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pIII^{CTX}, a Predicted CTXϕ Minor Coat Protein, Can Expand the Host Range of Coliphage fd To Include *Vibrio cholerae*

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CTXϕ is a filamentous bacteriophage that encodes cholera toxin. CTXϕ infection of its host bacterium, *Vibrio cholerae*, requires the toxin-coregulated pilus (TCP) and the products of the *V. cholerae tolQRA* genes. Here, we have explored the role of OrfU, a predicted CTXϕ minor coat protein, in CTXϕ infection. Prior to the discovery that it was part of a prophage, *orfU* was initially described as an open reading frame of unknown function that lacked similarity to known protein sequences. Based on its size and position in the CTXϕ genome, we hypothesized that OrfU may function in a manner similar to that of the coliphage fd protein pIII and mediate CTXϕ infection as well as playing a role in CTXϕ assembly and release. Deletion of *orfU* from CTXϕ dramatically reduced the number of CTXϕ virions detected in supernatants from CTXϕ-bearing cells. This defect was complemented by expression of *orfU* in *trans*, thereby confirming a role for this gene in CTXϕ assembly and/or release. To evaluate the requirement for OrfU in CTXϕ infection, we introduced fragments of *orfU* into *gIII* in an fd derivative to create OrfU-pIII fusions. While fd is ordinarily unable to infect *V. cholerae*, an fd phage displaying the N-terminal 274 amino acids of OrfU could infect *V. cholerae* in a TCP- and TolA-dependent fashion. Since our findings indicate that OrfU functions as the CTXϕ pIII, we propose to rename OrfU as pIII^{CTX}. Our data also provide new evidence for a conserved pathway for filamentous phage infection.

The molecular interactions that mediate the entry of *Escherichia coli*-derived filamentous phages into their hosts have been studied in considerable detail. pIII, a phage minor coat protein found on one tip of the virion, accounts for host specificity in both N-pilus (IKE and I2-2)- and F-pilus (Ff)-specific phages (fd, f1, M13) (3, 8, 11, 24). The initial step in Ff phage infection is mediated by the binding of pIII to the tip of the F pilus (19). The Ff phages do not absolutely require the F pilus for infection; however, Ff infection of F$^-$ *E. coli* occurs at much reduced frequencies (32). The binding of pIII to F is thought to result in pilus retraction, which brings the phage particle in close proximity to and perhaps through the bacterium's outer membrane. Subsequent translocation of Ff DNA through the periplasm and inner membrane requires the products of the *tolQRA* genes both in F$^+$ and in F$^-$ *E. coli* (32, 36, 37). Recent studies suggest that, in addition to binding to the pilus, pIII also binds to TolA, which serves as a coreceptor (4, 30). Although the exact role of TolQ and TolR in the infection process is unknown, they appear to interact with TolA through their inner membrane spanning regions (9, 21), and they may ordinarily constitute a channel for importing as yet unknown substrates across the inner membrane (42). Following the TolA-pIII interaction, pVIII, the Ff major coat protein, is inserted into the host cell inner membrane, and the phage DNA is translocated into the cytoplasm of the host cell (5, 33).

The 424-amino-acid Ff pIII is thought to consist of a leader sequence (2) and three domains, separated by glycine-rich regions, that serve distinct roles in phage entry and release. The first two pIII domains, designated g3p-D1 (residues 1 to 86) and g3p-D2 (residues 105 to 236), are required for Ff adsorption and entry (7, 30, 35), while the third domain, g3p-D3 (residues 270 to 406), is required for the assembly and release of Ff particles from host cells (6, 29). g3p-D1, the "penetration" domain, and g3p-D2, the "adsorption" domain, have been shown to bind to the Ff coreceptors TolA and F, respectively. Holliger and coworkers have proposed that following binding of g3p-D2 to F, the conformation of pIII changes and that this shift reveals the TolA binding site in g3p-D1. Thus, Ff infection is thought of as a two-step process in which different domains of pIII sequentially bind to F and then to TolA (16, 23).

The C terminus of pIII (g3p-D3) has been implicated in the assembly of Ff particles and in their release from the bacterial membrane. In the absence of pIII, Ff virions remain attached to the cell surface in long "polyphages" (6, 22, 28, 29). According to the model of Rakonjac et al. (28), pIII and pVI (another minor coat protein) are incorporated into assembling phage particles through interactions with pVIII to form a "pretermination complex." Only the last 83 amino acids of pIII are required to form this complex. A conformational change in g3p-D3 may disrupt the association of pIII with the inner membrane, enabling capping and release of virions. g3p-D3 is also required for the production of stable virions that are resistant to certain detergents (28).

CTXϕ is a *Vibrio cholerae*-derived lysogenic filamentous phage that contains *ctxAB*, the operon encoding cholera toxin, in its 7-kb genome. Nontoxigenic *V. cholerae* strains can be converted to toxigenicity by CTXϕ infection (41). As in Ff infection of *E. coli*, CTXϕ infection of *V. cholerae* requires a

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TABLE 1. List of strains and plasmids used in this study

