

Genomic Sequence and Receptor for the *Vibrio cholerae* Phage KSF-1Φ: Evolutionary Divergence among Filamentous Vibriophages Mediating Lateral Gene Transfer

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KSF-1Φ, a novel filamentous phage of *Vibrio cholerae*, supports morphogenesis of the RS1 satellite phage by heterologous DNA packaging and facilitates horizontal gene transfer. We analyzed the genomic sequence, morphology, and receptor for KSF-1Φ infection, as well as its phylogenetic relationships with other filamentous vibriophages. While strains carrying the *mshA* gene encoding mannose-sensitive hemagglutinin (MSHA) type IV pilus were susceptible to KSF-1Φ infection, naturally occurring MSHA-negative strains and an *mshA* deletion mutant were resistant. Furthermore, D-mannose as well as a monoclonal antibody against MSHA inhibited infection of MSHA-positive strains by the phage, suggesting that MSHA is the receptor for KSF-1Φ. The phage genome comprises 7,107 nucleotides, containing 14 open reading frames, 4 of which have predicted protein products homologous to those of other filamentous phages. Although the overall genetic organization of filamentous phages appears to be preserved in KSF-1Φ, the genomic sequence of the phage does not have a high level of identity with that of other filamentous phages and reveals a highly mosaic structure. Separate phylogenetic analysis of genomic sequences encoding putative replication proteins, receptor-binding proteins, and Zot-like proteins of 10 different filamentous vibriophages showed different results, suggesting that the evolution of these phages involved extensive horizontal exchange of genetic material. Filamentous phages which use type IV pili as receptors were found to belong to different branches. While one of these branches is represented by CTXΦ, which uses the toxin-coregulated pilus as its receptor, at least four evolutionarily diverged phages share a common receptor MSHA, and most of these phages mediate horizontal gene transfer. Since MSHA is present in a wide variety of *V. cholerae* strains and is presumed to express in the environment, diverse filamentous phages using this receptor are likely to contribute significantly to *V. cholerae* evolution.

Vibrio cholerae is the host for a variety of bacteriophages (vibriophages), which include virulent phages as well as temperate phages represented by the kappa-type phages produced by most strains of the El Tor biotype (18, 36). Another group of vibriophages includes the filamentous phages, which have a single-stranded DNA (ssDNA) genome (1, 8, 23, 25, 40). Several of the *V. cholerae*-specific filamentous phages have been implicated in virulence gene transfer among *V. cholerae* strains (2, 7, 40). Filamentous phages of *V. cholerae* have also been found to be distinct from the well-characterized filamentous coliphages in that some of these phages can form lysogens (1, 25, 40). The most remarkable of these phages is CTXΦ (40), which exists as a prophage in toxigenic *V. cholerae* and encodes cholera toxin (CT).

Mechanisms associated with the induction and propagation of CTXΦ and related phages have been a major area of interest in vibriophage biology (5, 6, 9–12). In toxigenic El Tor and

O139 strains of *V. cholerae*, CTX prophage is integrated in the bacterial genome arrayed in different tandem structures along with a related satellite phage RS1 (5, 6). The genome of RS1 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 (5–7, 39). Thus, satellite phage RS1 can replicate autonomously but depends on its helper phage CTXΦ for assembly and secretion of RS1 viral particles (6, 12, 39). Conversely, RS1 encodes the antirepressor RstC, which is not present in CTX prophage. This protein promotes transcription of CTXΦ genome and RS1 genes by counteracting the activity of the phage repressor RstR (6). We have shown previously that production of RS1Φ particles can also occur independent of CTXΦ but using functions encoded by another putative filamentous phage, called KSF-1Φ (13). RS1Φ produced via this process is capable of infecting recipient strains by a mechanism which is independent of the CTXΦ receptor toxin-coregulated pilus (TCP). In the present study, we examined the morphology, host range, genomic sequence,

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and receptor for KSF-1 Φ infection and analyzed phylogenetic relationships among diverse filamentous vibriophages which can mediate horizontal gene transfer.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *V. cholerae* host strains used for phage preparations or as recipients in transduction assays were from either clinical or environmental sources. Clinical strains were from patients who attended the treatment Center of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B), located in Dhaka. The environmental strains were isolated from surface waters in Dhaka. Strains were stored either in lyophilized form or in sealed deep nutrient agar at room temperature. Before use, the identities of the *V. cholerae* strains were confirmed by biochemical reaction and serology (42), and the presence or absence of genes encoding TCP and mannose-sensitive hemagglutinin (MSHA) pilus was ascertained using DNA probes or PCR assays as described below. The genetically marked phage genome pKSF1-Km was a derivative of the replicative-form (RF) DNA of KSF1 Φ , which was marked with a kanamycin resistance (Km^r) determinant as described below.

Probes and hybridization. The KSF-1 Φ genome was detected in naturally occurring *V. cholerae* strains by its positive hybridization with a probe derived from pKSF-1, the RF of the phage genome (13). Strand-specific oligonucleotide probes corresponding to the plus and minus strand of the KSF-1 Φ genome were also used to detect the presence of the single-stranded KSF-1 Φ genome. Colony blots or Southern blots were prepared using nylon filters (Hybond; Amersham Biosciences, Uppsala, Sweden) and processed by standard methods (29). The polynucleotide probes were labeled by random priming (16) using a random primers DNA labeling kit (Invitrogen Corporation, Carlsbad, CA) and [α - ^{32}P]dCTP (3,000 Ci/mmol; Amersham), and oligonucleotide probes were labeled by 3' tailing using terminal deoxynucleotidyl transferase and [α - ^{32}P]dCTP (Amersham). Southern blots and colony blots were hybridized with the labeled probes and autoradiographed by standard methods (29).

