JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6464–6471 Vol. 179, No. 20
0021-9193/97/$04.00+0

Filamentous Phage Infection: Required Interactions with the TolA Protein

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Received 30 May 1997/Accepted 7 August 1997

Infection of *Escherichia coli* by the filamentous phage f1 is initiated by binding of the phage to the tip of the F conjugative pilus via the gene III protein. Subsequent translocation of phage DNA requires the chromosomally encoded TolQ, TolR, and TolA proteins, after the pilus presumably has withdrawn, bringing the phage to the bacterial surface. Of these three proteins, TolA is proposed to span the periplasm, since it contains a long helical domain (domain II), which connects a cytoplasmic membrane anchor domain (domain I) to the carboxyl-terminal domain (domain III). By using a transducing phage, the requirement for TolA in an F^+ strain was found to be absolute. The role of TolA domains II and III in the infective process was examined by analyzing the ability of various deletion mutants of *tolA* to facilitate infection. The C-terminal domain III was shown to be essential, whereas the polyglycine region separating domains I and II could be deleted with no effect. Deletion of helical domain II reduced the efficiency of infection, which could be restored to normal by retaining the C-terminal half of domain II. Soluble domain III, expressed in the periplasm but not in the cytoplasm or in the medium, interfered with infection of a $tolA^+$ strain. The essential interaction of TolA domain III with phage via gene III protein appears to require interaction with a third component, either the pilus tip or a periplasmic entity.

The Ff class of single-stranded DNA filamentous bacteriophages (f1, M13, and fd) infect *Escherichia coli* containing the information for the F conjugative plasmid (for reviews, see references 30 and 44). Infection is initiated by the binding of one end of the phage to the tip of the conjugative pilus. Recognition of the pilus tip is the function of the amino-terminal portion of the phage gene III protein (pIII), a minor capsid protein found at one end of the phage particle (1, 13). Binding of the phage is thought to be followed by retraction of the pilus, bringing the pIII end of the particle near the surface of the bacteria (17). It is not known whether retraction is a result of normal polymerization-depolymerization cycles of the pilus or whether phage attachment triggers this process (11, 15). Once at the cell surface, most or all of the capsid protein integrates into the bacterial cytoplasmic membrane and the DNA is translocated into the cytoplasm. These latter processes require the products of the chromosomal *tolQ*, *tolR*, and *tolA* genes (36, 43).

The TolQ, TolR, and TolA proteins are part of the Tol system, which appears to be involved in maintaining the integrity of the outer membrane of *E. coli* (10, 22, 43). Mutations in any one of the contiguous genes *tolQ*, *tolR*, *tolA*, *tolB*, or *pal* cause bacteria to leak periplasmic proteins such as alkaline phosphatase and RNase I into the culture medium as well as to become extremely sensitive to detergents such as deoxycholate. The *tolQ*, *tolR*, *tolA*, and *tolB* gene products are also required for the import of the group A colicins (A, E1 to E9, K, L, N and S4) following binding of these bacteriocins to their receptors (7, 21). The genes have been sequenced and shown to be part of the gene cluster *orf-1 tolQRAB pal orf-2* located at 16.8 min on the *Escherichia coli* genetic map (22, 24, 33, 39, 41).

The TolQ, TolR, TolA, TolB, and Pal proteins have been localized to various parts of the bacterial cell envelope. TolQ has three transmembrane helices in the cytoplasmic membrane, with the major portion of the protein exposed to the cytoplasm (20, 39, 40). TolR is anchored in the cytoplasmic membrane at its amino terminus with the remainder in the periplasm (20, 32). TolA has a three-domain structure: an amino-terminal membrane anchor (domain I) and a periplasmic region consisting of a central long helical region (domain II) and a carboxyl-terminal region (domain III) required for activity (25, 26). The transmembrane regions of TolQ, TolR, and TolA have been shown to interact with each other (8, 23). TolB is a periplasmic protein (16) which interacts with Pal (5), an outer membrane lipoprotein associated with the peptidoglycan layer (22, 29). There is also evidence that the helical domain II of TolA may interact with outer membrane trimeric porins (9). The topologies and interactions of these proteins suggest that they form a complex which interacts with and thus forms a bridge between the cytoplasmic and outer membranes. The observation that the Tol proteins may be concentrated at contact sites between the membranes is consistent with the existence of such a complex (14).