Strains and plasmids	Description or genotype	Source or reference
Strains		
V. cholerae		
O395	O1 classical strain; Sm^r	26
TCP2	O395 Δ*tcpA*	14
Bah3	E7946 Δ*att*RS1 *recA*::*ctxB*	27
DH3	O395 *tolA*::pGP704	13
E. coli		
DH5αλpir	*endAl hsdR17 glnV44 thi-1 recA1 gyrA rel A1* Δ(*lacIZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ)M15*] λ*pir*$^+$	43
TG1	K12 Δ(*lac-pro*) *supE thi hsdD5*/F′ *traD36 pro*A^+B^+ *lacI*^{q} *lacZ*ΔM15	17
Plasmids		
pBAD24	Arabinose-inducible promoter vector; Ap^r	12
pDH5.52	*V. cholerae orfU* cloned into pBAD24	This study
pcDNA3.1/Myc-His(−)B	Vector used to add a *myc* epitope to the pIII-OrfU constructs	Invitrogen
pCTX-Kn	Replicative form of CTX-Knϕ	41
pCTX-KnΔ*orfU*	pCTX-Kn with an in-frame deletion of *orfU*	This study
fd phages		
fd-DOG	Phage display vector containing *Apa*LI/*Not*I restriction sites downstream of pIII leader sequence; tetracycline resistant	17
fd-pIII^{CTX}(15–274)	OrfU residues 15 to 274 followed by a myc epitope inserted downstream of pIII leader sequence in fd-DOG	This study
fdΔ1-pIII^{CTX}(15–274)	Derivative of fd-pIII^{CTX}(15–274) containing a deletion of g3p-D1 and g3p-D2	This study
fd-pIII^{CTX}(15–138)	OrfU residues 15 to 138 followed by a myc epitope inserted downstream of pIII leader sequence in fd-DOG	This study
fdΔ1-pIII^{CTX}(15–138)	Derivative of fd-pIII^{CTX}(15–138) containing a deletion of g3p-D1 and g3p-D2	This study
fd-pIII^{CTX}(117–274)	OrfU residues 117 to 274 followed by a myc epitope inserted downstream of pIII leader sequence in fd-DOG	This study
fdΔ1-pIII^{CTX}(117–274)	Derivative of fd-pIII^{CTX}(117–274) containing a deletion of g3p-D1 and g3p-D2	This study

cell surface pilus, toxin-coregulated pilus (TCP), and the periplasmic/inner membrane protein complex TolQRA (13). TCP is a type IV bundle-forming pilus that is also required for *V. cholerae* intestinal colonization (38). We and others previously found that inactivation of either *tcpA* or the *tolQRA* genes of *V. cholerae* reduced CTXϕ infection by more than 3 orders of magnitude (13, 41). Given the similarities of the requirements for CTXϕ infection of *V. cholerae* to those for Ff infection of *E. coli*, we hypothesized that CTXϕ might contain a virion coat protein that functions analogously to pIII and interacts with TCP and the TolQRA complex to enable CTXϕ infection of *V. cholerae.*

The CTXϕ core region encodes proteins for virion morphogenesis, including the putative major coat protein (Cep), three putative minor coat proteins (Psh, OrfU, and Ace), and Zot (20), which is similar to the Ff protein pI and is required for phage assembly and secretion. Although several of the putative CTXϕ gene products bear similarity to known filamentous phage proteins, none of the CTXϕ genes encode a protein similar in sequence to pIII. However, since pIII and OrfU have similar sizes and positions within their respective genomes (immediately 3′ of the gene encoding the major coat protein), we hypothesized that OrfU is the CTXϕ pIII homologue. Recently, Holliger and Reichmann determined the solution structure of g3p-D1 by nuclear magnetic resonance spectroscopy. They predicted that residues 18 to 86 of OrfU may fold into a β barrel similar to that of g3p-D1 (15). Their analysis lends additional credence to the hypothesis that OrfU is the CTXϕ pIII equivalent and to introducing the idea that these particular OrfU residues are involved in the binding of OrfU to the *V. cholerae* TolA (15). To test the hypothesis that OrfU mediates CTXϕ infection, we "displayed" OrfU on the surface of an Ff phage as an OrfU-pIII fusion protein. The resulting phage infected both F^+ *E. coli* and TCP^+ *V. cholerae*. Furthermore, OrfU was found to be required for CTXϕ production by *V. cholerae*. These findings indicate that OrfU functions as the CTXϕ pIII analogue, and we propose to rename OrfU as pIII^{CTX}.

MATERIALS AND METHODS

Strains, media, and antibiotics. The bacterial strains used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth at 37°C. To induce TCP production, the classical *V. cholerae* strain O395 was grown on a roller drum shaker at 30°C as previously described (13). To ensure F pilus production by *E. coli* strain TG1, the strain was maintained on M9 minimal medium plates containing glucose. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml (*V. cholerae*) and 100 μg/ml (*E. coli*); streptomycin, 200 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 15 μg/ml (*E. coli*) and 1 μg/ml (*V. cholerae*); tetracycline, 20 μg/ml (*E. coli*) and 1 μg/ml (*V. cholerae*). Arabinose (Ara) (0.02%) was added to LB broth to induce expression of genes under the control of the pBAD promoter.

