JOURNAL OF BACTERIOLOGY, Mar. 2000, p. 1739–1747 Vol. 182, No. 6
0021-9193/00/$04.00+0

CTXϕ Infection of *Vibrio cholerae* Requires the *tolQRA* Gene Products

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Received 4 October 1999/Accepted 21 December 1999

CTXϕ is a lysogenic filamentous bacteriophage that encodes cholera toxin. Filamentous phages that infect *Escherichia coli* require both a pilus and the products of *tolQRA* in order to enter host cells. We have previously shown that toxin-coregulated pilus (TCP), a type IV pilus that is an essential *Vibrio cholerae* intestinal colonization factor, serves as a receptor for CTXϕ. To test whether CTXϕ also depends upon *tol* gene products to infect *V. cholerae*, we identified and inactivated the *V. cholerae tolQRAB* orthologues. The predicted amino acid sequences of *V. cholerae* TolQ, TolR, TolA, and TolB showed significant similarity to the corresponding *E. coli* sequences. *V. cholerae* strains with insertion mutations in *tolQ*, *tolR*, or *tolA* were reduced in their efficiency of CTXϕ uptake by 4 orders of magnitude, whereas a strain with an insertion mutation in *tolB* showed no reduction in CTXϕ entry. We could detect CTXϕ infection of TCP$^-$ *V. cholerae*, albeit at very low frequencies. However, strains with mutations in both *tcpA* and either *tolQ*, *tolR*, or *tolA* were completely resistant to CTXϕ infection. Thus, CTXϕ, like the *E. coli* filamentous phages, uses both a pilus and TolQRA to enter its host. This suggests that the pathway for filamentous phage entry into cells is conserved between host bacterial species.

Vibrio cholerae is a gram-negative bacterium that causes cholera, a severe and sometimes lethal diarrheal disease. Humans become infected with *V. cholerae* after ingesting food or water that has been contaminated with the pathogen. *V. cholerae* is capable of colonizing and multiplying within the small intestine. This colonization requires production of a bundle-forming pilus, called toxin-coregulated pilus (TCP) (34). In addition to TCP, other virulence factors are expressed once the pathogen reaches the small intestine. One of these virulence factors is cholera toxin, a potent protein exotoxin that elicits a secretory response from intestinal epithelial cells. This response is the principle basis for the secretory diarrhea that is the hallmark of cholera (30).

Cholera toxin is an A-B-type toxin encoded by the *ctxAB* operon. This operon is part of the genome of CTXϕ, a 7-kb lysogenic filamentous bacteriophage (37). Lysogenic conversion of nontoxigenic strains to toxigenicity by CTXϕ infection appears to be a critical step in the evolution of fully pathogenic *V. cholerae*. The CTXϕ genome is subdivided into two regions: a 4.6-kb core region that includes *ctxAB* and a 2.4-kb region designated RS2 (38). The organization of the core-encoded genes and the deduced amino acid sequences of their products (with the exception of *ctxAB*) resemble those of filamentous phages derived from a variety of bacterial species. These similarities, along with experimental evidence, suggests that the CTXϕ core genes encode proteins required for virion morphogenesis. The CTXϕ core gene products include Cep, which is thought to be the virion major coat protein, and Psh, OrfU, and Ace, which are thought to be minor coat proteins. The core-encoded Zot protein is similar to protein pI of coliphage M13 (15) and is required for virion assembly and secretion but is not part of the phage particle. Although lacking similarity to any *Escherichia coli* filamentous phage DNA sequences, our data indicate that the RS2 region of the CTXϕ genome encodes the genes and noncoding sequences required for phage replication, integration, and transcriptional repression (14, 38).

The molecular steps involved in infection of *E. coli* by F pilus-specific filamentous phages (the Ff phages) such as f1 and M13 have been well characterized. The process begins when a domain of a minor coat protein (pIII) located on one end of the phage particle binds to the tip of the conjugative F pilus of *E. coli* (13). This interaction between pIII and F is thought to result in pilus retraction, which draws the phage through the bacterium's outer membrane. Subsequent phage translocation through the periplasmic space requires the *tolQRA* gene products (32, 33). Recent studies indicate that the periplasmic part of TolA binds to pIII and thereby serves as a coreceptor for phage entry into the bacterium (26). TolQ and TolR appear to interact with TolA via their inner membrane domains (8, 19), although their exact function in filamentous phage uptake remains unknown. Ff phage can infect *E. coli* lacking the F pilus, albeit at much lower frequencies than infection of F$^+$ cells (29). Following translocation of the phage through the periplasm via the TolQRA complex, the phage major capsid protein, pVIII, inserts into the inner membrane (4) and the single-stranded phage genome enters the cytoplasm and begins a new cycle of phage replication and infection.

The physiological role of the *tolQRAB* gene products remains uncertain. The *tolQRA* gene products of *E. coli* are thought to contribute to maintaining the integrity of the outer bacterial membrane. Disruption of these *tol* genes enhances the sensitivity of the bacteria to certain antibiotics and detergents and leads to leakage of periplasmic proteins into the extracellular surroundings (17, 18, 39). Mutations also prevent transfer of certain colicins into the cell (16). Disruption of a fourth *tol* gene, *tolB*, located immediately 3′ of *tolQRA*, generates cells with comparable membrane deficiencies; however, mutation of this *tol* gene has no detectable effect upon Ff phage uptake (32).

For filamentous phages that infect hosts other than *E. coli*, little is known concerning the molecular aspects of phage entry. We previously found that *V. cholerae* cells harboring deletions or particular amino acid substitutions in *tcpA*, which

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

Strain or plasmid	Description or genotype	Source or reference
Strains		
V. cholerae		
O395	O1 classical strain; Sm^r	23
DH1	O395 *tolQ*::pDH235	This study
DH2	O395 *tolR*::pDH107	This study
DH3	O395 *tolA*::pDH149	This study
DH4	O395 *tolB*::pDH270	This study
TCP2	O395 Δ*tcpA*	10
DH5	TCP2 *tolQ*::pDH235	This study
DH6	TCP2 *tolR*::pDH107	This study
DH7	TCP2 *tolA*::pDH149	This study
468-83	TCP TLC *attRS* CTXϕ$^-$	28
RV508	Rif^r $Spec^r$; derivative of classical strain 569B	37
E. coli		
Sm10λpir	*thi thr leu tonA lacY supE recA*::RP-2Tc::Mu Kn^r::λpir	23
TPS66	Missense mutation in *tolQ*/F$^+$	32
Plasmids		
pGP704	Suicide vector; *oriR6K mobRP4* Ap^r	23
pDH235	Internal fragment of *V. cholerae tolQ* (bp 72–381) inserted into pGP704	This study
pDH107	Internal fragment of *V. cholerae tolR* (bp 22–292) inserted into pGP704	This study
pDH149	Internal fragment of *V. cholerae tolA* (bp 79–530) inserted into pGP704	This study
pDH270	Internal fragment of *V. cholerae tolB* (bp 36–270) inserted into pGP704	This study
pBAD33	Ara-inducible promoter vector; Cm^r	9
pDH8	*V. cholerae tolQ* cloned into pBAD33	This study
pDH9	*V. cholerae tolQR* cloned into pBAD33	This study
pDH10	*V. cholerae tolR* cloned into pBAD33	This study
pDH11	*V. cholerae tolA* cloned into pBAD33	This study
pCTX-Kn	Replicative form of CTX-Knϕ	37
pMW1	pCTX-Kn Δ*orfU*	37
pMW2	pCTX-Kn Δ*zot*	37

