DNA of a novel filamentous bacteriophage VSK from *Vibrio cholerae* O139 into the host chromosomal DNA. *FEMS Microbiol Lett* **145**, 17–22.
- Karaolis, D. K. & Kaper, J. B. (1999). *Vibrio cholerae* TCP: a trifunctional virulence factor? Response. *Trends Microbiol* **7**, 393.
- Karaolis, D. K., Johnson, J. A., Bailey, C. C., Boedeker, E. C., Kaper, J. B. & Reeves, P. R. (1998). A *Vibrio cholerae* pathogenicity island associated

with epidemic and pandemic strains. *Proc Natl Acad Sci U S A* **95**, 3134–3139.

Kiefer, D. & Kuhn, A. (1999). Hydrophobic forces drive spontaneous membrane insertion of the bacteriophage Pf3 coat protein without topological control. *EMBO J* **18**, 6299–6306.

Kiefer, D., Hu, X., Dalbey, R. & Kuhn, A. (1997). Negatively charged amino acid residues play an active role in orienting the Sec-independent Pf3 coat protein in the *Escherichia coli* inner membrane. *EMBO J* **16**, 2197–2204.

Kimsey, H. H. & Waldor, M. K. (1998). CTX ϕ immunity: application in the development of cholera vaccines. *Proc Natl Acad Sci U S A* **95**, 7035–7039.

Kostrikis, L. G., Reisberg, S. A., Kim, H. Y., Shin, S. & Day, L. A. (1995). C2, and unusual filamentous bacterial virus: protein sequence and conformation, DNA size and conformation, and nucleotide/subunit ratio. *Biochemistry* **34**, 4077–4087.

Lee, C. A. (1999). *Vibrio cholerae* TCP: a trifunctional virulence factor? *Trends Microbiol* **7**, 391–392.

Marciano, D. K., Russel, M. & Simon, S. M. (1999). An aqueous channel for filamentous phage export. *Science* **284**, 1516–1519.

Marvin, D. A. (1998). Filamentous phage structure, infection and assembly. *Curr Opin Struct Biol* **8**, 150–158.

McMaster, G. K. & Carmichael, G. G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc Natl Acad Sci U S A* **74**, 4835–4838.

Mendez, C. & Chater, K. F. (1987). Cloning of *whiG*, a gene critical for sporulation of *Streptomyces coelicolor* A3(2). *J Bacteriol* **169**, 5715–5720.

Nakasone, N., Honma, Y., Toma, C., Yamashiro, T. & Iwanaga, M. (1998). Filamentous phage fs1 of *Vibrio cholerae* O139. *Microbiol Immunol* **42**, 237–239.

Rajanna, C., Wang, J., Zhang, D., Xu, Z., Ali, A., Hou, Y. M. & Karaolis, D. K. (2003). The vibrio pathogenicity island of epidemic *Vibrio cholerae* forms precise extrachromosomal circular excision products. *J Bacteriol* **185**, 6893–6901.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Serek, J., Bauer-Manz, G., Struhalla, G., van den Berg, L., Kiefer, D., Dalbey, R. & Kuhn, A. (2004). *Escherichia coli* YidC is a membrane insertase for Sec-independent proteins. *EMBO J* **23**, 294–301.

Thelin, K. H. & Taylor, R. K. (1996). Toxin-coregulated pilus, but not mannose-sensitive hemagglutinin, is required for colonization by *Vibrio cholerae* O1 El Tor biotype and O139 strains. *Infect Immun* **64**, 2853–2856.

Val, M. E., Bouvier, M., Campos, J., Sherratt, D., Cornet, F., Mazel, D. & Barre, F. X. (2005). The single-stranded genome of phage CTX is the form used for integration into the genome of *Vibrio cholerae*. *Mol Cell* **19**, 559–566.

Waldor, M. K. & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**, 1910–1914.

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