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Correspondence and requests for materials should be addressed to F.M.B. (e-mail: fmarbro@itsa.ucsf.edu). The structural coordinates are available from the Brookhaven Protein Databank (ID code: 1b89).

A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria

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The virulence properties of many pathogenic bacteria are due to proteins encoded by large gene clusters called pathogenicity islands[1,2], which are found in a variety of human pathogens including *Escherichia coli*, *Salmonella*, *Shigella*, *Yersinia*, *Helicobacter pylori*, *Vibrio cholerae*, and animal and plant pathogens such as *Dichelobacter nodosus* and *Pseudomonas syringae*[1–3]. Although the presence of pathogenicity islands is a prerequisite for many bacterial diseases, little is known about their origins or mechanism of transfer into the bacterium. The bacterial agent of epidemic cholera, *Vibrio cholerae*, contains a bacteriophage known as cholera-toxin phage (CTXΦ)[4], which encodes the cholera toxin, and a large pathogenicity island called the VPI (for *V. cholerae* pathogenicity island)[5] which itself encodes a toxin-coregulated pilus that functions as a colonization factor[6] and as a CTXΦ receptor[4]. We have now identified the VPI pathogenicity island as the genome of another filamentous bacteriophage, VPIΦ. We show that VPIΦ is transferred between *V. cholerae* strains and provide evidence that the TcpA subunit of the toxin-coregulated type IV pilus is in fact a coat protein of VPIΦ. Our results are the first description of a phage that encodes a receptor for another phage and of a virus–virus interaction that is necessary for bacterial pathogenicity.

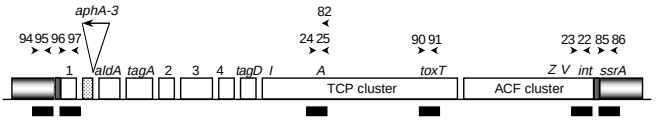

Figure 1 Diagram of the VPI. Arrows above genes indicate the PCR primers used and are identified by the number of the KAR primer series. Black bars, regions targeted for PCR; grey bars, common chromosomal flanking DNA; checked regions at each end of VPI denote *att* sites; dotted region (left) in the VPI represents the defective transposase (*xtn*). The *aphA*-3 gene encoding Km resistance is inserted into a *Bam*HI site between *xtn* and *aldA*.

Cholera is an ancient and life-threatening epidemic disease that occurs worldwide[7,8]. Virulent and epidemic strains of *V. cholerae* require two genetic elements to cause disease, CTXΦ[4] and VPI[5]. CTXΦ encodes the cholera toxin responsible for the severe secretory diarrhoea characteristic of the disease[4]. The VPI (Fig. 1) is required for the emergence of *V. cholerae* as it contains the toxin-coregulated pilus (TCP) gene cluster which encodes a type-IV pilus that functions both as an essential colonization factor[6,9] and as a CTXΦ receptor[4]. VPI has many features of bacterial pathogenicity islands (PAIs): it is large (~40 kilobases), contains genes associated with virulence, regulation and mobility, is inserted into a single chromosomal site (*att* site) adjacent to a tRNA-like gene, and it has a different G + C content compared with the host chromosome[4–6,10–13].

We previously identified one *V. cholerae* strain (E9120) that has lost the VPI (ref. 5). This strain contains *ctx* genes, suggesting that it previously had the VPI to allow infection by CTXΦ, and has an altered *att* site, indicating that the VPI had been excised. As the VPI contains a transposase-like gene and a phage-like integrase gene[5,13], we investigated other VPI genes using BLAST[14] to find ones whose predicted protein products share homology with phage or viral proteins. TagE has 30% identity and 55% similarity over 188 amino acids to Orf16 of *Staphylococcus aureus* bacteriophage Φ (Genbank accession number, AB009866); OrfZ has 26% identity and 41% similarity over 97 amino acids to an 'early' protein of rat cyto-