encodes the major subunit of TCP, are resistant to CTXϕ infection. This finding suggested that this type IV pilus serves as a receptor for CTXϕ (37). The CTXϕ ligand that binds TCP has been hypothesized to be the core-encoded protein OrfU, based both upon the size and relative position of this gene within the CTXϕ genome (37). Although OrfU does not have significant sequence similarity to Ff pIII, Holliger and Riechmann have predicted that the N-terminal portion of OrfU has structural similarity to the domain of pIII that interacts with *E. coli* TolA (11). In the current study, we investigated whether orthologues of the *E. coli tolQRAB* genes are encoded in the *V. cholerae* genome and whether the products of these genes are required for CTXϕ infection. We found that the *V. cholerae* genome contains four contiguous open reading frames (ORFs) predicted to encode proteins similar to *E. coli* TolQRAB and that disruption of the *V. cholerae tolQRA* genes severely reduces the efficiency of *V. cholerae* CTXϕ uptake. Further supporting the importance of *V. cholerae tolQRA* in CTXϕ uptake, we found that TCP$^-$ strains of *V. cholerae* can be infected by CTXϕ, albeit at greatly reduced frequencies, and that TolQRA are absolutely required for phage entry into TCP$^-$ cells.

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 (2) at 37°C. To induce TCP expression and the concomitant autoagglutination of classical *V. cholerae* strain O395, bacteria were cultured on a roller drum shaker at 30°C overnight as previously described (34). 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*); spectinomycin, 50 μg/ml; and rifampicin, 40 μg/ml. Arabinose (Ara) (0.02%) was added to LB broth to induce expression of genes under the control of the *E. coli* promoter, pBAD (9).

Construction of O395 *tolQRAB* mutant strains. Homologous recombination of suicide vectors containing internal fragments of *tolQ*, *tolR*, *tolA*, and *tolB* into their respective chromosomal genes was used to inactivate each of these genes in the *V. cholerae* O395 background. These gene fragments were amplified from O395 genomic DNA by PCR. The sequences of the primers used to amplify these internal *tol* gene fragments relative to the predicted start codon of each of these genes are shown in Table 2. These PCR products were subsequently cloned into the TA cloning vector pCRII-TOPO vector (Invitrogen, Carlsbad, Calif.) according to the manufacturer's protocol. An *Eco*RI fragment of each of the resulting plasmids which contained the cloned PCR product was then ligated to *Eco*RI-digested pGP704, a suicide vector encoding Ap^r which requires the product of the *pir* gene for replication (23). The resulting plasmids, pDH235, pDH107, pDH149, and pDH270 (Table 1), were subsequently introduced into *E. coli* Sm10λ*pir* and then mobilized into *V. cholerae* O395 and TCP2. Transconjugants (Sm^r and Ap^r colonies) were selected, and disruption of each *tol* gene in all of the resulting strains was confirmed by Southern analyses (data not shown).

Construction of *tolQRA*-complementing plasmids. The full-length *tolQ* gene was amplified by PCR with the forward primer tolQ-1 (5′ CCGAGAGCTTTG CCTCAGTTAATC 3′) located 43 bp upstream of the predicted start codon of *tolQ* and the reverse primer tolQ-2 (5′ TTTGGTTTGATAGCCAGCC 3′) ending 26 bp downstream of the predicted stop codon of *tolQ*. The PCR product was then cloned into the pCRII-TOPO vector. Following subcloning into pBluescript SK(−) (Stratagene, La Jolla, Calif.), a *Sac*I/*Kpn*I fragment containing the insert was ligated into *Sac*I/*Kpn*I-digested pBAD33 (9), resulting in pDH8.

The *tolQR* genes were amplified by PCR with the forward primer tolQ-1 *Kpn* (the tolQ-1 forward primer sequence with a *Kpn*I restriction site at the 5′ end) and the reverse primer tolR-2*Xba* (5′ TCTAGAATTTAAGGTCCGTGAGTA GCCCTAC 3′) which ends 2 bp downstream from the predicted stop codon of *tolR* and includes an *Xba*I site added to the 5′ end. The PCR product was first cloned into the pCRII-TOPO vector, and then a *Kpn*I/*Xba*I fragment containing the insert was ligated into *Kpn*I/*Xba*I-digested pBAD33 to yield pDH9.

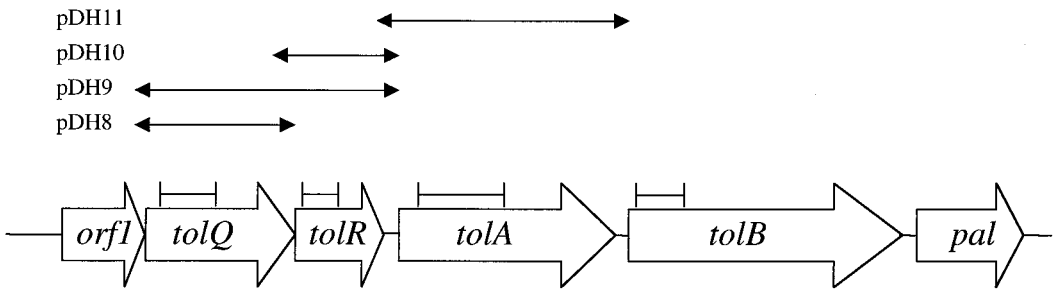
The *tolR* gene was amplified by PCR using the forward primer tolR-1 (5′ AGTTTCATACCATTCTCCACCGTC 3′) located 69 bp upstream of the predicted start codon of *tolR* and the reverse primer tolR-2, which has the same sequence and location as tolR-2*Xba* but lacks the *Xba*I site. The PCR product was subsequently cloned into the pCRII-TOPO vector, and then a *Hin*dIII/*Xba*I fragment containing the insert was ligated into *Hin*dIII/*Xba*I-digested pBad33, resulting in pDH10.

The *tolA* gene was amplified using the forward primer tolA-1 (5′ TCCTAAA GTAGGGCTACTCACGGAC3′) located 51 bp upstream of the predicted start codon of *tolA* and the reverse primer tolA-2 (5′ ACTAGCTCCAATGCCGCA TTC 3′) ending 97 bp downstream of the *tolA* predicted stop codon. Following cloning of the PCR product into the pCRII-TOPO vector and then subcloning into pBluescript SK(−), a *Pst*I/*Sal*I fragment containing the insert was ligated into *Pst*I/*Sal*I-digested pBAD33, resulting in pDH11.