PCR assays. All oligonucleotides used either as probes or PCR primers were synthesized commercially by Oswel DNA Service (University of Edinburgh, Edinburgh, United Kingdom), and PCR reagents and kits were purchased from Perkin-Elmer Corp. (Norwalk, CT). Presence of *tcpA* and *mshA* genes were detected by PCR assays as described previously (26, 34). The expected sizes of the PCR amplicons were ascertained by electrophoresis with agarose gels. Identities of all PCR products were further verified using specific oligonucleotide probes.

Construction of pKSF1-Km. The RF of KSF-1 Φ genome (pKSF-1), was isolated from the native host strain 55V71 (13) by standard methods (29), and a preliminary analysis of restriction endonuclease cleavage sites within pKSF-1 was done. The pKSF-1 DNA was then marked by inserting a Km^r marker. This was done by ligating XbaI-digested linearized pKSF-1 with a Km^r determinant derived from XbaI-cleaved pCTX-Km (40). A recipient *V. cholerae* strain, Env-002, was electroporated with the ligated DNA, and transformants were selected on Luria agar plates containing kanamycin (50 μ g/ml). Km^r colonies were analyzed for the presence of pKSF1-Km.

Preparation of phage. Overnight cultures of *V. cholerae* were diluted 100-fold in fresh LB medium and grown for 6 h at 30°C with shaking. Supernatant fluids were sterilized by filtration through 0.22- μ m-pore-size filters (Millipore Corporation, Bedford, MA). To confirm that the filtrates did not contain any bacterial cells, aliquots of the filtrates were streaked on Luria agar plates and incubated overnight at 37°C. For phage assays, aliquots of the sterile supernatant fluids were incubated with the recipient strains and plated on appropriate antibiotic plates as described in the following section.

For isolation and analysis of phage nucleic acids, the filtrates were mixed with one-fourth volumes of a solution containing 20% polyethylene glycol 6000 and 10% NaCl and centrifuged at 12,000 \times g to precipitate the phage particles. The precipitate was dissolved in a solution containing 20 mM Tris-Cl (pH 7.5), 60 mM KCl, 10 mM MgCl₂, and 10 mM NaCl and digested with pancreatic DNase I (100 units/ml) and RNase A (50 μ g/ml) at 37°C for 2 h to remove possible nucleic acids carried over from lysed bacterial cells. The solution was extracted with phenol-chloroform to disrupt phage particles, and the total nucleic acids were precipitated with ethanol. The nucleic acids were analyzed by Southern hybridization using appropriate probes (13) to detect the presence of relevant phage genomes.

Transduction assays. The susceptibilities of recipient strains to KSF- ϕ were assayed using the genetically marked phage KSF1-Km ϕ prepared from culture supernatants of Env-002 (pKSF1-Km). Phage particles were precipitated from 50-ml aliquots of filtered sterile supernatants, and the pellet was suspended in

100 μ l of TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (20 mM Tris-HCl [pH 7.5], 10 mM NaCl, 0.1 mM Na₂-EDTA). Recipient cells were grown in Trypticase soy broth (TSB) medium (Difco Laboratories, Becton Dickinson and Company, Sparks, MD) at 37°C, precipitated by centrifugation, and washed in fresh TSB. Approximately 10⁵ bacterial cells were mixed with 10 μ l of the phage preparation in a final volume of 100 μ l. Each mixture was inoculated into 5 ml of TSB and incubated for 1 h at 37°C, and aliquots of the culture were analyzed by plating on Luria agar plates containing kanamycin (50 μ g/ml) and on plates without kanamycin. The ratio of Km^r transduced colonies to the total number of colonies derived from the recipient strain was calculated and expressed as the susceptibility to KSF-1 Φ infection. To test the inhibition of phage infection with anti-MSHA antibody, dilutions of anti-MSHA monoclonal antibody HA17:10, provided by A.-M. Svennerholm (33), in parallel with TSB medium as a control were mixed with recipient cells and the mixtures were incubated at 37°C for 30 min prior to the addition of the phage.

Analysis of infected cells. Representative infected colonies were grown overnight in LB containing kanamycin (50 μ g/ml), and cells were precipitated by centrifugation. The supernatant fluids of the cultures were titrated for the presence of KSF1-Km ϕ particles using strain Env-002 as the recipient. Total DNA or plasmids were extracted from bacterial pellets by standard methods (29) and purified using microcentrifuge filter units (Ultrafree-Probind; Sigma Chemical Company, St. Louis, MO). Integration of the phage genome into the chromosome of the recipient cells was determined by comparative Southern blot analysis of total DNA and plasmid preparations from the phage-infected and the corresponding native strains as described previously (10, 12).

MSHA testing. Expression of MSHA was tested by slide agglutination as described previously (17) with *V. cholerae* grown in TSB medium (Difco) at 37°C, using chicken erythrocytes.