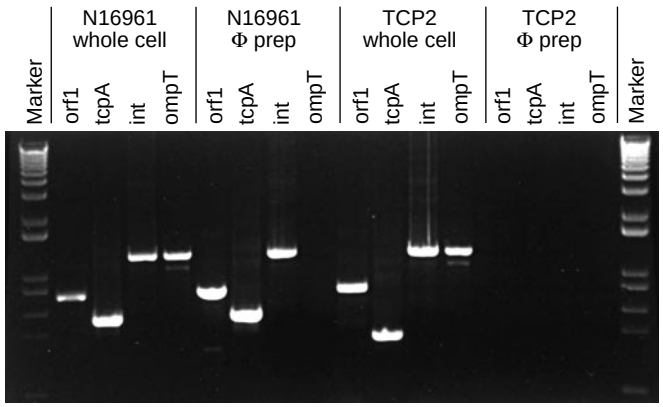

Figure 2 PCR products obtained from either whole-cell overnight cultures or cell-free phage (Φ) preparations of wild-type strain N16961 and TCP2. Note the presence of the VPIΦ genes *orf1*, *tcpA* and *int* in phage preparations of N16961 but not in the *tcpA* mutant strain TCP2. Markers are derived from a DNA ladder (0.1–12 kb; BRL).

Table 1 Detection of VPIΦ, CTXΦ and chromosomal genes

Strain	Sample	Detection of genes								
		VPIΦ				CTXΦ		Chromosomal		
		orf1	*tcpA*	*toxT*	*int*	*ctxB*	*zot*	flank VPI	*ompT*	*rfaD*
395										
	Whole cell	+	+	+	+	+	+	+	+	+
	Phage prep.	+	+	+	+	+	+	–	–	–
N16961										
	Whole cell	+	+	+	+	+	+	+	+	+
	Phage prep.	+	+	+	+	+	+	–	–	–
CVD110										
	Whole cell	+	+	+	+	+	–	+	+	+
	Phage prep.	+	+	+	+	–	–	–	–	–
TCP2										
	Whole cell	+	+	+	+	+	+	+	+	+
	Phage prep.	–	–	–	–	+	+	–	–	–
395-RF										
	Plasmid	+	+	+	+	+	+	–	–	–
N16961-RF										
	Plasmid	+	+	+	+	+	+	–	–	–
DK239										
	Whole cell	+	+	+	+	–	–	+	+	+
	Phage prep.	+	+	+	+	–	–	–	–	–

Genes were amplified by PCR from whole cell cultures, cell-free phage preparations and plasmid replicative-form preparations.

megalovirus (Genbank accession number, U62396); and OrfV has 32% identity and 47% similarity over 79 amino acids to the 'enhancin' protein of *Lymantria dispar* nucleopolyhedrovirus (Genbank accession number, AF019970). Even TcpA from the El Tor strain N16961 shows homology (31% identity, 48% similarity over 82 amino acids) to the product of a 769-amino-acid Orf from TT virus (Genbank accession number, AB011490). Taken together, these results indicated that the VPI could be the genome of a phage.

We tested this idea by examining phage preparations of *V. cholerae* strains N16961 and 395 for genes at the ends and centre of the VPI. By using the polymerase chain reaction (PCR) and sequencing, we found *orf1*, *tcpA*, *toxT* and *int* genes in both phage preparations (Fig. 1, 2 and Table 1), but no PCR products were amplified when primers targeted regions immediately outside the VPI, nor for *ompT* (which encodes an outer membrane protein) (Fig. 2) or *rfaD* (involved in lipopolysaccharide synthesis), which are also outside the VPI. A phage preparation derived from a VPI-negative strain prepared under identical conditions was also negative for the chromosomal *ompT* and *rfaD*, as well as for *orf1*, *tcpA*, *toxT* and *int* genes. As expected, PCR analysis of the preparations identified *ctxB* and *zot* genes on CTXΦ. Finding VPI genes in phage preparations after treatment with DNase and RNase indicated that the DNA was protected, presumably by a protein coat, and that this element was probably a bacteriophage (designated VPIΦ).

Phage DNA from strains N16961 and 395 was sensitive to digestion with S1 nuclease (specific for single-stranded DNA) and S1-treated phage DNA gave no PCR products. The same phage DNA was resistant to digestion with restriction endonucleases specific for double-stranded DNA and yielded VPI PCR products after digestion. Southern-blot analysis on phage DNA from DK238 (see below) and CVD110 using *tcpA* forward and reverse primers revealed that the phage DNA hybridized only with the primer that could hybridize to the positive strand (Fig. 3). Thus, VPIΦ, like CTXΦ (ref. 4), contains positive, single-strand DNA as its genome.