Assays of efficiency of CTXϕ infection. To compare the efficiency of CTXϕ infection of different mutant strains, both a previously described supernatant-based transduction assay (37) and a new coculture transduction assay were used. In the supernatant-based transduction assay, filtered supernatants from a strain harboring the kanamycin-marked CTXϕ replicative form, pCTX-Kn, were mixed with different recipients. Seventy-five microliters of recipient cells, which were autoagglutinated after overnight growth at 30°C, was vortexed and mixed with 75

TABLE 2. Sequences of the PCR primers used to generate the internal fragments for insertional mutations of *V. cholerae tolQRAB*

Primer name	Sequence	PCR product
tolQKO-1	5′ CCTTTTGGGAATGTCGGTTGC 3′	Internal fragment of *tolQ*; bp 72–381
tolQKO-2	5′ GCTGGTTTCGAGTGAATCAACTTC 3′	
tolRKO-1	5′ AAACGTGAGTGAAAGCAGA 3′	Internal fragment of *tolR*; bp 22–292
tolRKO-2	5′ GAACAATCACATCTTCGATG 3′	
tolAKO-1	5′ GCGATATTGCTCTGGGGAG 3′	Internal fragment of *tolA*; bp 79–530
tolAKO-2	5′ CGTTGCTGTTCTGCCTTTG 3′	
tolBKO-1	5′ TGCGGCATTGGAGCTAGTTATTAC 3′	Internal fragment of *tolB*; bp 36–270
tolBKO-2	5′ TGAATCGA CCCCCATAGATGTC 3′	



Predicted aa length *V. cholerae*	135	227	146	356	450	172
Predicted aa length *E. coli*	134	230	142	421	431	173
% identity	52%	64%	37%	29%	48%	60%
% similarity	69%	83%	58%	42%	67%	79%

FIG. 1. The organization of the *tol* gene clusters in *V. cholerae* and *E. coli* is identical. The predicted lengths of the *V. cholerae* Tol proteins were derived from an ORF map of the *V. cholerae* DNA sequence with MacVector. Percent identity and similarity were determined by comparing the predicted amino acid sequences of *V. cholerae* and *E. coli* Tol proteins with MacVector. The solid lines flanked by vertical bars represent the positions of the fragments of each *tol* gene that were used to construct the insertion mutations. The solid lines flanked by arrows represent the sequences cloned into pBAD33 used for the complementation studies.

μl of the cell supernatants containing CTX-Knϕ particles. The phage and recipient cells were gently mixed for 20 min at room temperature on a shaker. Then, each mixture was plated on LB agar containing streptomycin (for O395) or streptomycin and ampicillin (for the *tol* mutants) to enumerate the potential recipients and on LB agar containing Kn (for O395) or kanamycin and ampicillin (for the *tol* mutants) to enumerate the transductants. The frequency of infection was determined by dividing the number of transductants (Kn^r or Kn^r Ap^r CFU) by the number of recipients (Sm^r or Sm^r Ap^r CFU).

In the coculture transduction assay, RV508, a $Spec^r$ Rif^r derivative of 569B (37) harboring pCTX-Kn, was streaked on LB agar plates along with Sm^r potential recipient strains. After incubating at 30°C for 4.5 h, the cells were recovered from the plates in 3 ml of LB broth. The number of potential recipient cells was determined by counting the number of Sm^r CFU (the donor strain RV508 is Sm^s), and the number of transductants was determined by enumerating the Sm^r Kn^r CFU (for O395) or Sm^r Ap^r Kn^r CFU (for the *tol* mutants). Again, the frequency of infection was determined by dividing the number of transductants by the number of potential recipients.

Characterization of other phenotypes of the *tolQRAB* mutants. Immunoblot analysis of whole-cell lysates with polyclonal α-TcpA antiserum was carried out as previously described (23). For determination of the growth kinetics of the mutant strains, equivalent dilutions (based on optical density [OD] readings at 600 nm) of overnight LB broth cultures containing the appropriate antibiotics were used as the inocula for cultures. Aliquots were removed from these cultures at 30-min intervals for OD_{600} determination. At hourly intervals, these aliquots were also plated on LB agar with the appropriate antibiotics to enumerate the number of CFU.

The sensitivity of the *tol* mutants to deoxycholate (Sigma, St. Louis, Mo.) was assayed by growing the bacteria in LB broth plus the appropriate antibiotics, containing a range of deoxycholate concentrations from 0.025 to 12.4%. After approximately 14-h growth at 37°C, the turbidity of cultures was assayed visually, and cultures without apparent turbidity were scored as sensitive to deoxycholate. The starting inocula for these determinations were mid-log-phase cultures (OD_{600} of 0.5) of each strain tested.

RNase I leakage from the periplasm was assessed by plating bacteria on LB plates containing 1.0% (wt/vol) type VI RNA from Torula yeast (Sigma) as described by Lazzaroni and Portalier (18). After overnight growth, 0.5 N HCl was added to each plate to precipitate the RNA. Leakage of RNase I was detected by the appearance of a halo surrounding individual colonies after the addition of HCl. Leakage of β-lactamase was determined as follows. Supernatants of overnight cultures were assayed for β-lactamase activity by measuring the color change of nitrocefin (50 μg) (Calbiochem, San Diego, Calif.) per ml, a chromogenic substrate of β-lactamase which turns from yellow (390 nm) to red (486 nm) in the presence of β-lactamase; cleavage of substrate was monitored by a change in absorbance at 486 nm. β-Lactamase activity was measured in both the supernatant and periplasmic extracts of these cells. Periplasmic extracts were prepared by treating cells with NaCl, sucrose, and lysozyme to disrupt the outer membrane (7). The percentage of β-lactamase activity in the supernatant compared to cell associated β-lactamase activity was then calculated.

Molecular biology methods. Standard molecular biology methods were used in this study (2). Restriction enzymes and ligase were purchased from New England Biolabs (Beverly, Mass.) Southern hybridization was carried out with the ECL direct nucleic acid labelling and detection system (Amersham Pharmacia, Buckinghamshire, England) according to the manufacturer's instructions. The DNA probes for these blots were the internal fragments of the *tol* genes that were used for targeted disruption of these genes as described above. DNA sequencing was performed by dye terminator cycle sequencing with an Applied Biosystems 373A DNA sequencer at the Tufts Core Facility. The MacVector software package (Oxford Molecular Group) was used to assemble the *tolQRAB* sequence, and the BLAST programs (1) were used for comparing this sequence to the GenBank database. The hydrophobicity of TolQRAB was calculated with the Kyte-Doolittle algorithm in MacVector. The protein localization program P-sort (25) was used to assess protein localization.

RESULTS

The *V. cholerae tolQRAB* DNA sequence. Extrapolating from the model of filamentous phage entry into *E. coli*, we asked whether the *tolQRAB* gene cluster could be identified in the *V. cholerae* genome and if these gene products, TolQRA in particular, were necessary for entry of CTXϕ into *V. cholerae*. To address the first question, the amino acid sequence of each of the *E. coli* TolQRAB proteins was used to query the partial *V. cholerae* genome being sequenced by The Institute for Genomic Research (TIGR) for potential *V. cholerae tol* orthologues. Of the four *E. coli* sequences, *E. coli* TolR yielded the most significant similarity (E value, 2e-08) using the BLAST X algorithm (1). No clones overlapped with the relevant contig GVCCN44F at that time; consequently, we used inverse PCR to clone the adjacent sequences from classical *V. cholerae* strain O395, which we speculated might encode *V. cholerae tolQ* and *tolAB*. After several iterations of inverse PCR and DNA sequencing, a sequence spanning 4 kb and containing four ORFs which were similar to *E. coli* TolQRAB was generated. Our sequence of the O395 *tolQRAB* cluster has been deposited in GenBank with accession number AF187269. This sequence is virtually identical to the El Tor strain N16961 *tolQRAB* sequence determined by TIGR (http://www.tigr.org).