The molecular interactions involved in the functioning of the Tol system have thus far focused on the import of colicins. It is proposed that TolA is involved in forming the connection between the two membranes via its long helical domain II (26). This positioning would allow the carboxyl-terminal domain III of TolA to interact with the colicin molecule near the outer membrane receptor to which the colicin is bound. The observation that domain III is required for colicin activity (26) and directly interacts with colicins A and E1 (2) is consistent with this hypothesis.

It is still unclear how colicins are translocated across the outer membrane. It has been proposed that the binding of a colicin molecule to its receptor induces a conformational change allowing the colicin to enter the periplasm, perhaps through the porin portion of the receptor (21). Once inside the periplasm, the colicin would be able to interact with domain III of TolA and with periplasmic TolB. There is both in vitro and in vivo evidence for interactions between the amino-terminal

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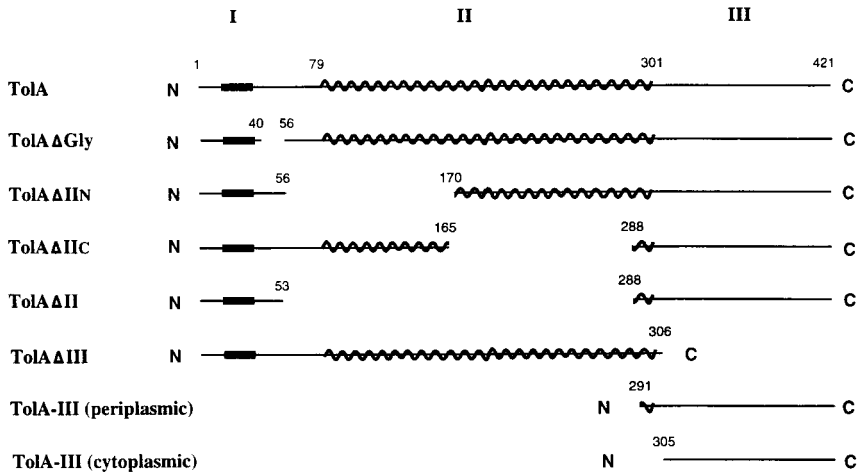

FIG. 1. Schematic diagram of TolA protein domains expressed from pET3c expression vector. I to III indicate protein domains which are delineated by the residue numbers above the TolA diagram. ▬, membrane-spanning region in N-terminal domain I; ∿, α-helical region of domain II (residues 79 to 301). The numbers above the diagrams of each of the mutant proteins delineate the edges of the deleted segments. The small number of non-TolA amino acids preceding the mature periplasmic form (26) and the cytoplasmic form (25) of soluble TolA-III are not depicted.

portion of colicins and these proteins (2, 6), and there is also evidence that the carboxyl-terminal region of colicin E3 requires TolB (18). Subsequent translocation of the colicin across the plasma membrane would require the TolQRA complex in a manner as yet unknown.

Colicin E1 is an exception to this pathway in that it does not require TolB or a porin to translocate into the inner membrane. It is postulated that colicin E1 may directly interact with the TolQRA complex via its unique requirement for TolC, a channel-forming outer membrane protein (3) involved in the Sec-independent export of α-hemolysin and colicin V (42, 45).