To determine whether VPIΦ has a plasmid replicative form (RF) in the cell, we analysed plasmid DNA from N16961 and 395 for VPI genes. RF preparations were sensitive to digestion with double-strand-specific *Pvu*II (which has a site in *tcpA*) as *Pvu*II-digested RF preparations failed to generate PCR fragments using *tcpA* primers (Fig. 4a), but were resistant to digestion with S1 nuclease and could still generate VPI PCR products after treatment with S1. PCR analysis revealed no chromosomal contamination as *ompT* and *rfaD* sequences were not amplified (Table 1). Southern-blot hybridization of RF preparations confirmed the presence of a VPIΦ replicative form. *Hae*III-digested RF from DK238 and CVD110, as well as N16961 chromosomal DNA, hybridized with a *tcpA* PCR product probe, whereas only chromosomal DNA hybridized with an *ompT* probe (Fig. 4b, c). These results indicate that, like other

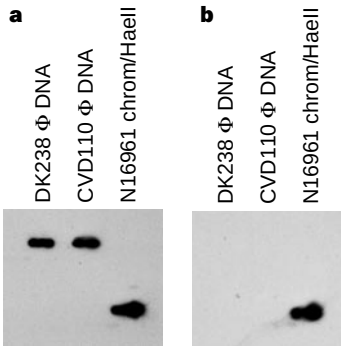

Figure 3 Southern blot of phage DNA preparations from DK238 and CVD110. **a**, Hybridization of the phage DNA with a single-stranded *tcpA* probe (KAR25) specific for the (+) strand. **b**, Lack of hybridization of phage DNA with a single-stranded *tcpA* probe (KAR24) specific for the (–) strand. Both probes hybridized to chromosomal DNA from *V. cholerae* N16961.

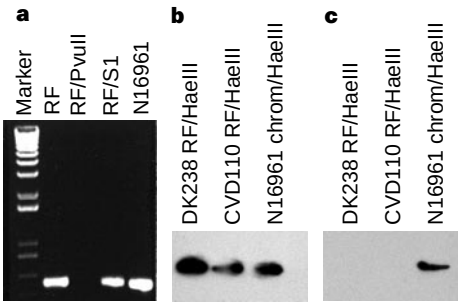

Figure 4 Analysis of the VPIΦ replicative form. **a**, PCR analysis of the RF using *tcpA* primers KAR24 and KAR25. Note that a *tcpA* product is not generated by using *Pvu*II-digested RF as template, whereas RF treated with S1 nuclease (RF/S1) generates a PCR product. N16961 chromosome was used as a control template. **b**, Southern-blot hybridization of *Hae*III-digested RF from DK238 and CVD110 probed with a *tcpA* PCR product; **c**, Southern-blot hybridization of *Hae*III-digested RF probed with an *ompT* PCR product shows hybridization only to the N16961 chromosomal control.

filamentous phages, VPIΦ forms a double-stranded-DNA plasmid replicative form in the cell.

The VPIΦ genome of a spontaneously streptomycin-resistant (Str) strain (N16961) was marked with the *aphA*-3 gene, which encodes resistance to kanamycin (Km) and neomycin (Neo), creating strain DK238. *aphA*-3 was inserted between *aldA* and *xtn* (Fig. 1), a region encoding a putative non-functional transposase[5]. We used whole cells and cell-free phage preparations from donor DK238 to determine whether VPIΦ could be transferred into the non-toxigenic VPI-negative strain DK236 (serogroup O10, nalidixic acid (Nal) resistant). After 1 hour of incubation at 37 °C of 3×10^7 DK236 recipient cells with either 3×10^7 donor DK238 cells or phage preparations from 2×10^7 DK238 cells, cultures were plated onto agar containing Nal/Neo. The use of a Nal-sensitive donor and Nal-resistant recipient enabled us to identify Nal-resistant tranductants that had acquired *aphA*-3 from VPIΦ. With DK238 cells, we obtained 1.3×10^4 transductants, suggesting that, on average, about 0.04% of recipient cells were transduced under these conditions; with phage preparations from 2×10^7 donor cells, we obtained $> 3 \times 10^8$ transductants per 3×10^7 recipients, indicating that VPIΦ made by one donor cell gives rise to at least 15 transductants. The successful transfer of the neomycin-resistance marker with cell-free DNase-treated phage preparations indicated that transduction, rather than conjugation, was the mechanism of DNA transfer.