Using the complete *V. cholerae* genome sequence recently released by TIGR, we found that the genes neighboring the *tolQRAB* gene cluster in *V. cholerae* are similar to those in *E. coli*. In both species, the cluster includes the *orf1*, *tolQ*, *tolR*, *tolA*, *tolB*, and *pal* genes (36), and the predicted *V. cholerae* TolQRAB sequences bear significant similarity to the *E. coli* sequences (Fig. 1). The proteins encoded by the *V. cholerae tol* genes also appear to resemble the *E. coli* proteins in secondary structure. For example, the Kyte-Doolittle hydrophobicity profiles of *V. cholerae* and *E. coli* TolQRAB predict that *V. cholerae* TolQ, TolR, and TolA, like the *E. coli* proteins, contain 3,

TABLE 3. Efficiency of CTXϕ infection in *V. cholerae* *tolQRAB* mutant strains[a]

Recipient[b]	Relevant genotype	No. of recipients/ml	No. of transductants/ml[c]	Frequency of infection[d]
O395	Wild type	3.7×10^9	9.0×10^6	2.4×10^{-3}
DH1	*tolQ*	3.0×10^8	1.3×10^2	4.3×10^{-7}
DH2	*tolR*	8.2×10^8	1.1×10^2	1.3×10^{-7}
DH3	*tolA*	5.9×10^8	0	0
DH4	*tolB*	1.1×10^8	9.1×10^5	8.2×10^{-3}

[a] The transduction assays were conducted at least four times, and the mean number of CFU from these experiments is presented. All standard deviations of these values were less than 10% of the means.
[b] All recipients are derivatives of O395 (see Table 1).
[c] Transductants were counted as the number of Kn^r CFU per milliliter.
[d] The frequency of infection was calculated by dividing the number of transductants per milliliter by the number of recipients per milliliter.

1, and 1 transmembrane domains, respectively (20, 21, 24, 35). The extended α-helical region with repeats of Lys, Ala, and Glu/Asp found in *E. coli* TolA (20) also appears to be present in *V. cholerae* TolA, based on an analysis of secondary structure performed with MacVector. Finally, the protein localization program P-sort (25) predicts that *V. cholerae* TolB, like *E. coli* TolB (12), is predominantly periplasmic. These structural similarities suggest that *V. cholerae* TolQRAB may perform functions similar to those of *E. coli* TolQRAB.

Role of *tolQRAB* in CTXϕ infection. To test the role of TolQRAB in CTXϕ infection, insertion mutations in each of these genes were generated within the classical biotype *V. cholerae* strain O395. We found that the strains harboring the *tolQ*, *tolR*, and *tolB* mutations grew heterogeneously on agar plates, forming either large or small colonies. The large colonies reproducibly restreaked only as large colonies, whereas the smaller colonies upon restreaking gave rise to a heterogeneous population of large and small colonies. DH3, which contains the *tolA* mutation, only grew as large colonies. These large colonies were confirmed by Southern analysis to have the correct integration. We suspect that a spontaneous, secondary mutation that enhances growth has arisen in the large colonies of all four of the strains harboring *tol* gene mutations. Because of the stability of the large colonies, we chose to use these homogenous populations for the studies of CTXϕ infection described below.

The ability of CTXϕ to infect each of these four mutant O395 derivatives was tested. Since CTXϕ infection of *V. cholerae* does not result in plaque formation, a transduction assay was initially used. In this assay, cell-free culture supernatants containing a kanamycin-marked CTXϕ (CTX-Knϕ) were used to transduce recipient strains to kanamycin resistance (Kn^r). We found that the O395 derivatives containing *tolQ*, *tolR*, and *tolA* mutations were dramatically less susceptible to CTXϕ infection than O395 (Table 3). More specifically, the *tolA* mutant strain could not be infected with this assay, and the *tolQ* and *tolR* mutants were approximately 4 orders of magnitude less efficient at CTXϕ uptake than wild-type O395. In contrast, the *tolB* mutation did not confer resistance to CTXϕ infection. All four mutant strains showed fewer CFU after overnight culture than the wild-type strain (Table 3, column 3); however, this growth difference is unlikely to account for the resistance of the *tolQRA* mutant strains to CTXϕ infection, since the growth defect of the *tolB* mutant did not inhibit phage infection. Overall, these results suggest that *V. cholerae* TolQRA proteins are important for uptake of CTXϕ, whereas TolB does not play an essential role in this process.

Complementation studies were performed to verify that the resistance of the *tolQ*, *tolR*, and *tolA* mutant strains to CTXϕ infection was due to the disruption of the targeted *tol* gene and not to polar effects or to spontaneous mutation in another gene. Plasmids containing each of the *tol* genes under the control of an Ara-inducible promoter, pBAD (9), were introduced into the corresponding mutant strain. Either in the presence or absence of inducer, DH1(pDH8), the *tolQ* mutant strain harboring the plasmid expressing *tolQ*, remained resistant to CTXϕ infection (Table 4). This suggested the possibility of a secondary mutation, or more likely, that the *tolQ* mutation in DH1 disrupted the expression of the downstream gene, *tolR*. To address this latter possibility, a plasmid containing both *tolQ* and *tolR*, pDH9, was introduced into the *tolQ* mutant strain. This plasmid rendered this strain nearly as susceptible to CTXϕ infection as the wild-type strain (Table 4). We conclude that the *tolQ* mutation in DH1 eliminates expression of TolR and that TolR is required for CTXϕ infection. To discern whether *tolQ* is also required in this process, a plasmid encoding a functional TolR, pDH10 (see below), was introduced into the *tolQ* mutant. Even in the presence of Ara, no complementation was seen (Table 4). Thus, *tolQ*, like *tolR*, is necessary for the efficient uptake of CTXϕ. Although supplying a functional TolR in *trans* did not complement the *tolQ*

TABLE 4. *tol* gene complementation of CTXϕ infection defects in *V. cholerae*[a]

Recipient strain[b]	Relevant genotype	Plasmid encoding gene(s)	Arabinose[c]	No. of recipients/ml	No. of transductants/ml[d]	Frequency of infection[e]
O395	Wild type		−	4.9×10^9	5.1×10^6	1.0×10^{-3}
DH1(pDH8)	*tolQ*	*tolQ*	−	6.5×10^8	1.0×10^2	1.5×10^{-7}
			+	1.0×10^8	1.3×10^2	1.3×10^{-6}
DH1(pDH9)	*tolQ*	*tolQR*	−	3.6×10^8	4.4×10^4	1.2×10^{-4}
			+	4.8×10^9	1.6×10^6	3.3×10^{-4}
DH1(pDH10)	*tolQ*	*tolR*	−	5.9×10^8	1.4×10^2	2.4×10^{-7}
			+	6.4×10^8	1.1×10^2	1.7×10^{-7}
DH2(pDH10)	*tolR*	*tolR*	−	8.9×10^8	1.1×10^3	1.2×10^{-6}
			+	5.1×10^9	7.5×10^6	1.5×10^{-3}
DH3(pDH11)	*tolA*	*tolA*	−	1.7×10^9	2.6×10^6	1.5×10^{-3}
			+	5.2×10^8	4.2×10^5	8.0×10^{-4}

[a] The transduction assays were conducted at least three times, and the mean number of CFU from these experiments is presented. All the standard deviations of these values were less than 10% of the means.
[b] All recipients are derivatives of strain O395.
[c] +, addition of 0.02% Ara; −, no Ara.
[d] Transductants were counted as the number of Kn^r CFU per milliliter.
[e] Frequency of infection is calculated by dividing the number of transductants (Kn^r recipients per milliliter) by the number of recipients.