DNA sequencing and analysis of genomic sequence. Nucleotide sequencing was performed with an automated DNA sequencing system (ABI Prism 310; PE Applied Biosystems, Foster City, CA) using BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems). Initially, overlapping subclones of pKSF-1 were constructed in pUC18 and were sequenced using universal sequencing primers (Invitrogen). The nucleotide sequences of both strands of pKSF-1 were further determined by primer walking with primers derived from the preliminary sequencing of pKSF-1 subclones in pUC18. Sequences were processed using the Sequencher alignment program, Version 4.0 (Gene Codes Corporation, Inc., MI). The nucleotide sequence of KSF-1 was compared to sequences in the GenBank databases, and the protein homology search was done using the National Center for Biotechnology Information BLAST server program. Different phage gene sequences were aligned using the CLUSTAL-W multiple sequence alignment program (3), and phylogenetic analyses were conducted using Molecular Evolutionary Genetics Analysis software (MEGA version 2.1; Arizona State University, Tempe, Arizona) (27). Trees were constructed by neighbor joining using the Jukes-Cantor distance method. Bootstrap values were calculated based on 1,000 computer-generated trees.

Nucleotide sequence accession number. The sequence of the KSF-1 Φ genome has been assigned GenBank accession number AY714348.

RESULTS AND DISCUSSION

KSF-1 Φ is viable and infectious. The importance of filamentous phages in *V. cholerae* evolution is beginning to be understood in more detail with the discovery of new phages involved in lateral gene transfer. We previously observed the presence of extrachromosomal pKSF-1 DNA with non-O1/non-O139 strain 55V71 and a single-stranded version of pKSF-1 in phage DNA preparations from the same strain. This led us to assume that pKSF-1 is the genome of a filamentous phage. Furthermore, pKSF-1 supported morphogenesis of the *V. cholerae* satellite phage RS1 (13).

In the present study, electron microscopic examination of KSF-1 Φ showed that the phage was indeed a filamentous particle (Fig. 1). Filamentous phages which belong to the genus *Inovirus* of the family *Inoviridae* are known to be long proteinaceous tubes ranging in length between 0.8 and 2 μ m and containing a single-stranded circular DNA genome. The single-stranded DNA is converted to a double-stranded RF in

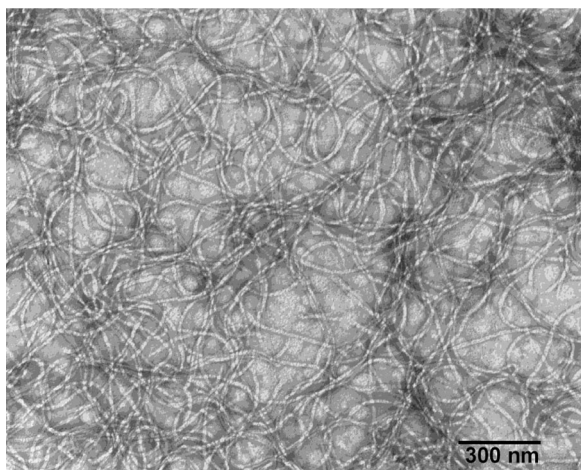


FIG. 1. Electron micrograph of KSF-1 phage particles. Phage particles were isolated from the culture supernatant of strain Env-002 infected with KSF1-KmΦ. Phage preparations from the native strain Env-002 did not show any detectable phage particles.

infected cells. Consistent with this definition, the length of KSF-1Φ particle was 1.2 μm, with a width of 7 nm, and the particle contained a single-stranded DNA genome. The size of KSF-1Φ is similar to that of a previously described filamentous phage, fs2, of *V. cholerae* O139 (23). In this study, we further demonstrate that KSF-1 phage is able to mediate transfer of its own genome to recipient cells by the formation of infectious particles. To monitor the production of phage particles, we constructed a genetically marked derivative of the phage genome which carried a Km^r determinant. The Km^r also allowed us to conveniently monitor phage infection, since infected cells became resistant to kanamycin. We found that a variety of *V. cholerae* strains, including *V. cholerae* O1 and non-O1 strains, were infected by the phage (Table 1). The infected strains produced moderate to high titers (2.7×10^3 to 1.5×10^6) of new infectious particles. Production of the phage particles by infected cells was not associated with cell lysis, and KSF-1Φ did not form plaques on a lawn of susceptible bacteria.

MSHA pilus is the receptor in KSF-1Φ infection. Filamentous phages are known to utilize pili as their receptors, and *V. cholerae* produces several types of pili and carries the genes for many pilin-like proteins (19). The most remarkable of these is the CTXΦ receptor TCP, which is also the major intestinal colonization factor. We previously found that RS1Φ produced by KSF-1Φ-positive strains, presumably using KSF-1Φ virion proteins, did not use the CTXΦ receptor TCP for infecting recipient cells (13). In the present study, we investigated whether a different type IV pilus, MSHA, which is produced by a wide variety of *V. cholerae* strains, could act as a receptor in KSF-1Φ infection. First, we identified groups of naturally occurring *V. cholerae* strains which were (i) negative for the *mshA* gene encoding MSHA and (ii) positive for the *mshA* gene and produced MSHA. These strains were exposed to KSF1-KmΦ, the genetically marked derivative of KSF-1Φ. While strains which were negative for *mshA* were resistant to the phage, the MSHA-positive strains were found to be infected (Table 1). The susceptibility varied between 2.1×10^{-5} to 6.2×10^{-2} for different strains. Classical biotype strains are mostly hemagglu-

TABLE 1. Susceptibility of *V. cholerae* strains carrying different combinations of MSHA and TCP genes to a genetically marked derivative of KSF-1Φ^a