Construction of pCTX-KanΔ*orfU* and an *orfU* expression plasmid. An in-frame deletion of *orfU* in pCTX-Kan was constructed using the PCR technique of splicing by overlapping extension (18). The two outside primers were DelUA (5′ GCGATAAGGGGCTTTATTGGTATG 3′) and DelUD (5′ CATTCGTG ATGATGTGACGGC 3′). The two internal primers were DelUB2 (5′ GAGC ATGGCGGCCAGAAAAAAGGGCGAACG 3′) and DelUC2 (5′ CGTTCG CCCTTTTTTCTGGCCATGCTC 3′). After subcloning of the deletion PCR product into pCRII-TOPO, an *Asc*I/*Pml*I fragment from this plasmid was ligated

TABLE 2. Primers used in construction of *orfU-gIII* fusions

Primer name	Sequence	*orfU* sequence amplified[a]	Amino acid sequence[b]
OrfU15F	5′ GGGCCCGTGCACCATCGGTAACGGCTTCC 3′	44–818	PSVTAS. . .
OrfU274R	5′ TCTAGAGCCCCTGTATAATTGGGGCTGCT 3′		PNYTGAL
OrfU15F	5′ GGGCCCGTGCACCATCGGTAACGGCTTCC 3′	44–413	PSVTAS. . .
OrfU138R	5′ TCTAGAGCAGACTTAAACGAGCTGCTCAG 3′		SSFKSAL
OrfU117F	5′ GGGCCCGTGCACCCTTACCACCTTCACCG 3′	350–413	PLPPSP. . .
OrfU274R	5′ TCTAGAGCCCCTGTATAATTGGGGCTGCT 3′		PNYTGAL

[a] Numbers represent the base pairs in *orfU* which were amplified. Base pair 1 is the first base pair of the ATG start codon of *orfU*.
[b] The first six amino acids of the first line are the first amino acids of OrfU located downstream of the pIII leader sequence. The first six amino acids of the second line are the last OrfU amino acids in the construct. The L is the leucine which is incorporated into each construct due to our cloning strategy; it is followed by the amino acids of the Myc tag, which are not shown.

with *Asc*I/*Pml*I-digested pCTX-Kn. The resulting plasmid lacks all but the last nine amino acids of OrfU. An *orfU* complementing plasmid, pDH5.52, was constructed by amplifying *orfU* with the forward and reverse primers OrfU1F (5′ ATGCGCTATTTTCTACTGTTTTTG 3′) and OrfU4R (5′ TCTAGAAAATC ACCTAAACAAAATGAGCATG 3′). After subcloning of the PCR product into pCRII-TOPO (Invitrogen, Carlsbad, Calif.), an *Xba*I/*Eco*RI fragment from this plasmid was ligated with *Xba*I/*Eco*RI-digested pBAD24.

Transduction assay. CTX-Knϕ and CTX-KnΔ*orfU*ϕ transducing particles were enumerated using a previously described transduction assay (13). Briefly, filtered supernatants were prepared from cultures grown to an optical density at 600 nm (OD_{600}) of approximately 0.8. A 75-μl volume of the filtered supernatants from these cultures was mixed with 75 μl of TCP$^+$ O395 cells for 20 min at room temperature on a shaker. Each mixture was then plated on LB agar containing either streptomycin, to enumerate the number of recipient cells, or kanamycin, to enumerate the transductants. The transduction frequency was calculated by dividing the number of transductants by the number of recipients. Similar transduction assays were used to calculate the numbers of the fd-derived transducing phages we studied here. To prepare these phages, DH5αλpir harboring the different fd-derived phages was grown overnight in LB broth and tetracycline. Phage particles were then precipitated from filtered supernatants with 5% polyethylene glycol (PEG) in 2.5 M NaCl overnight at 4°C, followed by centrifugation at 10,000 × *g* for 30 min. The particles were then resuspended in LB broth. To enumerate transductants, cell mixtures were plated on LB agar plates containing tetracycline, and to enumerate recipients, the mixtures were plated on LB plates either alone (for TG1) or with streptomycin (for the *V. cholerae* strains). The transduction frequency was calculated as described above.

Detection of phage DNA in supernatants. Southern blot analysis was used to detect phage DNA in filtered supernatants from mid-log-phase cultures of Bah3 harboring pCTX-Kn derivatives. The phage particles were PEG precipitated as described above and then resuspended in a sodium dodecyl sulfate (SDS)-containing buffer (1% SDS, 1× Tris-acetate-EDTA [TAE], 5% glycerol, 0.25% loading dye) at 70°C for 20 min to disrupt virions (29). These preparations were then subjected to 1% agarose electrophoresis, and CTXϕ DNA was then detected by Southern analysis using the CTXϕ *rstB* gene as a probe. The *rstB* gene was amplified with primers RstBF (5′ ATGAAATTATGGGTGATTAAT 3′) and RstBB (5′ CCACCGCGAAATAGAGCGCCT 3′). For the nondenaturing Southern blots, the NaOH treatment of the gel was omitted prior to transfer.

Construction of fd-DOG derivatives containing OrfU-pIII fusions. Our strategy was to amplify different regions of *orfU* and subclone them into the pcDNA3.1/Myc(−)B vector to add a *myc* epitope to the 3′ end of the construct. We then inserted this downstream of the leader sequence of *gIII* in fd-DOG. The primers used for these constructs are listed in Table 2. After the first round of amplification, the PCR products were ligated into the TA cloning vector pCRII-TOPO according to the manufacturer's protocol. An *Apa*I/*Xba*I fragment from each of the resulting plasmids was then ligated to *Apa*I/*Xba*I-digested pcDNA3.1/Myc(−)B vector. These myc fusion constructs were PCR amplified with the original forward primer and the reverse primer MycR (5′ GCGGCCGCCAGA TCCTCTTC 3′). Following subcloning into pCRII-TOPO, an *Apa*LI/*Not*I fragment was ligated into *Apa*LI/*Not*I-digested fd-DOG. This scheme resulted in the insertion of *orfU* fragments, an extra CTA (leucine), and a *myc* tag immediately 3′ of the pIII signal sequence (see Fig. 2B).

pIII-OrfU fusions that lacked the adsorption (g3p-D2) and the penetration (g3p-D1) domains of pIII were also generated. First, a *Not*I site was introduced into the pIII-OrfU fusion construct just upstream of the linker sequence which separates g3pD2 and g3pD3. The *Not*I site was added by using the QuikChange XL Site-Directed Mutagenesis kit and the *Not*IF primer (5′ CCTCCTGTC AATGCGGCCGCAGGCTCTGGTGGTGG 3′) and its reverse complement, *Not*IR. The resulting plasmids were digested with *Not*I to release the g3pD1D2 fragment and were then religated to form the fdΔ1 constructs.