TABLE 5. Influence of *tcpA* and *tol* mutations on CTXϕ transfer in a coculture transduction assay[a]

Recipient[b]	Mutant gene(s)	No. of recipients/ml	No. of Kan^r recipients/ml	Frequency of infection[c]
O395	Wild type	2.2×10^9	6.3×10^7	2.9×10^{-2}
TCP2	*tcpA*	2.1×10^9	1.6×10^3	7.6×10^{-7}
DH1	*tolQ*	1.1×10^9	2.9×10^3	2.6×10^{-6}
DH5	*tcpA* and *tolQ*	4.9×10^8	0	0
DH2	*tolR*	7.5×10^8	2.1×10^3	2.8×10^{-6}
DH6	*tcpA* and *tolR*	1.3×10^8	0	0
DH3	*tolA*	7.1×10^8	4.4×10^2	6.2×10^{-7}
DH7	*tcpA* and *tolA*	1.2×10^8	0	0
DH4	*tolB*	2.2×10^8	2.2×10^7	1.0×10^{-1}

[a] Donor strain RV508 (pCTX-Kn) was cross-streaked with the indicated recipient strain on LB agar plates. Transfer of CTX-Knϕ to the recipient was then measured after 4.5 h. These assays were conducted at least three times, and the mean number of CFU from these experiments is presented. The standard deviation of these values was less than 10% of the means.

[b] All recipient strains are derivatives of O395.

[c] Frequency of infection was calculated by dividing the number of Kn^r recipients by the total number of recipients. The ratio of donors (no. of Spec^r Rif^r CFU) to recipients was constant for each assay.

mutation, it was sufficient to complement the mutation in *tolR* in DH2 (Table 4). Similarly, expression of TolA rendered the *tolA* mutant strain, DH3, susceptible to CTXϕ (Table 4). The results of these complementation studies confirm that TolQ, TolR, and TolA are required for infection of *V. cholerae* by CTXϕ and that the spontaneous, secondary mutations which allowed these *tol* mutant strains to grow as large colonies on LB agar plates were not responsible for these strains' resistance to CTXϕ infection.

Requirement for *tolQRA* in CTXϕ infection of TCP$^-$ *V. cholerae*. *E. coli* lacking the F pilus can be infected by Ff phage, although at greatly reduced frequencies than F$^+$ *E. coli*. F-independent infection of *E. coli* by Ff phage is dependent on the *E. coli tolQRA* gene products (29). With our standard liquid suspension CTXϕ transduction assay, which relies upon cell-free filtered supernatants containing a marked CTXϕ to transduce recipient cells, we have not been able to detect transductants of TCP$^-$ *V. cholerae* recipients (37). However, we found that we could circumvent the requirement for TCP in CTXϕ infection if a CTXϕ producing donor strain was grown in close proximity to a potential recipient strain. When a strain harboring the CTX-Knϕ replicative form was cross-streaked with a TCP$^-$ recipient strain, O395 derivative TCP2 (10), we found that CTX-Knϕ transfer to the recipient was detectable, albeit approximately 100,000-fold less frequently than CTX-Knϕ transfer to O395 in this assay (Table 5). Transfer of the CTXϕ genome under these conditions still required formation of functional virions, since mutant forms of CTX-Knϕ that are maintained as plasmids but do not give rise to virions (pMW1 and pMW2 [37]) were not transferred. This result indicates that CTXϕ transfer was mediated by CTXϕ virions rather than by an alternative route, such as conjugation.

Using this protocol, we found that TCP$^+$ strains containing a mutation in any one of the *tolQRA* genes could be infected by CTXϕ at very low frequencies, similar to those observed for infection of the TCP$^-$ strain (Table 5). This suggests that, individually, none of the *V. cholerae tolQ*, *tolR*, or *tolA* gene products is absolutely required for CTXϕ infection. However, mutations in any of these three genes in combination with the *tcpA* mutation rendered the recipient strains completely resistant to CTXϕ infection in this assay (Table 5). In contrast, as in the liquid-based transduction assay, a *tolB* mutation did not significantly affect the ability of the recipient strain to be infected by CTXϕ. None of the *tol* mutations impaired transfer of an RP4-derived conjugal plasmid (5) from *E. coli* (data not shown). The finding that disruption of the *V. cholerae tol* genes did not reduce the ability of cells to act as recipients in conjugation is an additional indication that transfer of CTXϕ genes under these assay conditions is dependent upon infection of recipients by virions, rather than an alternative mechanism of gene transfer. In conclusion, these experiments demonstrate that, similar to Ff phage infection of *E. coli*, TCP$^-$ cells can be infected by CTXϕ and that the *tolQRA* gene products are absolutely required for infection of TCP$^-$ cells. However, unlike *E. coli*, a single mutation in *V. cholerae tolQ*, *tolR*, or *tolA* does not render these cells completely resistant to CTXϕ infection.

Other properties of *V. cholerae* strains with *tolQ*, *tolR*, *tolA*, and *tolB* mutations. A potential explanation for the inefficiency of CTXϕ entry in *V. cholerae* strains with *tolQ*, *tolR*, and *tolA* mutations is that these strains do not express TCP. This possibility is unlikely because DH1, DH2, and DH3, and DH4 cells autoagglutinated after overnight growth at 30°C, a property dependent on TCP production. To further confirm that these *V. cholerae tol* mutant strains synthesize TCP, immunoblot assays were carried out. As shown in Fig. 2, all four of the *tol* mutant strains expressed approximately wild-type amounts of a 20.5-kDa polypeptide that stained with α-TcpA antiserum. This is comparable to *E. coli*, where *tol* mutations have been shown not to interfere with production of F pili (32).