Strain	Description	Presence of		Susceptibility to KSF1-KmΦ
		<i>mshA</i>	<i>tcpA</i>	
AK-31047	O1	+	+	5.4×10^{-3}
C6706	O1	+	+	3.2×10^{-2}
KHT46	C6706Δ <i>mshA</i>	-	+	0
AL-11089	O139	+	+	2.3×10^{-2}
Env-99	O139	+	+	7.5×10^{-3}
Env-002	O1	+	+	6.3×10^{-3}
O395	O1	+	+	2.7×10^{-5}
TCP-2	O395 Δ <i>tcpA</i> Δ <i>ctxA</i>	+	-	2.1×10^{-5}
55V71	Non-O1, non-O139	+	-	4.2×10^{-2}
761V1325	Non-O1, non-O139	+	-	3.4×10^{-2}
775V1346	Non-O1, non-O139	+	-	6.2×10^{-2}
825V1409	Non-O1, non-O139	-	-	0
824V1406	Non-O1, non-O139	-	-	0
828V1412	Non-O1, non-O139	-	-	0
819V1399	Non-O1, non-O139	+	-	5.7×10^{-2}
820V1401	Non-O1, non-O139	+	-	6.3×10^{-2}
883V1486	Non-O1, non-O139	+	-	9.9×10^{-3}
886V1491	Non-O1, non-O139	+	-	9.2×10^{-3}

^a Presence of different genes was detected using DNA probes and PCR assays. Susceptibility to the phage was determined using a genetically marked phage (see text for details). The values are the proportions of total cells infected (number of colonies growing on kanamycin plates divided by the total number of colonies recovered). Values are averages from three independent assays.

tinin negative. However, they carry the *mshA* pilin gene and express the gene, although at a very low level (24). In agreement with this previous observation, the classical biotype strain O395 and its TCP-deleted derivative TCP2 (37) were infected by KSF-1 phage at a low frequency (Table 1).

The biological activity of MSHA is known to be inhibited by D-mannose. We, therefore, presumed that if MSHA is the receptor for KSF-1 phage, the addition of D-mannose to block the receptor should render MSHA-positive strains resistant to the phage. We found that the addition of increasing concentrations of D-mannose solution to recipient cells, prior to the assay for susceptibility to KSF-1Φ, progressively inhibited infection of the MSHA-positive strains (Fig. 2). These findings indicated that MSHA pilus can act as a receptor in KSF-1Φ infection. We further tested this assumption by using an isogenic pair of strains, in one of which the *mshA* gene was deleted (38). While the wild-type strain was infected by KSF-1Φ at high efficiency, the mutant was completely resistant to the phage (Table 1). Finally, we used a monoclonal antibody against MSHA to block the putative receptor and test whether the antibody inhibited infection of MSHA-positive cells by KSF-1Φ. In our assay, incubation with the antibody for 30 min prior to the addition of phage particles completely inhibited infection of otherwise susceptible cells by KSF-1Φ. Taken together, these results suggested strongly that MSHA is the receptor in KSF-1Φ infection. Several other filamentous vibriophages, including fs1, fs2, 493, and VGJ, reportedly use MSHA as their receptor (2, 8, 24). Most of these phages have been either presumed or demonstrated to be involved in lateral gene transfer in *V. cholerae*. This study shows that KSF-1Φ, which also mediates horizontal gene transfer, constitutes another example of a filamentous phage using MSHA pili as receptors in infecting recipient strains.

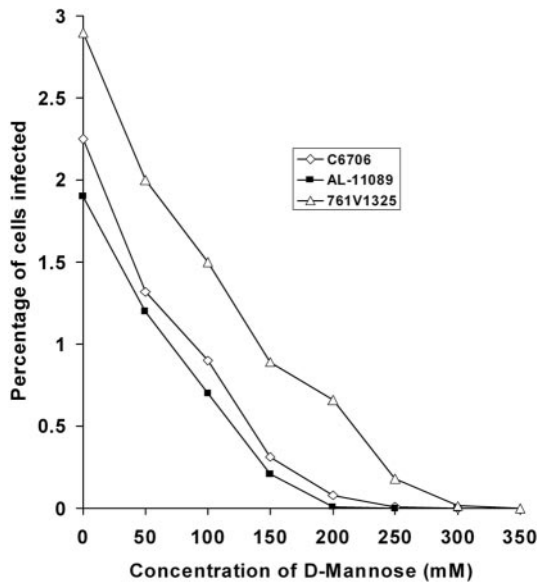


FIG. 2. Effect of D-mannose on the susceptibility of *V. cholerae* strains to KSF1-KmΦ. The identity (and serotype) of strains tested are C6706 (O1, El Tor), AL-11089 (O139), and 761V1325 (non-O1/non-O139).

Genomic organization of KSF-1Φ. Determination of the nucleotide sequence of the KSF-1Φ genome revealed that the phage genome comprises 7,107 nucleotides and contains 14 open reading frames (ORFs; ORFI through ORFIV) as shown in Fig. 3. The ORFs were preceded by purine-rich consensus sequences (Shine-Dalgarno sequence) for potential ribosome binding sites. Of the 14 ORFs, 13 were located on one of the

strands, and we assumed that this strand was the viral strand (plus strand) of the single-stranded KSF-1Φ genome. This was subsequently confirmed by hybridizing phage DNA preparations with single-strand-specific oligonucleotide probes. A homology search showed that 4 of the 14 predicted protein sequences of KSF-1Φ were homologous to previously reported phage proteins. The percent homology with the sequence of various previously reported phage proteins varied from 25% to 100%. Detailed findings of the homology search are presented in Table 2.