Translocation of f1 phage is proposed to be similar to that for colicins, and in particular that of colicin E1, since neither requires TolB. Since the pilus emanates from the inner membrane and protrudes through the outer membrane, retraction of the pilus would potentially bring the gene III end of the phage directly to the TolQRA complex. However, interactions between the inner membrane-bound Tol proteins and f1 phage that are required for infection have not been characterized. Of the three Tol proteins, TolA has the greatest structural potential to initiate contact with the incoming phage particle. In this study, we investigated the requirement for discrete domains of TolA in phage infection. The results indicate that the carboxyl-terminal domain III of TolA is essential for infection. The long helical domain II of TolA is not required, although the carboxyl half of this domain does enhance the rate of infection. We also show that binding of the phage to the F-pilus appears to be required to allow phage pIII to interact with domain III of TolA.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K91 (HfrC) and K17 (F^-) were obtained from Marjorie Russel (The Rockefeller University). GM1 (F′ *lac pro*) was obtained from D. Steege (Duke University). K17DE3 is K17 lysogenized with lambda DE3 carrying the inducible gene for T7 RNA polymerase (25). K17DE3/F^+ contains the F′ *lac pro* of GM1 introduced by conjugation. K17DE3*tolA*/F^+ and K91*tolA* (previously designated K91A1 [25]) each contain a mini-Tn*10* insertion near nucleotide 200 of the *tolA* gene.

TolA plasmids. TolA and TolA deletion proteins (Fig. 1) were expressed in pET3clF (25) or in pET3clR. These vectors contain the pUC18 multiple-cloning sequence inserted into pET3c in the forward and reverse orientation, respectively, downstream of the T7 promoter and ribosome binding sequence. Previously described plasmids include pSKL10 (25) encoding wild-type TolA, pSKL19 encoding domain III of TolA [TolA-III (cytoplasmic)] and pSKL21 (26) encoding domain III of TolA behind a signal sequence [TolA-III (periplasmic)]. Plasmids p*tolA*Δ1, p*tolA*Δ2, and p*tolA*Δ3 encode deletions of the N-terminal half, the C-terminal half, and all of domain II of TolA, respectively (37). These proteins are designated TolAΔIIN (p*tolA*Δ1), TolAΔIIC (p*tolA*Δ2), and TolAΔII (p*tolA*Δ3) in this paper (Fig. 1). Plasmid pEMC18 is a derivative of pET3clF which expresses no protein.

Plasmid pEMC30 expresses TolAΔGly in which residues 41 to 55 (SAGGGGGSSIDAVMV) have been deleted. The deletion was constructed in a pET3clF derivative expressing the wild-type *tolA* gene but lacking vector *Hin*dIII and *Eco*RI sites. An *Eco*RI site was introduced into the *tolA* gene at residue 56 by replacing the *Bam*HI-*Not*I fragment with the same fragment of pSK[3.5+2.3] (37). Digestion of the resulting plasmid with *Hin*dIII and *Eco*RI, followed by Klenow filling and religation, resulted in an in-frame deletion of 15 residues of TolA which included the polyglycine sequence.

Plasmid pEMC33 expresses TolAΔIII in which the C-terminal domain III of TolA has been deleted. To construct plasmid pEMC33, the *tolA* gene in pSKL10 was digested with *Bsm*I and blunted with T4 DNA polymerase and the *Nde*I-*Bsm*I fragment encoding residues 1 to 306 of TolA was recloned into the *Nde*I-*Sma*I sites of pET3clR. The vector adds the amino acid sequence GVPSSN to the C terminus of TolAΔIII.

Media and chemicals. Bacteria were grown in TY medium as described previously (39). Unless otherwise stated, antibiotics were used at the following concentrations: ampicillin, 60 μg/ml; chloramphenicol, 15 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Research Organics. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and Boehringer Mannheim. Antiserum to M13 phage was purchased from Pharmacia.