Molecular biology methods. Standard molecular biology methods were used in this study (1). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.). For the Western blot analyses, the anti-myc antibody was purchased from Invitrogen and the anti-pVIII antibody was purchased from Stratagene (La Jolla, Calif.). Southern hybridization was carried out with the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia, Little Chalfont, Buckinghamshire, England) according to the manufacturer's instructions. The low-complexity regions of pIII and OrfU were analyzed by the "Predictprotein" program at the EMBL Heidelberg website (www.embl-heidelberg.de/predictprotein/predictprotein.html) (44).

RESULTS

Requirement for OrfU for CTXϕ production. To begin to study the role of OrfU in CTXϕ virion morphogenesis and infectivity, we made an in-frame *orfU* deletion in pCTX-Kn, the plasmid form of a kanamycin-marked CTXϕ. This deletion removed all of *orfU* except for the sequence encoding the final nine amino acids. This deletion mutant was electroporated into the classical strain O395, which contains a defective prophage, and Bah3, which lacks a CTX prophage and a phage attachment site. Compared to Bah3(pCTX-Kn), no infectious particles were detected in filtered supernatants of Bah3(pCTX-

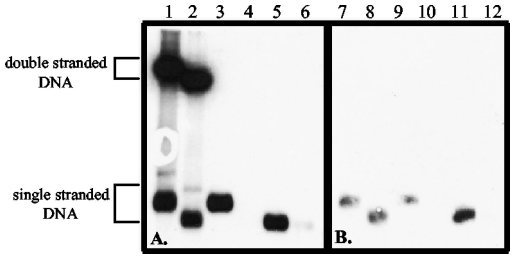

FIG. 1. Comparison of the amounts of phage and plasmid DNA present in *orfU*$^+$ and *orfU* mutant CTXϕ derivatives by Southern blot analysis using an *rstB* probe. The samples shown in panel A were denatured in NaOH prior to transfer to the nylon membrane; samples in panel B were not denatured. Lanes 1 and 7 and lanes 2 and 8 contain *Pml*I-digested plasmid DNA of pCTX-Kn and pCTX-KnΔ*orfU*, respectively. The remaining lanes are preparations of phage DNA from filtered supernatants: lanes 3 and 9, Bah3(pCTX-Kn); lanes 4 and 10, Bah3(pCTX-KnΔ*orfU*); lanes 5 and 11, Bah3(pCTX-KnΔ*orfU*/pDH5.52 + 0.02% Ara); lanes 6 and 12, Bah3(pCTX-Kn Δ*orfU*/pDH5.52 without Ara). After a longer exposure, a faint band with an apparent molecular weight identical to that of the lower band seen in lane 2 was evident in lane 4.

A

OrfU

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MRYFLLFLTL LFLSPSVTAS AINCDPNTTT SHQLLFGFGS PIVQSVLFDG 50
CMLDIEKDDY GFVWSCLSNE NGDYCKGLYK PRFSQGVSPN WPMCDLSGAS 100
AERCIYPYCP EGEECVPLPP SPPSDSPVDG LSSSFKSAFN QVYKNQSEMA 150
STLNHVSGQV SHSQDMVQLN TKFHADRVLE SVTAVNNRLG GQMEYLEEIR 200
IDVWDTQREV RKAKDELYSR VAAVSYDVLY SELNVLRAID ELKDSLGGTV 250
VPPNPDQPNP TPPDSSSPNY TGALNTISKK LNTLETISQQ LDTMNTALSG 300
RCSNPERCQF PIREAETELE TAQQNLKQMI NEKITQSALH QFKGSAAVPS 350
FCSYVEAFGY NLCFDFSLFS ENLHIIRMIV LAMAYILAAM LILFR 395
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pIII

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MKKLLFAIPL VVPFYSHSAE TVESCLAKPH TENSFTNVWK DDKTLDRYAN 50
YEGCLWNATG VVVCTGDETQ CYGTWVPIGL AIPENEGGGS EGGGSEGGGS 100
EGGGTKPPEY GDTPIPGYTY INPLDGTYPP GTEQNPANPN PSLEESQPLN 150
TFMFQNNRFR NRQGALTVYT GTVTQGTDPV KTYYQYTPVS SKAMYDAYWN 200
GKFRDCAFHS GFNEDPFVCE YQGQSSDLPQ PPVNAGGGSG GGSGGGSEGG 250
GSEGGGSEGG GSEGGGSGGG SGSGDFDYEK MANANKGAMT ENADENALQS 300
DAKGKLDSVA TDYGAAIDGF IGDVSGLANG NGATGDFAGS NSQMAQVGDG 350
DNSPLMNNFR QYLPSLPQSV ECRPFVFSAG KPYEFSIDCD KINLFRGVFA 400
FLLYVATFMY VFSTFANILR NKES 424
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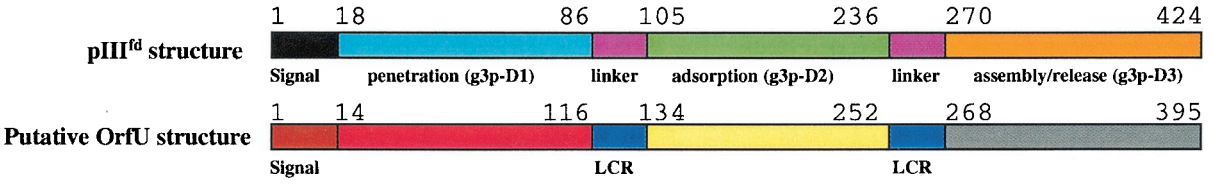
B