The peptide encoded by ORFI of KSF-1Φ is homologous to the replication proteins of previously described phages VGJ, VSK, VSKK, and fs1 (1, 8, 25). ORFI is also similar in terms of position and size to genes of previously reported filamentous phages, which mapped at the same relative position as the *gII* gene of M13 phage (Fig. 3). This gene encodes the pII protein, which is necessary for rolling-circle replication of the phage genome (31). The deduced amino acid sequence of the putative peptide encoded by ORFII is homologous to peptides of previously described filamentous vibriophages that mapped at the same position as the ssDNA-binding protein of Ff phages (f1, M13, and fd), and its size was similar to that of this protein (Fig. 3). We therefore assume that ORFI and ORFII of KSF-1 phage are homologues of the *gII* and *gV* genes of Ff phages and thus constitute the putative replication module of KSF-1 phage. ORFIII through ORFVII of KSF-1Φ map at the same relative position as *gVII*, *gIX*, *gVIII*, *gIII*, and *gVI*, respectively, of the M13 phage, although there was no significant homology of the putative peptides encoded by these ORFs with that of M13. Of these five ORFs, the first four had sizes comparable to the corresponding genes of previously described Ff phages (31), and these genes are known to encode the minor and

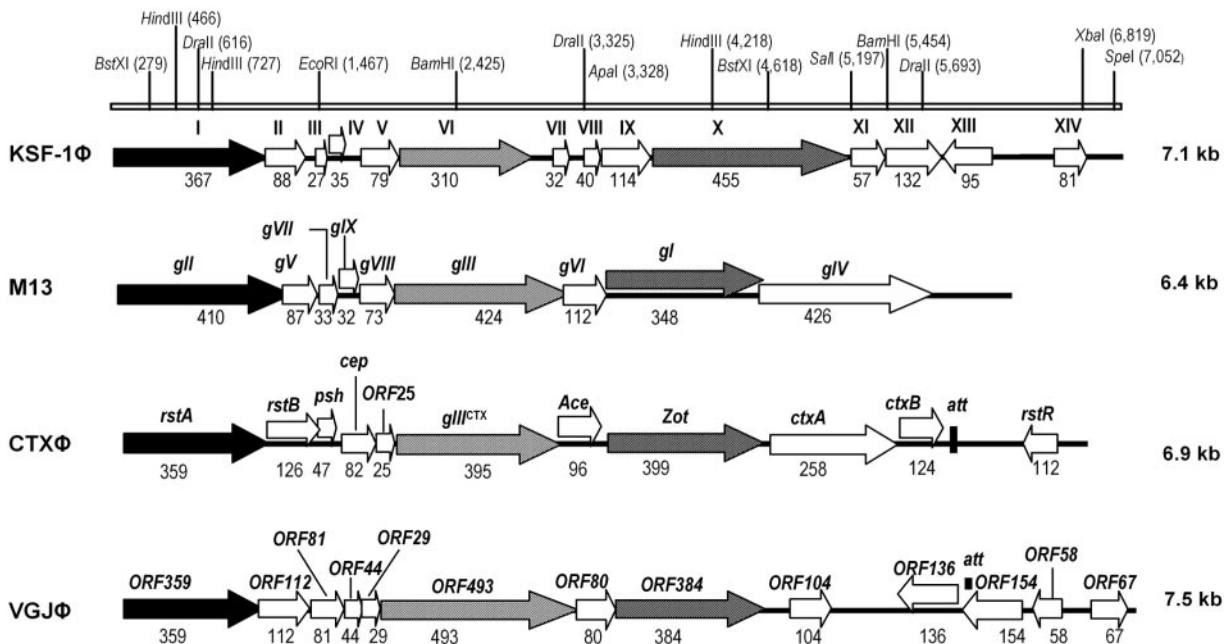


FIG. 3. Genomic organization of KSF-1Φ. Linear ORF maps of M13, CTXΦ, VGJΦ, and KSF-1 phages, aligned by using the first base of the replication initiator gene. The restriction map of KSF-1Φ is shown on top. ORFs or genes are represented by arrows oriented in the direction of transcription, and filled or striped arrows represent genes included in the phylogenetic analyses.

TABLE 2. Results of BLAST search showing reported homology of ORFs and intergenic regions of KSF-1 phage with those of other phages or bacteria