Transfer of VPIΦ from donor to recipient was confirmed for one transductant, DK239, by using O antigen agglutination, antibiotic-susceptibility testing, ribotyping, colony hybridization with *aphA*-3 and *tcpA* probes, and PCR analysis of *aphA*-3, *tcpA*, *toxT* and *int* genes (Table 1). PCR on DK239 using primers for the right VPI junction showed that the VPI had integrated into the same site as wild-type VPI-positive strains. PCR on phage preparations from DK239 indicates that, although DK239 produces VPIΦ, CTXΦ was not acquired (Table 1).

Not all *V. cholerae* strains could act as donors or recipients of VPIΦ. Although we detected VPIΦ particles in phage preparations of classical strain 395, no VPIΦ was transferred when a derivative of 395 (also marked by *aphA*-3 in the VPI) was used as donor, suggesting that this strain is unable to transfer VPIΦ efficiently, or that different conditions are needed. With N16961 as donor, transfer occurred into the O10 strain DK236 but not into the non-toxigenic VPI-negative strain DK237 (serogroup O1). As both DK236 and DK237 have a vacant *att* site[5], these strains may differ in their ability to acquire VPIΦ, possibly explaining the limited number of toxigenic serogroups. Epidemic and pandemic cholera is associated with O1 and O139 strains. Although we have shown that VPI^+ non-O1/non-O139, potentially toxigenic strains can be created *in vitro*, VPI^+CT^+ O1 strains may have an advantage *in vivo* or in the environment over VPI^+CT^+ non-O1 strains, explaining the predominance of O1 strains in epidemic and pandemic disease. Our results highlight the potential for serogroups of *V. cholerae* other than O1 and O139 to acquire the VPI and become pathogenic and even epidemic and pandemic strains.

Concentrated phage preparations of several VPI-positive *V. cholerae* strains were viewed under electron microscopy. Immunoelectron microscopy of preparations from strain 395 using rabbit anti-TcpA antibodies revealed many gold particles bound to parallel bundles of VPIΦ particles (Fig. 5a). El Tor strain CVD110, which has the *ctxA*, *zot*, *ace* and *orfU* genes deleted[15], cannot produce CTXΦ or CTXΦ-encoded genes, but does contain VPIΦ genes (Table 1). Our CVD110 preparation contained many phage particles, some of which formed a 'braided' network of filaments, presumably representing VPIΦ (Fig. 5b). Immunoelectron microscopy of phage preparations from El Tor strain N16961, like 395, revealed numerous gold particles bound to filamentous phage (Fig. 5c).

As VPI is a bacteriophage and the structure of type-IV pili resembles that of a filamentous bacteriophage[16], we investigated whether the TCP pilin subunit, TcpA, could be a VPIΦ coat protein. TCP2 is a derivative of strain 395 with a large internal deletion in the *tcpA* gene that prevents it from producing TCP[17]. No VPIΦ genes were detected in phage preparations from TCP2, indicating that TcpA is required for VPIΦ production; in contrast, CTXΦ genes were evident (Table 1 and Fig. 2). In immunoprecipitation experiments on these phage preparations, rabbit anti-TcpA-peptide antibodies and mouse anti-rabbit agarose beads bound VPIΦ, allowing selective removal of the complex. PCR analysis on N16961 and 395 immunoprecipitates revealed VPIΦ-encoded genes (Fig. 6). We