	Construct Name	Schematic Structure (*Apa*LI | *Not*I)	Transduction Frequency: *V. cholerae* TCP$^+$	*V. cholerae* TCP$^-$	*E. coli* F$^+$
1	fd-DOG		UD	UD	2.0×10^{-1}
2	fd-pIII^{CTX}(15-274)		2.7×10^{-3}	1.7×10^{-6}	2.0×10^{-1}
3	fdΔ1-pIII^{CTX}(15-274)		5.0×10^{-1}	6.7×10^{-6}	UD
4	fd-pIII^{CTX}(15-138)		1.1×10^{-4}	3.3×10^{-5}	1.6×10^{-1}
5	fdΔ1-pIII^{CTX}(15-138)		1.6×10^{-4}	5.3×10^{-5}	UD
6	fd-pIII^{CTX}(117-274)		UD	UD	2.2×10^{-1}
7	fdΔ1-pIII^{CTX}(117-274)		UD	UD	UD

FIG. 2. (A) Both pIII and OrfU contain two regions of low secondary structure according to the computer algorithm "Predictprotein" located at www.embl-heidelberg.de/predictprotein/predictprotein.html (44). The amino acids in red are these low-complexity regions (LCRs). The areas of LCR for OrfU are from residues 116 to 134 and 252 to 268, and those for pIII are from residues 86 to 104 and 246 to 272. These LCRs of pIII have been previously designated as the linkers that separate the three domains of pIII (15). (B) (Top) Schematic representations of three pIII domains and the three regions in OrfU separated by LCRs. The boundaries for the LCRs of OrfU were determined from the analysis described

KnΔ*orfU*) (Table 3, third and fourth rows). This defect in CTXφ production could be complemented in *trans* by producing OrfU from a defective CTX prophage (in the classical strain O395) or from a plasmid, pDH5.52 (Table 3, second and sixth rows). For the latter experiments, we expressed *orfU* from the arabinose-inducible promoter pBAD on pDH5.52 in Bah3 (pCTX-KnΔ*orfU*). When arabinose was added to Bah3(pCTX-KnΔ*orfU*/pDH5.52), infectious CTX-Knφ was produced at levels comparable to those observed for wild-type CTXφ virions (Table 3; compare sixth row with third row). As expected, virions produced following introduction of exogenous OrfU still carried the *orfU* deletion and continued to be dependent upon a complementing OrfU for virion production in new hosts infected with CTX-KnΔ*orfU*φ (data not shown). These results indicate that OrfU is required for the production of infectious CTXφs.

Since pIII is required for Ff phage assembly and release, the absence of Kn-transducing particles in supernatants from Bah3 (pCTX-KnΔ*orfU*) was potentially attributable to a lack of production of CTX-KnΔ*orfU* virions by this strain. An alternate possibility was that Bah3(pCTX-KnΔ*orfU*) produced virions that were incapable of infecting a recipient strain. To distinguish between these alternatives, we tested supernatants of Bah3 cells harboring either pCTX-Kn or pCTX-KnΔ*orfU* for the presence of single-stranded phage DNA by Southern blot analysis. Bah3(pCTX-Kn) supernatants contained abundant CTXφ-hybridizing DNA that could be detected even if the DNA was not denatured with NaOH after electrophoresis, suggesting that the hybridizing species was single-stranded phage DNA (Fig. 1, lanes 3 and 9). In marked contrast, very little CTXφ-hybridizing DNA was detected in supernatants from cells harboring pCTX-KnΔ*orfU* (Fig. 1, lanes 4 and 10). However, as in the previous experiment, expression of *orfU* in *trans* restored levels of secreted DNA to wild-type levels (Fig. 1, lanes 5 and 11). The reduction in phage production by Bah3 (pCTX-KnΔ*orfU*) supernatants was not due to a replication defect of pCTX-KnΔ*orfU*, since pCTX-KnΔ*orfU* replicated as well as pCTX-Kn. Approximately equal amounts of both the single- and double-stranded forms of phage DNA were detected in plasmid preparations of Bah3(pCTX-Kn) and Bah3 (pCTX-KnΔ*orfU*) (Fig. 1, lanes 1 and 2). These data suggest that the lack of infectious CTX-Knφ in supernatants from Bah3(pCTX-KnΔ*orfU*) results primarily from a failure in the assembly and/or release of CTXφ particles. However, since the small number of virions secreted from Bah3(pCTX-KnΔ*orfU*) did not yield any transductants, it appears that OrfU is also necessary for CTXφ to infect a new host.

The role of OrfU in CTXφ infection. To investigate whether OrfU functions in CTXφ infection, we introduced fragments of *orfU* into *gIII* in a tetracycline-marked fd derivative, fd-DOG, that was previously used as a phage display vector (17). This resulted in display of OrfU fused to pIII on the surface of fd-DOG. We adopted this approach for three reasons. First, this method circumvents the requirement for OrfU in CTXφ production by *V. cholerae* because the recombinant fd phages utilize the pIII portion of the fusion protein to be released from *E. coli*. Second, fd virions containing pIII fusion proteins often retain normal levels of infectivity for F$^+$ *E. coli*, so phages that failed to infect *V. cholerae* could still be titered on *E. coli* (34). Third, we knew that although fd alone cannot infect *V. cholerae*, it is capable of replication in *V. cholerae* when introduced by electroporation (unpublished data). Thus, by producing different pIII-OrfU fusion proteins on the surface of fd, we tested whether various portions of OrfU could enable fd to infect *V. cholerae*.