ORF/intergenic region	Nucleotide position	Homology to gene products of other bacteriophage or bacterium		Percent identity	Accession number
		Phage	Host bacterium		
I	23–283	VGI	<i>V. cholerae</i>	60	NP835472
I	1–1101	VSKK	<i>V. cholerae</i>	98	NP536619
I	1–1101	VSK	<i>V. cholerae</i>	89	NP542355
I	1–498	fs1	<i>V. cholerae</i>	99	NP695201
I	40–1083	CTX	<i>V. cholerae</i>	38	NP231097
I	40–1083	Vf33, Vh12, f237, Vfo3K6, Vfo4K68,	<i>V. parahaemolyticus</i>	38	BAA33512, BAA33520, NP797930, NP059531, NP059541
I	40–1095		<i>Vibrio vulnificus</i>	34	NP936848
I	292–1101	VGIphi	<i>V. cholerae</i>	100	NP835472
I	408–491	VSK	<i>V. cholerae</i>	65	NP542355
I	469–972		<i>V. cholerae</i>	45	AAP84006
I	496–1101	fs1	<i>V. cholerae</i>	99	NP695202
II	1109–1327	VGI	<i>V. cholerae</i>	98	NP835473
II	1109–1327	VSKK, fs1	<i>V. cholerae</i>	98	NP536620 NP695203
II	1136–1327	VSK	<i>V. cholerae</i>	92	NP542356
II	1166–1324	Vf33, Vh12, f237, Vfo3K6, Vfo4K68	<i>V. parahaemolyticus</i>	44	BAA33506, BAA33514, NP797931, NP059532, NP059542
Intergenic	1333–1449	VGI	<i>V. cholerae</i>	69	NP835473
Intergenic	1333–1449	fs1	<i>V. cholerae</i>	69	NP695203
Intergenic	1333–1425	VSKK	<i>V. cholerae</i>	77	NP536620
Intergenic	1333–1425	VSK	<i>V. cholerae</i>	77	NP542356
Intergenic	1333–1425	Vf33, Vh12	<i>V. parahaemolyticus</i>	48	BAA33506, BAA33514
Intergenic	1333–1425	Vfo3K6, Foa4K68, f237	<i>V. parahaemolyticus</i>	45	NP059532, NP059542, NP797931
IX	3453–3782	Vfo3K6, f237, Vfo4K68	<i>V. parahaemolyticus</i>	27	NP059536, NP797936, NP059546
X	3800–5164	Vfo3K6, Vfo4K68, f237	<i>V. parahaemolyticus</i>	39	NP059537, NP059547, NP797937
X	3800–4807	CTX	<i>V. cholerae</i>	25	AAL09680
X	3800–4762		<i>Pseudomonas aeruginosa</i>	23	ZP00138787
X	3800–4612	PH	<i>Pseudomonas aeruginosa</i>	26	NP039606
Intergenic	6942–7106	VGI	<i>V. cholerae</i>	58	NP835484
Intergenic	6955–7032	VGI	<i>V. cholerae</i>	69	NP835484

major capsid proteins. ORFVI of KSF-1 Φ is located at the same relative position as *gIII* of F ϕ phages and the *gIII^{CTX}* of CTX phage and is similar in size (20). The *gIII* gene encodes pIII, a minor capsid protein that recognizes and interacts with the receptors and coreceptors of these phages. It thus appears that ORFIII through VII of KSF-1 phage encodes putative structural proteins of the phage, including a pIII-like protein involved in receptor binding. Protein encoded by ORFX of KSF-1 Φ was found to have a modest level of homology with the Zot-like protein of *Vibrio parahaemolyticus* phages f237, and VfO4K68, as well as to the Zot protein of CTX phage (Table 2). The relative position and size of the *zot* gene is similar to the *gI* gene, which encodes pI in F ϕ phages and is needed for assembly and secretion of the viral particles (30, 35). Thus, it is likely that the product of ORFX plays a similar role in KSF-1 Φ .

Additionally, some filamentous phages encode transcriptional repressors, such as RstR of CTX Φ and vpf122 of Vf33 phage, which regulate the expression of other phage genes (6). In CTX Φ and in VJG Φ , the repressor genes are transcribed in the direction opposite that of most other genes. We identified an ORF (ORFXIII) in KSF-1 Φ , which also transcribes in the opposite direction, although it was not clear whether this ORF encodes a functional repressor. The functions of putative peptides encoded by ORFIX, ORFXI, ORFXII, and ORFIV are not known. The KSF-1 genome also contains an 18-bp *att*-like sequence, CAAGCCGATACTGCGCGA, which is similar but not identical to that of other integrative filamentous phages. It also remains to be established whether this site is involved in possible phage integration. Generally, the genome of a filamentous phage is organized in a modular structure, in which functionally related genes are grouped (21). These include the replication module containing the genes coding for rolling-circle replication protein and the ssDNA-binding protein, the structural module containing the major and minor coat protein-encoding genes, and the assembly and secretion module containing genes for morphogenesis and extrusion of the virus particles. Although the functions of all the gene products encoded by the putative ORFs of KSF-1 Φ are not clearly known and, overall, KSF-1 Φ appears to have a mosaic genomic structure, the genetic organization of other filamentous phages appear to be preserved in KSF-1.

Evolutionary relationships of KSF-1 Φ and other filamentous vibriophages. The coevolution of *V. cholerae* as a pathogen and genetic elements that mediate the transfer of virulence genes is becoming increasingly evident. Since the discovery of CTX Φ , a number of filamentous phages have been reported to be involved in lateral gene transfer (2, 13, 14, 32). However, the phylogenetic relationships among these filamentous phages have not been adequately explored. Construction of phylogenetic trees using different regions of the genomic sequence of KSF-1 Φ and corresponding regions of previously described filamentous vibriophages allowed us to assess evolutionary relatedness and divergence between KSF-1 Φ and other filamentous phages of *V. cholerae* as well as *V. parahaemolyticus*. The current trend towards using sequence data for phylogenetic analysis has raised the question of identifying the most appropriate level for analysis. Lawrence and colleagues (28) have discussed the problems associated with viral genomes having mosaic structures and that mosaicism of viral genomes under-