Phage infection and membrane integrity assays. The rate of infection was determined by the addition of f1 phage at a multiplicity of infection of 1 to bacteria growing exponentially at 2×10^8 cells/ml. At various times after infection, aliquots were mixed with 1 μl of anti-M13 phage antiserum and rapidly chilled. Appropriate dilutions were plated on a lawn of K91. In competition experiments with soluble TolA-III, log-phase cultures of K17DE3/F^+/pLys containing the appropriate plasmid were induced at 2×10^7 cells/ml with 0.04 mM IPTG for 1.5 to 2 h. The concentration of bacteria was adjusted to 2×10^8 cells/ml with warm medium, and then f1 phage was added at a multiplicity of infection of 1. At various times after infection, 10-μl aliquots were added to 10 μl of anti-M13 phage antiserum in 80 μl of TY at 0°C. Aliquots (0.1 ml) were then plated on a lawn of K91. Infection with transducing phage was performed essentially as described by Russel et al. (36). Specifically, an aliquot (0.1 ml) of exponentially growing cells at 2×10^8 cells/ml was infected for 10 min with the Amp^r transducing phage pD8 at a multiplicity of infection of 1 for transducing particles. Antiserum to M13 phage (1 μl) was added, and the culture was rapidly chilled. The entire sample (or appropriate dilutions) was plated on agar containing 120 μg of ampicillin per ml overlaid just prior to use with an equal volume of agar containing no antibiotic. The titer of transducing particles was 2.7×10^{10}, and the titer of helper phage was 8.2×10^9. Sensitivity to 0.2% deoxycholate and leakage of periplasmic RNase I were assayed as previously described (26).

TolA-III purification and antiserum preparation. The cytoplasmic form of soluble TolA-III, prepared as described by Levengood et al. (25), was used to

TABLE 1. Complementation of *tolA* mutant with TolA deletion proteins

Expt.	Strain	Plasmid	Protein[a]	Infectivity[b]		Deoxycholate sensitivity[c]	RNase I leakage[d]
				f1 plaques	f2 plaques		
1	K91		TolA(chrom)	161 (++++)	263	−	−
	K91*tolA*			0	97	+	+
	K91*tolA*	pEMC30	TolAΔGly	197 (++++)	111	−	−
2	K17DE3/F$^+$		TolA(chrom)	219 (++++)	338	−	−
	K17DE3/F$^+$	pBR322	TolA(chrom)	229 (++++)	330	−	−
	K17DE3*tolA*/F$^+$	pBR322		0	248	+	+
	K17DE3*tolA*/F$^+$	pSKL10	TolA	191 (+++)	259	−	−
	K17DE3*tolA*/F$^+$	p*tolAΔ1*	TolAΔIIN	197 (+++)	305	−	−
	K17DE3*tolA*/F$^+$	p*tolAΔ2*	TolAΔIIC	122 (++)	337	+	−
	K17DE3*tolA*/F$^+$	p*tolAΔ3*	TolAΔII	35 (+)	194	+	+
	K17DE3*tolA*/F$^+$	pEMC33	TolAΔIII	0	175	+	+

[a] Relative to chromosomal TolA [TolA(chrom)], plasmid expression of TolA, TolAΔIIN, and TolAΔIIC was fourfold greater whereas expression of TolAΔII and TolAΔIII was approximately twofold greater, as determined by quantitative Western blotting of at least two separate experiments (see Fig. 2). Expression of TolAΔGly was not quantitated.

[b] Number of plaques formed when stationary-phase bacteria containing the indicated plasmids were mixed with 200 to 300 PFU of f1 or f2 bacteriophage and then plated on TY agar containing appropriate antibiotics. Relative plaque size: ++++, normal-sized plaques; +++, slightly smaller; ++, definitely smaller; +, tiny.

[c] +, little or no growth on agar containing 0.2% deoxycholate (sensitivity); −, normal growth (resistance).