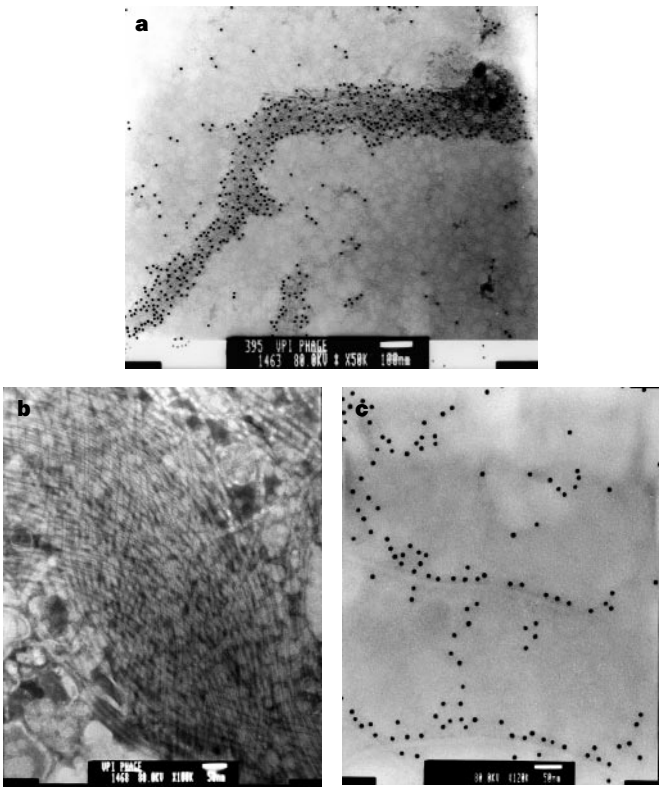

Figure 5 Electron micrographs of VPIΦ. **a**, Phage preparations from 395 incubated with rabbit anti-TcpA antibody and 10-nm colloidal gold-conjugated goat anti-rabbit IgG (original magnification, ×50,000; scale bar, 100 nm); **b**, phage preparations from CVD110 (magnification, ×100,000; scale bar, 50 nm). **c**, Phage preparations from N16961 incubated with rabbit anti-TcpA antibody and 10-nm colloidal gold-conjugated goat anti-rabbit IgG (magnification ×120,000; scale bar, 50 nm).

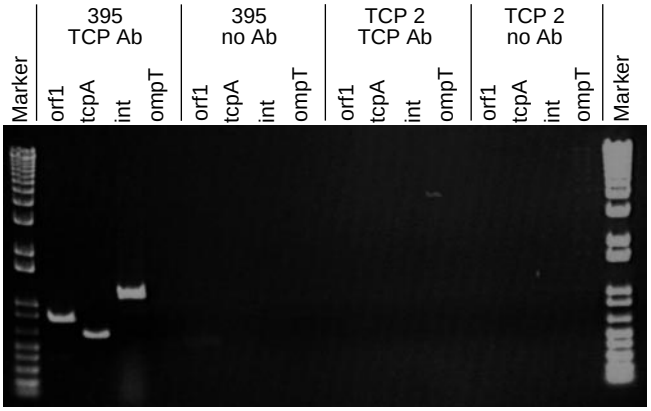

Figure 6 PCR products of immunoprecipitated phage preparations from strains 395 and TCP2, following incubation with and without anti-TCP antibodies (Ab), demonstrate specificity of anti-TCP antibody for VPIΦ. Genes amplified by PCR are indicated above each well.

Table 2 Detection of genes in phage immunoprecipitation reactions

Strain	Sample	Detection of genes							
		orf1	*tcpA*	*toxT*	*int*	*ctxB*	flank VPI	*ompT*	*rfaD*
395									
	Anti-TCP	+	+	+	+	–	–	–	–
	Bead only	–	–	–	–	–	–	–	–
TCP2									
	Anti-TCP	–	–	–	–	–	–	–	–
	Bead only	–	–	–	–	–	–	–	–

PCR was used on phage preparations from 395 (wild type) and TCP2 (*tcpA* mutant) immunoprecipitated in the presence or absence of anti-TCP antibody.

demonstrated antibody specificity for TcpA and VPIΦ by the lack of PCR products in similar experiments done without anti-TcpA antibodies (Table 2). PCR on phage preparations from an independent *tcpA* mutant, RT4032, which contains an in-frame (non-polar) deletion in *tcpA*, also failed to generate VPI genes; however, the *tcpA* mutants TCP2 and RT4032 could be complemented by supplying *tcpA* on a plasmid (pRT198) because PCR detected VPI genes in phage preparations of both transformants (Fig. 7). These results together support the idea that TcpA is an important coat protein of VPIΦ.