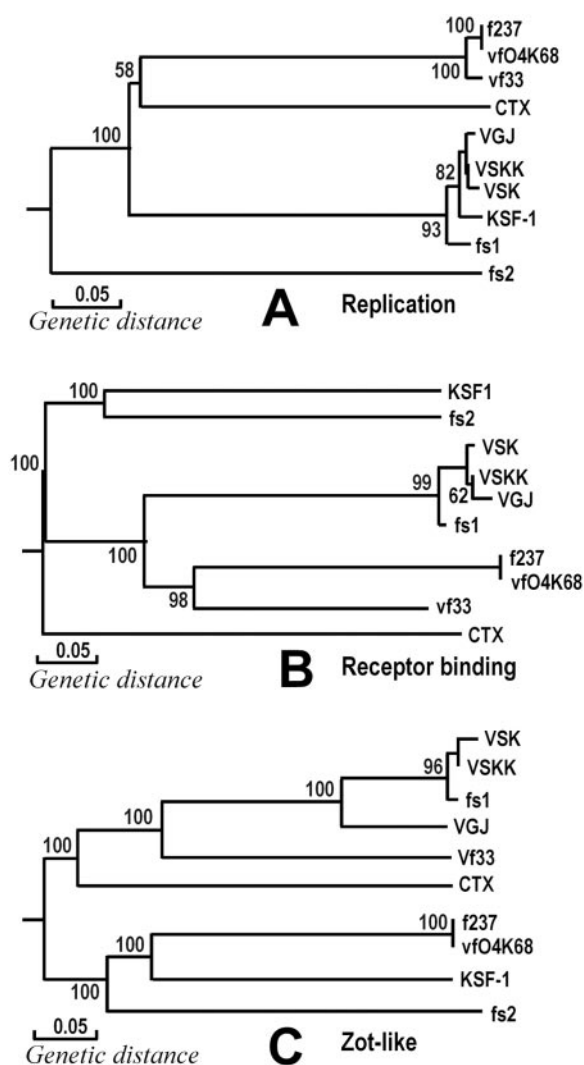


FIG. 4. Phylogenetic relationships among gene sequences of putative replication proteins (A), Zot-like proteins (B), and pIII-like receptor-binding coat proteins (C) of 10 different filamentous vibriophages. The trees were constructed from pairwise Jukes-Cantor distances by using the neighbor-joining method. Bootstrap values based on 1,000 computer-generated trees are indicated at the nodes, and only values greater than 60 are shown.

mines the validity of approaches based on comparison of whole phage genomes. It has also been suggested that phylogenetic analyses should be performed at the level of individual genes, since these represent the functional units of highly mosaic viral genomes (41). We therefore selected three essential genes or corresponding ORFs encoding putative replication proteins, pIII-like receptor-binding coat proteins, and Zot-like phage assembly proteins for phylogenetic analysis. These three ORFs in the KSF-1 Φ genome all showed modest to high levels of homology with corresponding genes of one or more previously described filamentous vibriophages (Table 2). Estimates of genetic relationships based on the sequence of these genes among 10 different filamentous vibriophages are shown in Fig. 4.

Based on the sequence of replication protein genes, the *V.*

parahaemolyticus phages clustered together, but at least three distinct branches were observed among the *V. cholerae* phages (Fig. 4). These branches were represented by CTX phage, fs2 phage, and a group of five other phages, including KSF-1, VGJ, VSK, VSKK, and fs1. Thus, KSF-1 appears to be closely related to VGJ, VSK, VSKK, and fs1 in its replication protein gene. However, in the phylogenetic tree based on Zot-like protein genes, KSF-1Φ appeared more closely related to phages f237 and vfO4K68 of *V. parahaemolyticus* than to *V. cholerae*-specific filamentous phages. Similarly, phage vf33 of *V. parahaemolyticus* belonged to a different branch, closer to a cluster *V. cholerae* phages. CTX phage and fs2 phage also belonged to distinct branches of this phylogenetic tree. The most notable feature of this analysis is the divergent clustering of pIII-like protein genes (Fig. 4) carried by phages sharing the same receptor. This gene encodes a minor capsid protein that recognizes and interacts with the phage receptor. Understandably, CTXΦ, which uses TCP as its receptor, belongs to a branch distinct from those of the rest of the *V. cholerae* phages. However, KSF-1 and another five *V. cholerae* phages, fs1, fs2, VGJ, VSK, and VSKK, which reportedly use MSHA as their receptors, did not cluster together but, rather, belonged to four different branches. Interestingly, phage fs2, which uses the same receptor as KSF-1, fs1, VSK, VSKK, and VGJ phages, is diverged from these phages in all three of the genomic regions examined. Overall, we found that *V. cholerae* phages KSF-1, fs1, VSK, VSKK, and VGJ belong to a closely related cluster in terms of their replication proteins, but KSF-1Φ is considerably diverged from this cluster in the sequence of receptor-binding proteins and Zot-like proteins. The overall sequence homology of KSF-1Φ with other filamentous phages was also not high and ranged between 34.3% for CTXΦ to 52.5% for VSK phage. These results are consistent with the mosaic genomic structure of KSF-1Φ, which seems to have evolved through extensive horizontal exchange of genetic materials among these phages and, possibly, other unidentified phages.

In contrast to the well-characterized filamentous coliphages, several of the filamentous phages of *V. cholerae* integrate into the host chromosome (1, 2, 40). KSF1Φ genome was also occasionally found to integrate into the chromosome of susceptible *V. cholerae* (Fig. 5). This is consistent with the presence of genomic sequence encoding a putative protein with homology to that of an integrase protein of phage VSK described previously (25). However, the mechanisms related to this integration event are not clear from this study. As mentioned above, the KSF-1Φ genome also contains an 18-bp *att*-like sequence which is similar but not identical to the *att* site of VGJΦ and other integrative filamentous phages. Further studies are under way to characterize the chromosomal junctions and understand the mechanisms leading to KSF-1Φ integration. Phages VGJ and KSF-1 have been recently shown to participate in the transfer of CTXΦ or the RS1 satellite phage in a TCP-independent manner. Horizontal transfer of CTX and RS1 by VGJ phage involves site-specific cointegration and formation of a hybrid phage genome (2). On the other hand, transfer of RS1 by KSF-1Φ seems to occur by heterologous packaging of excised RS1 element into viral particles (13). Thus, the mechanisms involved in lateral gene transfer by these two phages appear to be different.