[d] +, appearance of a clear zone surrounding the colony following trichloroacetic acid precipitation of agar containing 1.5% RNA (leakage); −, no clear zone (no leakage).

make rabbit antiserum to TolA-III. For inhibition studies, exponentially growing K17DE3/F$^+$/pLysS/pSKL21 bacteria expressing periplasmic TolA-III were induced for 2 h with 0.04 mM IPTG. Proteins precipitated from 3 liters of medium with ammonium sulfate (70% saturation) were dissolved in 10 mM Tris-HCl (pH 7.4)–1 mM phenylmethylsulfonyl fluoride and passed over DEAE-cellulose resin equilibrated in 10 mM Tris-HCl (pH 7.4) at 4°C. The TolA-III protein, present in the flowthrough fraction at a concentration of 0.6 mg/ml, was essentially pure as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and Western blotting. Cells from 1 ml of cell culture were suspended in 0.1 ml of 4% SDS–0.25 M Tris (pH 6.8) and boiled for 10 min. The protein concentration was determined by the bicinchoninic acid assay (Pierce). A 20-μg portion of protein was run on SDS-PAGE (10% acrylamide), transferred to nitrocellulose, and reacted either with antisera against TolA domains II and III (25) or with affinity-purified antibodies against TolA-III followed by ^{125}I-protein A (ICN). The blot was quantitated with a Molecular Dynamics phosphorimaging screen and the Image Quant Program.

RESULTS

Analysis of *tolA* deletion mutants for infection by f1 bacteriophage. TolA is anchored to the inner membrane by an amino-terminal transmembrane region (domain I) followed a helical domain II attached to a more globular domain III (Fig. 1). Between the membrane-anchoring domain I and the helical region of domain II is a region of five sequential glycines which might be required to give flexibility between these two regions. The long helical region is proposed to act as a tether, allowing domain III to interact with components of the outer membrane (26). Since the pilus may bring the tip of the phage through the outer membrane and close to the cytoplasmic membrane, it was of interest to determine which regions of the long TolA molecule were required for infection.

A series of deletion mutations of *tolA* were constructed in a pET3c expression vector (Fig. 1). Each plasmid was placed in a bacterial strain containing a mini-Tn*10* near nucleotide 200 of the *tolA* gene (25). The ability of these deletion mutants to support phage infection was determined by analyzing whether the phage could form plaques on bacteria containing plasmids expressing one of the TolA deletion proteins (Table 1). Removal of the polyglycine region from TolA (TolAΔGly) had no effect on the ability of bacteria to support phage infection (Table 1). Also, these bacteria remained resistant to deoxycholate and did not leak periplasmic material into the medium.

Three different deletions of domain II were tested. The absence of the amino-terminal half of domain II (TolAΔIIN) had no effect on the number or size of plaques compared to plaques formed on bacteria producing intact TolA from pSKL10 (Table 1). Bacteria containing TolA lacking the carboxyl half of domain II (TolAΔIIC) gave fewer plaques, and the plaque size was somewhat smaller. In addition, these bacteria were sensitive to deoxycholate. Infection of bacteria producing TolA lacking the entire domain II region (TolAΔII) resulted in the appearance of less than 20% of the expected number of plaques, and the plaques were extremely small. In addition, bacteria producing TolAΔII were sensitive to deoxycholate and leaked periplasmic proteins into the media. No plaques were formed when TolA lacking domain III (TolAΔIII) was expressed in the *tolA* mutant bacteria. These bacteria also exhibited the periplasmic leakage and deoxycholate sensitive phenotype of the *tolA* null mutation. The effects of these *tolA* deletions on plaque number and size were quite reproducible, even when the amount of *tolA* mutant expression was increased by the addition of IPTG or when the infection and replication were slowed by growth at the lower temperature of 34°C (data not shown).