TABLE 3. *orfU* is required for formation of CTX-Knφ transducing particles

Strain[a] from which supernatant is derived	Frequency of infection[b]
O395 pCTX-Kn	4.0×10^{-4}
O395 pCTX-KnΔ*orfU*	1.5×10^{-5}
Bah3 pCTX-Kn	6.4×10^{-4}
Bah3 pCTX-KnΔ*orfU*	$<4.0 \times 10^{-9}$
Bah3 pCTX-KnΔ*orfU*/pDH5.52 with no arabinose	$<3.0 \times 10^{-9}$
Bah3 pCTX-KnΔ*orfU*/pDH5.52 + 0.02% arabinose	9.6×10^{-4}

[a] O395 harbors a defective CTX prophage that produces OrfU, while Bah3 lacks all CTXφ sequences.

[b] Calculated by dividing the number of transductants (Kn^r colonies) by the number of O395 recipients. The transduction assays were conducted at least three times, and the mean frequency is presented. The standard deviations of these values were less than 10% of the means.

We compared the amino acid sequences of OrfU and pIII in order to choose segments of OrfU to fuse to pIII. The three functional domains of pIII are separated by stretches of glycine-rich residues that are thought to constitute regions of minimal structural complexity functioning as flexible linkers (16). No such glycine-rich stretches are found in the OrfU sequence; however, there are two regions rich in prolines and serines that could separate OrfU into functionally distinct domains similar in size to those found in pIII. According to the computer algorithm "Predictprotein," these regions (residues 116 to 134 and 252 to 269) in OrfU are unlikely to form a secondary structure (Fig. 2A) and therefore may act as linkers between OrfU domains. This algorithm accurately defined the residues that constitute the linker within pIII (Fig. 2A).

We initially fused the first two-thirds of the *orfU* coding region to *gIII* in fd-DOG. As shown at the top of Fig. 2B, this segment of OrfU included the amino acids through the end of the second putative low-complexity region (Fig. 2A). This region was chosen because only the N-terminal two-thirds of pIII, which includes g3p-D1 and g3p-D2, mediates Ff infection of *E. coli* (29, 30, 35). The *orfU* N-terminal fragment was introduced 3′ of the *gIII* signal sequence and 5′ of the complete *gIII* coding sequence in fd-DOG, generating fd-pIII^{CTX}(15-

in the legend to panel A. The residues for the linkers of pIII are as previously described. (Bottom) Schematic of the six pIII-OrfU fusions used to investigate the regions of OrfU required to expand the host range of fd to include *V. cholerae*. Light blue boxes represent the *myc* tag added to the 3′ end of *orfU* in each of the fusions. The transduction frequencies were calculated by dividing the number of transductants (tetracycline-resistant colonies) by the number of recipient cells. The TCP$^+$ and TCP$^-$ *V. cholerae* recipients were O395 and TCP2, respectively. TG1 was the F$^+$ *E. coli* recipient. The transduction frequencies represent the means from at least three experiments, and all standard deviations were less than 10%. UD, undetectable.

274) (Fig. 2B, row 2). A *myc* tag was inserted at the same time to facilitate detection of the fusion protein (Fig. 2B, row 2). The fd-pIII^{CTX}(15-274) virion was predicted to contain part of OrfU linked to pIII on one tip of the fd virion (10, 25). The OrfU fragment fused to pIII in fd-pIII^{CTX}(15-274) did not alter this phage's ability to infect *E. coli*; fd-pIII^{CTX}(15-274) infected the F$^+$ *E. coli* strain TG1 as well as fd-DOG did (Fig. 2B; compare rows 1 and 2). However, unlike supernatants from a strain producing fd-DOG (Fig. 2B, row 1), filtered supernatants from an *E. coli* strain harboring fd-pIII^{CTX}(15-274) could be used to infect *V. cholerae* (Fig. 2B, row 2). Thus, the N-terminal two-thirds of the OrfU polypeptide fused to pIII is sufficient for expanding the host range of a phage that was previously restricted to F$^+$ *E. coli*. The ability of fd-pIII^{CTX}(15-274) to infect *V. cholerae* was dependent on TCP and TolA, the known CTXϕ coreceptors, as the efficiency of infection by fd-pIII^{CTX}(15-274) of a *V. cholerae* strain lacking TCP was reduced by 3 orders of magnitude (Fig. 2B, row 2). The infection of a *tolA* mutant strain (O395 *tolA*::pGP704) was below the levels of detection for this assay ($<10^{-9}$). Taken together, these data suggest that OrfU mediates CTXϕ infection of *V. cholerae* and that amino acids 15 to 274 of OrfU contain regions that interact with TCP and TolA.