Our findings help explain how non-pathogenic bacteria can become pathogens. Many bacterial pathogens contain clusters of genes that encode virulence factors responsible for inducing disease. We have shown that the large cluster of genes essential for the epidemic properties of *V. cholerae* originates from viral (bacteriophage) DNA that has become incorporated into the bacterial chromosome, and that transfer of the unusually large filamentous bacteriophage VPIΦ confers new virulence on the recipient. Type IV pili like TCP are expressed by a variety of human and animal pathogens, including enteropathogenic *E. coli*, *Neisseria gonorrhoeae*, *P. aeruginosa* and *D. nodosus*[18–21], and may also have a bacteriophage origin and transferable genes that endow the bacterium with virulence factors. It is not understood how TCP serves as both a bacteriophage and colonization factor, particularly as it has not been shown to bind directly to intestinal tissue or to cultured epithelial cells. The type-IV pilus of enteropathogenic *E. coli*, BFP, which shares significant homology with TCP, was recently reported to function in colonization by mediating bacteria-to-bacteria adherence, thereby increasing the bacterial mass that colonizes the intestine[22]. TCP likewise mediates interbacterial adherence (autoagglutination is a standard assay for this structure), so released VPIΦ/TCP pili might help colonization by serving as a bridge between phage/pili bound to different bacteria. TCP is also the receptor for CTXΦ (ref. 4), creating a situation in which one phage serves as the receptor for a second phage in a sequential infection process that results in bacterial virulence. Our results highlight the potential contribution of virulence-conferring phage ('pathophage') in the emergence of pathogens, now and in the future. □

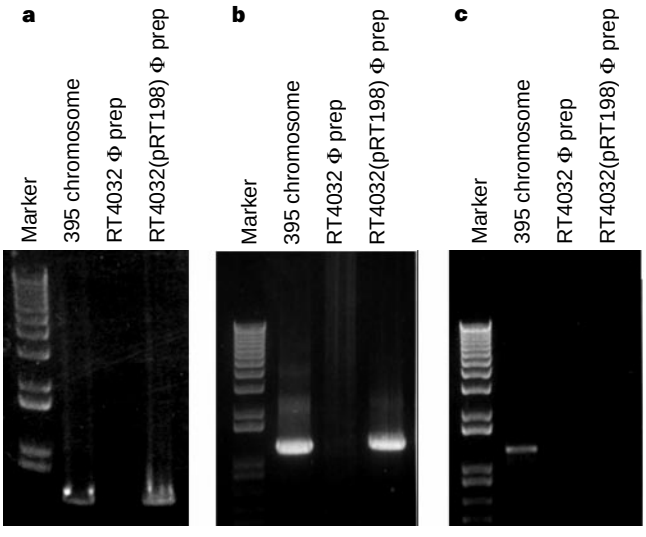

Figure 7 PCR analysis of VPI genes from a *tcpA* mutant and mutant complemented with *tcpA*. **a**, PCR done with *tcpA* primers KAR24 and KAR82; **b**, PCR done with *orf1* primers; **c**, PCR done with *rfaD* primers. Note the absence of a *tcpA* and *orf1* PCR product in phage preparations from RT4032 (owing to its inability to make VPIΦ) and the presence of products when RT4032 is complemented with pRT198 supplying *tcpA*.