In our studies of the requirement of the *tolQRAB* gene products in CTXϕ infection, we noted that there were reproducibly fewer viable cells in overnight cultures of *tol* mutant strains than in O395 (Table 3). Further studies were carried out to characterize the growth properties of the *V. cholerae tol* mutant strains. The change of the OD_{600} over time of the O395 derivatives with either *tolQ*, *tolR*, *tolA*, or *tolB* mutations during growth in LB broth did not dramatically differ from O395 (Fig. 3A). However, when these cultures were plated on LB agar to determine the number of CFU, there were far fewer colonies in the *tol* mutant strains (Fig. 3B). This discrepancy between the OD_{600} and the recovery of colonies on LB agar plates was most noticeable during the initial lag phase of growth. This reduced plating efficiency may be explained at least in part by

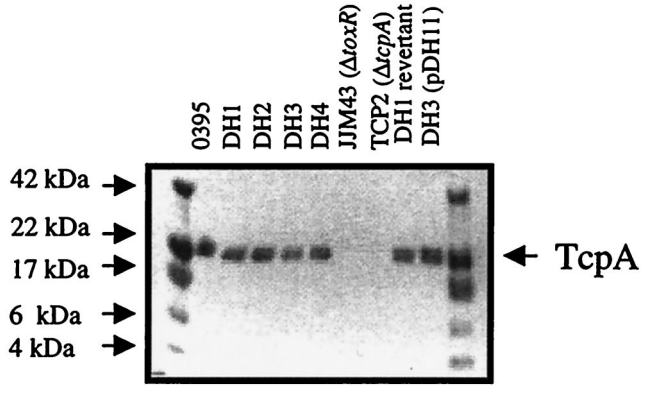


FIG. 2. *V. cholerae tolQRAB* mutants (DH1, DH2, DH3, and DH4) produce amounts of TcpA similar to those produced by O395. All strains are derivatives of O395. The DH1 revertant was made by growing a small colony of DH1 in LB broth overnight at 37°C in the absence of ampicillin. The excision of pDH235 from *tolQ* in this revertant strain was confirmed by Southern analysis. All strains were grown in LB broth at 30°C. Whole-cell lysates were prepared in sample buffer as previously described (23) and run on an 4 to 12% Tris-Bis gradient gel (Novex, San Diego, Calif.). The proteins were then transferred to nitrocellulose and probed with anti-TcpA polyclonal antiserum.

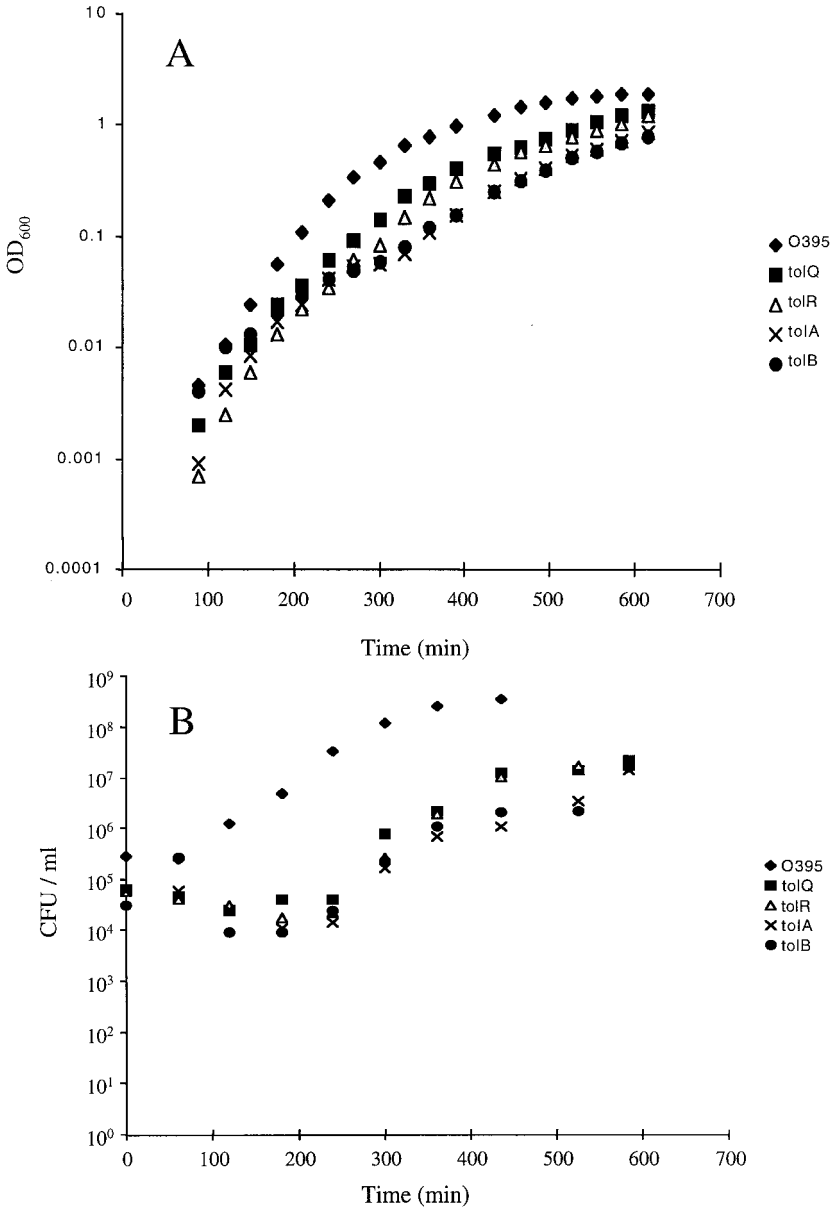


FIG. 3. Kinetics of growth of O395 and O395-derived *tol* mutant strains. All strains were grown in LB broth with the appropriate antibiotics at 37°C and their OD_{600} (A) and CFU (B) were determined over time.

the fact that all four *V. cholerae tol* mutants formed extensive filaments during growth (Fig. 4). This phenotype was most notable during the lag phase of growth. None of the *tol*-complementing plasmids, with the exception of the *tolR*-encoding plasmid pDH10, completely eliminated filamentation of the *tol* mutant strains. Since direct interactions between TolQRA have been demonstrated in *E. coli* (8, 19), our failure to fully complement the growth defect in the *tol* mutants may reflect our inability to restore the correct stoichiometry of the TolQRA proteins in these strains.

In addition to their inability to facilitate entry of filamentous phages, *E. coli tol* mutants exhibit reduced stability of their outer membranes, resulting in leakage of periplasmic proteins into the extracellular environment (39). In *E. coli*, this leakiness has been assessed by assays for the extracellular presence of periplasmic proteins, such as β-lactamase and RNase I, and by increased sensitivity to detergents like deoxycholate. The parental strain O395 and its *tolQRAB* derivates (DH1, DH2, DH3, and DH4) were tested for similar phenotypes. Two *E. coli* control strains, MG1655 (wild-type *E. coli* K-12) and TPS66 (32) (an *E. coli* strain harboring a missense mutation in *tolQ*), were also analyzed. As reported for *E. coli*, we saw significant leakage of β-lactamase (about 30% of the total) into the culture supernatants from *V. cholerae tol* mutants. However, unlike wild-type strains of *E. coli* containing pBR322 (a plasmid encoding the β-lactamase gene), which have very low levels of β-lactamase (about 1.5%) in supernatants (40), *V. cholerae* O395, harboring the *bla*-containing plasmid pCRII, had significant amounts of β-lactamase (about 15 to 20% of the total) present in culture supernatants. Thus, the effect of the *tol* mutations in leakage of β-lactamase in *V. cholerae* was difficult to ascertain. To ensure that the β-lactamase detected in the supernatants of O395 and DH1-4 was not due to cell lysis, we also assayed the supernatants for the presence of the