To be certain that the pIII portion of the OrfU-pIII fusion protein did not contribute to infection of *V. cholerae* by fd-pIII^{CTX}(15-274), we constructed a derivative of this phage, fdΔ1-pIII^{CTX}(15-274), that lacks g3p-D1 and g3p-D2 (Fig. 2B, row 3). Elimination of these pIII domains did not prevent fdΔ1-pIII^{CTX}(15-274) from infecting *V. cholerae* (Fig. 2B, row 3). In fact, this deletion appears to enhance the infectivity of fdΔ1-pIII^{CTX}(15-274) relative to fd-pIII^{CTX}(15-274) for both TCP$^+$ and TCP$^-$ *V. cholerae* (Fig. 2B; compare rows 2 and 3). Thus, the pIII adsorption (g3p-D2) and penetration (g3p-D1) domains do not contribute to the ability of fd-pIII^{CTX}(15-274) to infect *V. cholerae*. The reason why fdΔ1-pIII^{CTX}(15-274) infected *V. cholerae* at a greater efficiency than fd-pIII^{CTX}(15-274) is not clear. It is not likely that unequal numbers of virions were added to the *V. cholerae* recipients. Western blot analysis of phage proteins with anti-pVIII antisera suggested that approximately equivalent numbers of virions were used in each infection experiment (Fig. 3, bottom panel, first and fifth lanes; also data not shown). Perhaps residues in g3pD1D2 in the pIII-OrfU(15-274) fusion protein sterically hinder OrfU residues from interacting with TCP and/or TolA.

The data presented above suggest that residues 15 to 274 of OrfU contain a region(s) that interacts with both TCP and TolA. Based on the sequence analysis shown in Fig. 2A and on experimental studies of pIII, we investigated whether these 259 amino acids could be subdivided into an adsorption domain, for binding the pilus, and a penetration domain, for binding TolA. To test whether the segments flanked by the low-complexity regions can function as autonomous domains, we constructed recombinant fd phages containing OrfU residues 15 to 138 [fd-pIII^{CTX}(15-138)] and 117 to 274 [fd-pIII^{CTX}(117-274)] fused to pIII (Fig. 2B, rows 4 and 6, respectively) by using the same cloning strategy that we followed to construct fd-pIII^{CTX}(15-274). Derivatives of these new phages that lacked g3p-D1 and g3p-D2 were also generated and designated fdΔ1. Both fd-pIII^{CTX}(15-138) and fdΔ1-pIII^{CTX}(15-138) were capable of infecting TCP$^+$ *V. cholerae* (Fig. 2B, rows 4 and 5), although at lower efficiencies than fd-pIII^{CTX}(15-274) and fdΔ1-pIII^{CTX}(15-274). However, unlike the fd derivative that displayed amino acids 15 to 274 of OrfU (Fig. 2B, rows 2 and 3), these new derivatives infected TCP$^+$ and TCP$^-$ *V. cholerae* at similar frequencies (Fig. 2B, rows 4 and 5). Neither fd-pIII^{CTX}(15-138) nor fdΔ1-pIII^{CTX}(15-138) could infect *V. cholerae* harboring a mutation in *tolA*. Together with previous data, these results suggest that a TolA binding domain of OrfU lies within amino acids 15 to 138, since this region is sufficient to mediate TolA-dependent phage infection. They also suggest that amino acids 15 to 138 in and of themselves do not contain a TCP binding domain, since TCP does not influence the infectivity of the fd derivatives displaying these amino acids (Fig. 2B, rows 4 and 5). Interestingly, fd-pIII^{CTX}(15-138) and fdΔ1-pIII^{CTX}(15-138) infect TCP$^-$ *V. cholerae* better (approximately 10-fold) than comparable phages that contain a fusion protein containing OrfU residues 15 to 274 (Fig. 2B; compare rows 4 and 5 with rows 2 and 3). These findings suggest the possibility that OrfU amino acids 139 to 274 can inhibit infection of TCP$^-$ *V. cholerae*.

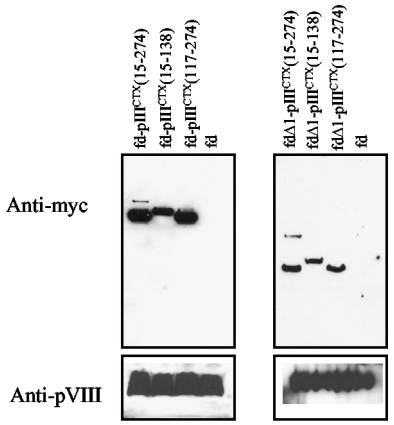


FIG. 3. Comparison of the amounts of pVIII phage protein and pIII-OrfU fusion proteins in phage preparations by Western blot analysis using an anti-myc (top) or anti-pVIII (bottom) antiserum. Phage preparations were resuspended in LB medium, prepared in sample buffer as previously described (1), run on a 10% Bis-Tris gel, and transferred to a nitrocellulose membrane.

In contrast to the fd derivatives bearing OrfU residues 15 to 138, those fd derivatives that displayed only residues 117 to 274 of OrfU could not infect *V. cholerae* (Fig. 2B, rows 6 and 7). As judged from the Western blots in Fig. 3, the failure of fd-pIII^{CTX}(117-274) and fdΔ1-pIII^{CTX}(117-274) to infect *V. cholerae* was not due to low viral titers or degradation of the pIII-OrfU fusion protein. Phages displaying OrfU residues 117 to 274 appear to be as abundant as the other fd derivatives, and they contain a gIII fusion protein of the expected size. It is therefore likely that these fd derivatives were not able to infect *V. cholerae* because they do not contain OrfU residues 15 to 139 and consequently cannot bind to TolA. Ff derivatives that lack the pIII TolA binding domain are similarly unable to infect *E. coli*. It is not clear whether fd-pIII^{CTX}(117-274) and fdΔ1-pIII^{CTX}(117-274) are able to bind to TCP. The experiments presented above suggest that OrfU amino acids 117 to 274 are necessary for binding to TCP, but we have not yet determined whether these amino acids are sufficient.