Methods

Bacterial strains and plasmids. Strain 395 is a representative of the sixth pandemic clone (classical biotype) of *V. cholerae*; strain N16961 is an isolate from the current seventh ('El Tor') pandemic which began in 1961 in Indonesia. CVD110 is a derivative of a seventh pandemic strain (E7946) which is deleted in its *ctxA*, *zot*, *ace* and *orfU* genes[15]. The environmental isolates DK236 and DK237 are non-toxigenic VPI-negative serogroup O10 and O1 strains, respectively, and are Nal-resistant owing to selection of a spontaneous resistant mutant. To construct DK238, the *xtn–aldA* region of the chromosomally integrated N16961 VPI was amplified by PCR with primers KAR166 (located in *orf1*) and KAR167 (located in *aldA*). This 1.8-kb fragment was ligated into pGEM-T, creating pDK40. This plasmid was digested with *Bam*HI and the *aphA*-3 gene (encoding Km/Neo resistance) was inserted, creating pDK42, which was digested with *Sph*I/*Sac*I; the *xtn–aldA*::*aphA*-3 fragment was ligated into the suicide vector pCVD442, creating pDK43. Plasmid pDK43 was used in an allelic exchange procedure to introduce the *aphA*-3-containing fragment into the homologous region of the N16961 chromosome, creating strain DK238. Strain TCP2 is derived from 395, has amino-acid residues 119–154 deleted from its *tcpA* gene and does not produce the TCP structure[17]. RT4032 (from R. Taylor) is an in-frame *tcpA* deletion mutant of 395 in which the codons encoding amino acids +1 of mature TcpA through the TAA stop codon and one additional T nucleotide are removed. Plasmid pRT198 is a pBR322-based plasmid with a 2-kb *Hin*dIII fragment containing *tcpA* of 395 cloned into the *Hin*dIII site of pBR322.

PCR and sequencing. PCR was performed as described[23] under the following conditions: denature at 96 °C for 3 min; annealing, 48 °C, 30 s; extension, 72 °C, 2 min for 1 cycle, then denature 96 °C, 30 s; annealing, 48 °C, 30 s; extension, 72 °C, 2 min for 30 cycles. Primers used to determine the presence and sequence of *V. cholerae* genes were: *orf1*, KAR96, 5′-TGCTACTTACCCAATGGCAC-3′ and KAR97, 5′-GAGCCAGGCTTATTTGGGCG-3′; *tcpA*, KAR24, 5′-AAAACCGGTCAAGAGGG-3′ and KAR25, 5′-CAAAAGCTACTGTGAATGG-3′ for seventh pandemic strains and KAR82, 5′-CAAATGCAACGCCGAATGG-3′ for sixth pandemic strains; *toxT*, KAR90, 5′-ATAACTTTACGTGGATGGC-3′ and KAR91, 5′-AAAATCAGTGATACAATCG-3′; *int*, KAR22, 5′-GATAAAGAGATCAAAGCC-3′ and KAR23, 5′-ATCTGCTTCCATGTGGG-3′. Additional primers included KAR94, 5′-TATGATACTGAAAACACCTC-3′ and KAR95, 5′-GATGCTAACAGCAGAGCATA-3′ (outside left VPI junction); KAR85, 5′-CGCCTGCGAACCGACACGC-3′, and KAR86, 5′-GCAGCAAGCCTCCACTCCG-3′ (outside the right VPI junction); K898, 5′-GAATTCTGTCGGGTTGTAATCCTG-3′ and K643, 5′-GCCATACTCAGCATATACAC-3′ (*ompT*); K371, 5′-CGGGATCCGAGCTCATTACCTACACTAGTG-3′ and K369, 5′-CGGGATCCGACAGGCTATAATGCGTGCAAC-3′ (*rfaD*); KAR161, 5′-AAAATTCCTTGACGAATACC-3′ and KAR162, 5′-TTGCTTCTCATCATCGAACC-3′ (*ctxB*); KAR166, 5′-GACAGGATTACTGAGATATCTG-3′ and KAR167, 5′-AACCAAGGTGAGGTTTGTACC-3′ (*xtn-aldA* region). Primers were synthesized using an Applied Biosystems DNA synthesizer and sequenced

with a *Taq* Dye-Terminator kit (Perkin-Elmer) and an automated 373A DNA sequencer (Applied Biosystems).