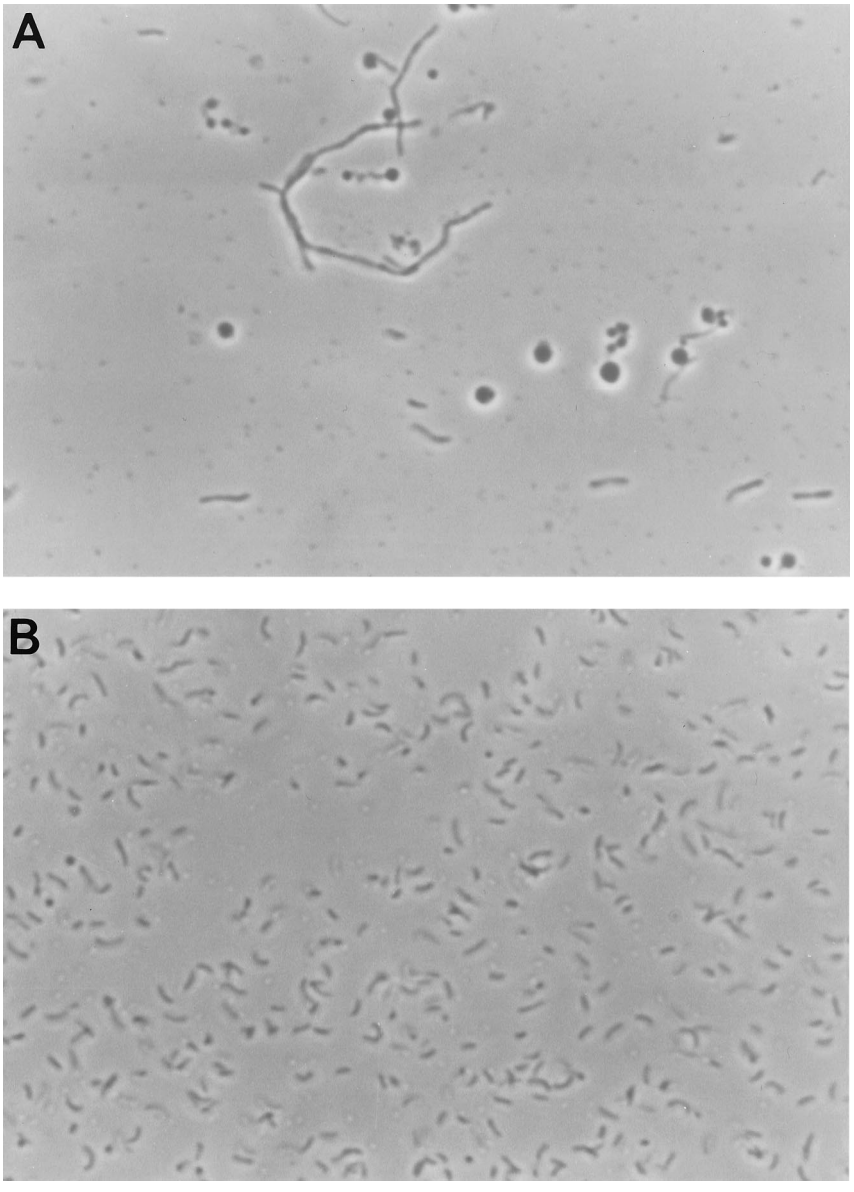


FIG. 4. Filamentous morphology of *V. cholerae tol* mutant strain DH4 (A) compared with O395 (B). O395 derivatives with insertions in *tolQ*, *tolR*, and *tolA* also exhibited filamentation.

cytoplasmic enzyme, β-galactosidase (β-Gal). We found that β-Gal activity in the supernatants of these strains was less than 5% of total β-Gal activity. A similar low percentage of β-Gal activity in supernatants of MG1655 and TPS66 was also measured.

A similar difficulty was encountered when the leakage of RNase I was assayed. Both TPS66 and the four *V. cholerae tol* mutants (DH1 to DH4) exhibited a halo around colonies indicative of leakage of RNase I. While the wild-type *E. coli* strain showed no such halo, O395 did, although the size of its halo was slightly smaller than those around the colonies of DH1 to DH4. Thus, the O395 derivatives with *tol* mutations leaked slightly more RNase I than O395; however, due to the significant basal level of leakiness found in O395, this difference was not as dramatic as that seen between TPS66 and MG1655.

The sensitivity of DH1, DH2, DH3, DH4, and O395 to deoxycholate was compared. The *V. cholerae tol* mutants showed an increased sensitivity to deoxycholate as these strains had no growth at concentrations of deoxycholate eightfold less than the inhibitory concentration for O395. Thus, as observed in *E. coli* (39), mutations in the *V. cholerae tolQRAB* genes increase the sensitivity of these strains to deoxycholate.

DISCUSSION

Our work indicates that there are significant parallels between the pathways used by CTXϕ for entry into *V. cholerae* and by Ff phage for entry into *E. coli*. Like Ff phage entry into F^+ *E. coli*, we found that efficient entry of CTXϕ into *V. cholerae* requires both a pilus, TCP, and the products of the *tolQRA* genes but not the product of *tolB*. Most likely, in both instances, binding of the phages to their respective pilus receptors constitutes the initial event in infection. We found that CTXϕ, like the coliphages, does not absolutely require its pilus receptor for uptake into *V. cholerae*. Therefore, it is reasonable

to suggest (as Reichmann and Holliger proposed for the coliphages [26]) that the initial binding of CTXϕ to TCP enables the phage to bind to *V. cholerae* at a significant distance from the cell surface. Binding to TCP somehow then directs CTXϕ to the *V. cholerae* outer membrane where it can interact with the TolQRA complex, which facilitates its traversal of the periplasmic space. In the absence of TCP, however, occasionally CTXϕ particles can contact the outer membrane and interact with the TolQRA complex. This model is supported by our data that mutations in both *tcpA* and one of the *tolQRA* genes render the target cell resistant to CTXϕ infection.

The similarity of *V. cholerae* TolQRA to *E. coli* TolQRA, together with the requirement for *V. cholerae* TolQRA in CTXϕ entry into *V. cholerae*, suggests that elements of the recently proposed model of Ff phage infection of *E. coli* apply to CTXϕ infection of *V. cholerae*. Based on structural and biochemical data, Riechmann and Holliger have proposed that the requirement for both the F pilus and TolQRA in the entry of Ff phages into *E. coli* is based on the dual-binding specificities of pIII, a minor coat protein located on one end of Ff phages (26). Domain 2 of pIII (g3p-D2) binds to the F pilus, and domain 1 (g3p-D1) binds to the carboxyl terminus of TolA (TolA-III). Binding of g3p-D2 to F is thought to lead to pilus retraction and to exposure of g3p-D1 for binding to TolA-III. After g3p-D1 binds to TolA-III, the subsequent steps leading to internalization of the Ff phage are not known, though g3p may, along with TolQRA, form a channel in the inner membrane (26).