DISCUSSION

We found that OrfU is required for assembly and/or release of CTX virions by *V. cholerae* and that display of a part of OrfU on fd is sufficient to enable fd to enter *V. cholerae*. These results suggest that OrfU functions as the pIII equivalent for CTXφ. *orfU* was first described as the open reading frame immediately 5′ of *ace* several years prior to the discovery of CTXφ (40); it was designated *orfU* because its function was unknown. Given our findings, we propose to rename OrfU as pIII^{CTX} and *orfU* as *gIII^{CTX}* in order to remain consistent with the Ff nomenclature.

As with pIII^{fd}, different segments of pIII^{CTX} appear to mediate distinct steps in phage infection. pIII^{CTX} residues 15 to 138 were sufficient to allow fd entry into *V. cholerae* in a TolA-dependent and TCP-independent manner, suggesting that, as with pIII^{fd}, the amino-terminal end of pIII^{CTX} constitutes its penetration domain. Inclusion of pIII^{CTX} residues 139 to 274 as well as residues 15 to 138 increased infection of *V. cholerae* by up to 3 orders of magnitude in a TCP-dependent fashion, suggesting that this pIII^{CTX} region includes residues that interact with TCP. However, we were unable to demonstrate a direct interaction between fd-pIII^{CTX}(117-274) and TCP (data not shown). It is not clear whether residues 139 to 274 can function alone as an adsorption domain or whether additional amino acids from pIII^{CTX} are needed as well. If residues 139 to 274 are sufficient to bind TCP, it is possible that proper folding of this region requires additional OrfU residues. In pIII^{fd}, Deng et al. were able to show functional pilus binding activity for isolated g3p-D2 only after expressing this domain with g3p-D1 and then separating the two domains by protease treatment (7). Thus, the TCP binding activity of pIII^{CTX} residues 139 to 274 may be detectable only when pIII^{CTX} residues 17 to 274 are produced together.

It has been proposed that pIII^{fd} interacts sequentially with F, then TolA, and that binding to F induces a conformational change in pIII that reveals the TolA binding site (16). Some of our data suggest that a similar multistep process may occur during pIII^{CTX}-mediated infection of *V. cholerae*. We observed that fd-pIII^{CTX}(15-138) and fdΔ1-pIII^{CTX}(15-138) infected TCP$^-$ *V. cholerae* nearly 1 order of magnitude more efficiently than did fd-pIII^{CTX}(15-274) and fdΔ1-pIII^{CTX}(15-274), which both appear to contain a TCP binding domain. Thus, in the absence of TCP, the putative pIII^{CTX} adsorption domain (residues 139 to 274) may inhibit the penetration domain (amino acids 15 to 138) from binding to TolA. If this model is correct, it suggests that the underlying mechanisms of CTXφ and Ff infection of their respective hosts are conserved. However, given the lack of similarity in the amino acid sequences of pIII^{CTX} and pIII^{fd}, such conservation would probably be based on similarities in the molecular structures of these virion minor coat proteins.

pIII^{CTX} is usually required for CTXφ assembly and release, as well as for CTXφ infection; however, we were unable to localize the assembly and release functions to a particular segment of the protein. While production of full-length pIII^{CTX} from a plasmid could facilitate release of CTX-KnΔ*orfU* particles from a strain harboring pCTX-KnΔ*orfU* (Fig. 1), production of the C-terminal two-thirds of pIII^{CTX} did not (data not shown). Therefore, most or even all of pIII^{CTX} seems to be required for assembly and release of CTXφ particles; in contrast, only the C-terminal 80 amino acids of pIII are required for fd assembly and release (28).

Since the C-terminal domain of pIII (g3p-D3) is required for fd virion assembly and release, investigation of the role of g3p-D3 in fd infection has not been carried out. Our finding that fdΔ1-pIII^{CTX}(15-274), which contains the adsorption and penetration domains of pIII^{CTX} fused to g3p-D3, infects *V. cholerae* suggests that the C terminus of pIII^{CTX} does not play a role in infection. However, it is also possible that g3p-D3 substituted for this part of pIII^{CTX} in fdΔ1-pIII^{CTX}(15-274) to allow infection of *V. cholerae*. Model and colleagues have proposed that g3p-D3 may mediate fusion of the virion with the membrane during entry (28). Similarly, the role of pVIII in fd infection is not clear. pVIII is synthesized as an inner membrane protein (31), and during infection, this virion protein has been shown to be "recycled" into the inner membrane of the recipient cell (33, 39). Our data demonstrate that besides pIII^{CTX}, other CTXφ proteins are not required for fd infection of *V. cholerae*. These data could suggest that the major coat protein is not required for infection, or that pVIII's role in fd infection of *E. coli* is conserved in infection of *V. cholerae*.

Our findings that the host range of fd can be altered to include a new genus by the relatively simple technique of either adding or substituting an alternative filamentous phage infection protein for pIII^{fd} may prove useful in the development of bacteriophage as therapeutic agents. For example, it may be possible to create a library of fd-based phages that are able to infect a broad range of bacterial pathogens. CTXφ may also be a useful starting platform from which to engineer such libraries. Furthermore, in addition to using CTXφ-based phages as antimicrobial agents, introduction of antigen-encoding DNA sequences into *gIII^{CTX}* may enable display of antigenic polypeptides in CTXφ virions. Production of such recombinant CTXφs during human infection by live attenuated *V. cholerae* vaccine strains could prove to be an effective method for delivery of heterologous antigens to the gut immune system.

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