The CT-converting phage CTXΦ is also known to carry a

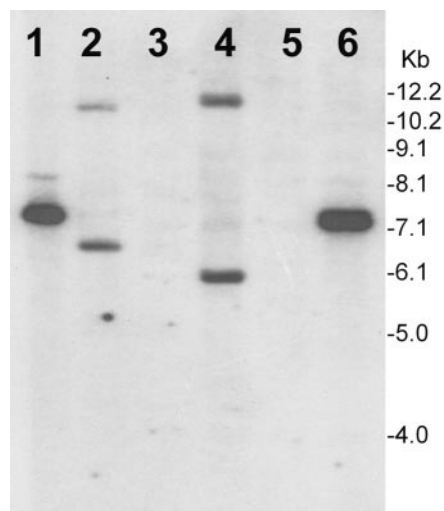


FIG. 5. Southern hybridization analysis of total genomic DNA or plasmids isolated from *V. cholerae* strains infected with KSF1-KmΦ, demonstrating phage integration. Total DNA or plasmids were digested with XbaI and hybridized with the pKSF-1 probe. Lanes 1 and 6, pKSF linearized with XbaI; lanes 2 and 4, total DNA from infected strains N16961 and SA406, respectively; lanes 3 and 5, plasmid preparations of strains N16961 and SA406, respectively. Numbers indicating molecular sizes of bands correspond to a 1-kb DNA ladder (Invitrogen). The absence of bands in the lanes containing plasmid preparations and the positive hybridization of restricted chromosomal DNA of the transductants suggest integration of the KSF1-KmΦ genome into the chromosome of recipient strains.

phage repressor gene, *rstR*, which encodes the RstR protein that provides immunity to superinfection by the same phage. We did not detect an ORF with a predicted protein product homologous to RstR in KSF-1, and strains carrying KSF-1Φ were susceptible to superinfection by the same phage. For example, strain 55V71, which carried a native KSF-1Φ, was also susceptible to superinfection by the genetically marked KSF1-Km phage (Table 1). Interestingly, however, the large intergenic region between ORFXIII and ORFXIV of the KSF-1Φ genome contained a 27-bp sequence (bases 6098 to 6124) which was identical to part of the nucleotide sequence of *rstR* gene, suggesting that KSF-1Φ might have been derived from an ancestral phage which had functional RstR.

Significance of MSHA and filamentous phages in lateral gene transfer. Whereas CTXΦ uses the TCP pili for infecting recipient cells, at least four other phages, including fs1, fs2, VGJ, and KSF, use the MSHA pili as their receptor. It is interesting that fs1, fs2, KSF-1, and VGJ have considerable divergence in the sequences of their putative receptor-binding proteins (Fig. 4). Thus, MSHA pili can act as a receptor of genetically diverse filamentous phages, and several of these phages have been shown to mediate horizontal gene transfer. In previous studies, the *mshA* gene encoding MSHA was found to be present in a wide variety of *V. cholerae* strains (15, 34). Hence, this phage receptor is apparently ubiquitous among *V. cholerae* strains, and filamentous phages mediating gene transfer could spread rapidly among these strains.

Phage VGJ, which also uses MSHA as its receptor, has been reported to facilitate the transfer of CTX phage as well as RS1 satellite phage genome by forming hybrid genomes (9). In our

study, however, RS1 was transferred through alternative packaging of circularized single-stranded RS1 instead of the KSF-1 genome. Particularly because of their flexible capsid structure, filamentous phages may be uniquely suited to mobilizing genes and larger elements. Hence, one or more of these vibriophages are also likely to package possible other DNA elements carrying the appropriate signals, and thus we speculate that the range of DNA transfer mediated by filamentous phages may be considerably more than currently appreciated. In this regard, it is also remarkable that a gene (*orf8*) associated with a filamentous vibriophage has been linked exclusively to strains associated with the "pandemic" O3:K6 clone of *Vibrio parahaemolyticus* (22, 32).

Little is known about the expression of TCP in the aquatic habitats of *V. cholerae*, but it is reasonable to assume that it would be low compared to that in the intractant environment. On the other hand, MSHA pili have been shown to be involved in adherence of *V. cholerae* to zooplankton (4). Therefore, MSHA is likely to be expressed more in the natural aquatic habitat of *V. cholerae*.

The consequences of acquisition of genes by horizontal transfer is only beginning to be understood in molecular detail. The acquisition of virulence genes and pathogenicity islands contributes to the improved evolutionary fitness of *V. cholerae* as a pathogen and this, in turn, may promote its more efficient seeding of the environment and establishment of endemicity. In the present study, we show that phages involved in horizontal gene transfer may be diverse in terms of their evolutionary lineage and mechanism of gene transfer but can share common receptors. Moreover, lack of homoimmunity for such phages as KSF-1 Φ can allow superinfection of the same host by more than one KSF-1-derived phage particle carrying different DNA elements, which could accumulate and result in quantum leaps in the evolution of *V. cholerae*. Clearly, further studies are required to understand how vibriophages interact with *V. cholerae* to promote this organism's acquisition of the critical genes which alter its virulence or adaptation to its environmental niche.

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