Given the requirement for *V. cholerae* TolQRA in CTXϕ infection and the predicted structural similarity of domain 1 of the CTXϕ g3p orthologue, OrfU, with domain 1 of g3p (11), it is reasonable to suggest that this domain of OrfU binds to *V. cholerae* TolA after OrfU binds to TCP. If the *E. coli* model applies, the initial binding of CTXϕ to TCP (presumably via OrfU domain 2) results in retraction of TCP and exposes OrfU domain 1 for binding to the C terminus of TolA. TCP retraction has not been demonstrated experimentally in *V. cholerae* but is suggested by our data. A structural prediction of this model is that after CTXϕ particles bind TCP, OrfU is in sufficient proximity to TolA to enable these proteins to interact. If this is the case, then two distinct types of pili, F and TCP, are similarly distributed relative to the TolQ, TolR, and TolA proteins in *E. coli* and *V. cholerae*. It remains to be shown whether there is a direct physical interaction between TolQRA and F in *E. coli* and TolQRA and TCP in *V. cholerae*.

Our demonstration of CTXϕ infection of TCP^- *V. cholerae* cells may provide an explanation for the origin of $CTX\phi^+$ TCP^- *V. cholerae* isolates that have been reported. In the laboratory, we were able to infect TCP^- CTXϕ-*V. cholerae* O1 isolate 468-83 (28) with CTX-Knϕ by coculturing it with a CTXϕ donor strain. As with TCP2, a laboratory-derived TCP^- strain, the frequency of infection of 468-83 by CTX-Knϕ was very low (approximately 2.6×10^{-9}) in this assay. Thus, the experimental evidence indicates that infection of TCP^- strains by CTXϕ is possible, but it is likely to be a rare event. As would be expected in light of these results, relatively few $CTX\phi^+$ TCP^- strains have been found among environmental isolates.

Although the host molecules required for CTXϕ infection of *V. cholerae* are similar to molecules required for Ff infection of *E. coli*, our data suggest that there are differences in the importance of TolQRA in CTXϕ infection. Unlike *E. coli*, a mutation in any one of the *V. cholerae tolQRA* genes, including *tolA*, did not render the target cell completely resistant to CTXϕ. There are a number of potential explanations for this difference. First, there could be an alternate route for uptake of CTXϕ, mediated by different proteins. Since cells with mutations in both *tcpA* and one of the *tolQ*, *tolR*, or *tolA* genes could not be infected with CTXϕ, this alternative pathway must be dependent (at least indirectly) on the expression of TCP. Ongoing studies are aimed at determining if *V. cholerae* carries other genes that might play a role in uptake of CTXϕ. Second, because our mutations in *tolQ*, *tolR*, and *tolA* were plasmid integrations into the middle of these genes, it is possible that truncated TolQRA proteins were sufficient to permit the low levels of infection we observed with these mutations. Finally, it may be that our alternative assay for transduction, which relied upon coculture of donor and recipients on a semisolid surface, is more sensitive than the liquid transduction assays used for *E. coli* and Ff phages. If so, this could account for the residual CTXϕ infection of the *V. cholerae tol* mutant strains.

The similarities between the *tol* gene products of several gram-negative species, including *E. coli* (32, 33), *Haemophilus influenzae* (31), *Pseudomonas putida* (27), and *Pseudomonas aeruginosa* (6), suggest that the structure and function of the *tol* gene products, although as yet not completely defined, is conserved between species. In spite of this similarity, however, differences exist in the phenotypes exhibited by *E. coli*, *P. aeruginosa*, and *V. cholerae* strains containing mutations in their *tolQRAB* genes. In *P. aeruginosa*, viable strains containing mutations in the *tolQRAB* genes could not be obtained, leading to the suggestion that TolQRAB proteins are essential in this bacteria (6). Although the *tol* genes were not essential in *V. cholerae*, most mutants grew slowly, giving rise to small colonies in the absence of presumed secondary mutations. In addition, we found that our small-colony *V. cholerae* strains with mutations in *tolQ*, *tolR*, and *tolB* were not viable at 42°C. In *E. coli*, TolQRAB proteins have not been reported to be essential for growth at any temperature. Also, the defect in efficiency of plating exhibited by *V. cholerae* strains with *tolQRAB* mutations has not been reported in *E. coli*, although it has recently been reported that a mutation in *tolA* in *E. coli* impairs septation and cell division (22). The molecular bases for these differences in phenotypes are not known.

In *E. coli*, the transcriptional organization of the *tolQRAB* gene complex consists of two operons: *orf1 tolQRA*, which has a promoter upstream of *orf1*, and *tolB pal*, which has a promoter upstream of *tolB* (37). Similar to *E. coli*, the data from our complementation studies suggest that *V. cholerae tolQ* and *tolR* are transcriptionally linked, as the insertion mutation in *tolQ* was polar on *tolR* and could only be complemented with the addition of both *tolQ* and *tolR*. Another possibility, demonstrated for *E. coli* (36), is that translational control of *tolR* expression by TolQ exists in *V. cholerae*. Since the *tolR* mutation in DH2 was not polar on *tolA*, these results suggest that in *V. cholerae* there is no linkage of *tolQR* transcription to *tolA* transcription but rather that *tolA* may have its own promoter. Another explanation is that the mutation introduced in *tolR* by pDH107 integration is only partially polar on *tolA* and that the resultant reduced levels of *tolA* message are sufficient to enable CTXϕ infection. Further studies are required to determine the exact transcriptional organization of *V. cholerae tolQRAB*.

Our finding that the *V. cholerae tolQRA* products are required for CTXϕ uptake suggests a way to improve the biosafety of live-attenuated ($CTX\phi^-$) *V. cholerae* vaccine strains. These strains can be reinfected by CTXϕ and thereby revert back to toxigenicity. However, a mutation in either *tolQ*, *tolR*, or *tolA* in a vaccine strain should render it relatively resistant to CTXϕ infection. A potential difficulty with this approach is that the pleiotropic effects of these mutations may diminish the strains' capacity to colonize the intestine, a prerequisite for antigenicity (10). Colonization defects have been reported in a

Salmonella enterica serovar Typhimurium strain harboring a mutation in *tolB* (3). Our preliminary results suggest that the *tolQRA* mutations in DH1, DH2, and DH3 significantly attenuate colonization; however, these results are difficult to interpret given the plating inefficiency of these strains. Even if the *V. cholerae tol* gene products are required for colonization, it may be possible to isolate specific *tolQ*, *tolR*, or *tolA* mutations which render the resulting strains resistant to CTXϕ infection but which do not significantly attenuate intestinal colonization. For this approach to work, the essential functions of TolQRA must be structurally separable from the activity of these proteins as phage coreceptors.

ACKNOWLEDGMENTS

We thank N. Golden for construction of the *tolR* mutation and A. Kane, B. Davis, B. Hochhut, H. Kimsey, A. Camilli, and C. Moyer for critical reading of the manuscript. We are grateful to Robert Webster for sending strain TPS66. We thank A. Kane and the GRASP Center Intestinal Microbiology Core for preparation of media and M. Berne of the Tufts Core Facility for DNA sequencing.

This work was supported by NIH grant AI 42347. M.K.W. is a Pew Scholar in the Biomedical Sciences.

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