Isolation of phage and replicative form. Isolation of phage from *V. cholerae* was done essentially as described[24]. In brief, 1-litre Luria broth cultures were grown overnight at either 30 °C (395) or 37 °C (N16961). Cultures were centrifuged twice at 10,000 *g* and the supernatant was passed through a 0.45-μm low-protein-binding filter. DNase I and RNase I (Boehringer Mannheim) were added to the filtrate at a final concentration of 1 μg ml^{-1} and incubated at room temperature for 3 h. NaCl and PEG 8000 were added to a final concentration of 1 M and 10% w/v, respectively, and the mixture was left to precipitate overnight at 4 °C. The supernatant was centrifuged at 11,000 *g* for 20 min and the pellet resuspended in 4 ml of SM buffer. PEG was removed with an equal volume of chloroform. This supernatant was layered onto a $CsCl_2$ step gradient consisting of 2 ml each of $CsCl_2$ in SM buffer ($d = 1.7$, 1.5 and 1.45). After centrifugation at 25,000 r.p.m. for 2 h in an SW41 rotor (Beckman), the lower phage band ($1.45 \leqslant d \leqslant 1.5$) was extracted and dialysed against two changes of TM buffer. The phages were further concentrated by the addition of PEG 8000 (10% w/v) and placed on ice for 2 h. The phage preparation was centrifuged at 14,000 *g* for 20 min and the resulting pellet (containing phage particles) was resuspended in 100 μl of SM buffer. PEG was removed with an equal volume of chloroform. 5 μl of the phage preparation was used for PCR.

The replicative form was isolated from 1 litre of Luria broth culture as described[24].

Although filamentous phage do not lyse the cell or have a 'burst size', we calculated the approximate number of VPIΦ and CTXΦ released per cell and the RF copy number. The number of phage released was calculated by determining the amount of phage DNA (from its absorbance) in 1 litre of overnight culture of DK238 and CVD110 containing $\sim 10^{12}$ cells. As strain DK238 is positive for VPIΦ and CTXΦ and CVD110 is positive for only VPIΦ, the difference in amount (in μg) between the two strains should reflect the number of CTXΦ genomes (7 kb) and the balance the number of VPIΦ genomes (40 kb) released per cell. We estimate that 280 and 200 μg l^{-1} of ssDNA is present in a phage preparation from 1-litre cultures of DK238 and CVD110, respectively. If 1 μg of 1-kb ssDNA contains 1.8×10^{12} molecules, then 1 μg of 7-kb ssDNA contains 2.6×10^{11} CTXΦ molecules and 80 μg contains 2×10^{13} molecules, so 2×10^{13} molecules/10^{12} cells indicates that an average of 20 CTXΦ are made per cell during overnight culture. Likewise, for VPIΦ we calculate that an average of 9 VPIΦ are produced per cell. Our calculations all assume that no other phage (or RF) is produced and that DNA extraction is 100% efficient, which is unlikely, so values could be underestimates.

We estimated that 57 and 39 μg l^{-1} of dsDNA are present in RF preparations from 1-litre cultures of DK238 and CVD110, respectively, which yields an average CTXΦ RF copy number of 2 per cell after overnight culture; the average VPIΦ RF copy number is similarly estimated as 1 per cell.

Immunoprecipitation. DNase- and RNase-treated phage preparations were incubated with rabbit anti-TcpA peptide antibody (1:10,000) at 37 °C and with vigorous shaking for 1 h. Mouse anti-rabbit IgG (whole molecule) agarose beads (Sigma) were added and the reaction was incubated at 4 °C overnight with gentle rotation. After several low-speed centrifugations and washings in deionized water, the pellet was resuspended in deionized water.

Electron microscopy. Phage preparations of CVD110 and 395 were placed on a carbon–formvar-coated 300-mesh copper grid (Electron Sciences) for 2 min then negatively stained with 1.5% phosphotungstic acid for 1 min and analysed by electron microscopy. In addition, equal volumes of 395 and N16961 phage preparations were separately incubated with rabbit anti-peptide TcpA antibody (1:10,000) at 37 °C for 20 min, after which this suspension was placed on a grid for 5 min, and 10 μl of 10-nm colloidal gold-conjugated goat anti-rabbit IgG (ICN Biomedicals) was added before negative staining.

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Structure of Cdc42 in complex with the GTPase-binding domain of the 'Wiskott–Aldrich syndrome' protein

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The Rho-family GTP-hydrolysing proteins (GTPases), Cdc42, Rac and Rho, act as molecular switches in signalling pathways that regulate cytoskeletal architecture, gene expression and progression of the cell cycle[1]. Cdc42 and Rac transmit many signals through GTP-dependent binding to effector proteins containing a Cdc42/Rac-interactive-binding (CRIB) motif[2]. One such effector, the Wiskott–Aldrich syndrome protein (WASP), is postulated to link activation of Cdc42 directly to the rearrangement of actin[3].