The differences observed in plaque-forming efficiency of cells containing the different TolA deletions is not due to a reduced expression of the TolA deletion proteins from their respective plasmids. Equal amounts of protein from bacteria containing each of the plasmids were subjected to SDS-PAGE and Western blot analysis with antibodies to TolA as shown in Fig. 2. All of the plasmids directed the synthesis of a TolA molecule which migrated at the position expected for the particular deletion. Quantitation of the bands with a PhosphorImager showed that the amount of TolA, TolAΔIIN, or TolAΔIIC produced from their respective plasmids was about fourfold greater than the amount of TolA synthesized from the chromosomal gene. The amount of TolAΔII or TolAΔIII was approximately twice the amount of chromosomally encoded TolA. Also, the decrease in plaque-forming efficiency caused by the mutants is not due to mislocalization of the mutant proteins. Cellular fractionation of K17DE3*tolA*/F$^+$ bacteria expressing either TolAΔII, TolAΔIII or wild-type TolA from plasmids under the conditions in Table 1 showed that essen-

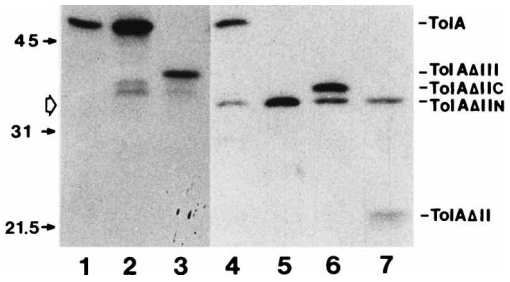

FIG. 2. Relative expression of TolA proteins. Equal amounts of total cellular protein (20 μg), obtained from cultures grown in the absence of inducer, were quantitated by Western blotting with antiserum to TolA domains II and III (lanes 1 to 3) or antiserum to TolA-III (lanes 4 to 7), followed by ^{125}I-protein A. Lanes: 1, K17DE3/F$^+$ (chromosomal TolA); 2 to 7, K17DE3*tolA*/F$^+$ containing plasmids pSKL10 (lanes 2 and 4, TolA), pEMC33 (lane 3, TolAΔIII), p*tolAΔ1* (lane 5; TolAΔIIN), p*tolAΔ2* (lane 6, TolAΔIIC), or p*tolAΔ3* (lane 7, TolAΔII). The open arrow indicates a nonspecific anti-TolA-III immunoreactive band comigrating with TolAΔIIN. The relative mobilities of protein standards with molecular masses in kilodaltons are indicated on the left.

tially all of each protein was localized to the inner membrane (data not shown).

The presence of the mutant TolA proteins did not affect the production of the F pilus, since the RNA bacteriophage f2, which uses the side of the pilus as a receptor, was able to form plaques on all the strains synthesizing the mutant TolA molecules (Table 1). These results suggest that the decrease in plaque number and size observed with bacteria producing certain TolA deletions could simply be the result of a lower efficiency of infection directed by these mutant TolA proteins. This would be reflected in a lower than normal rate of infection. To test this, bacteria expressing the various TolA mutations were infected with f1 at a multiplicity of 1. Aliquots, taken at various times after infection, were incubated with anti-M13 antibody to sequester free phage, and then the cells were plated on a lawn of K91 (wild-type *tolA*) to determine the number of infected bacteria. As shown in Fig. 3A, infection of wild-type K17DE3/F$^+$ increased over time, with about 2.5 to 4% of bacteria being infected by 15 min. For all these experiments, 4 to 10% of wild-type bacteria were infected by 15 min. When TolA was supplied from plasmid pSKL10 in the *tolA* mutant K17DE3*tolA*/F$^+$, the rate of infection was approximately half that observed for wild-type K17DE3/F$^+$ (Fig. 3A, TolA chrom). Presumably, this is the result of an imbalance in the ratio of TolA, TolQ, and TolR due to the fourfold excess of TolA produced from the plasmid (Fig. 2).

Figure 3B (inset) shows that TolAΔIIN lacking the amino-terminal half of domain II was just as effective in supporting infection as was the same amount of wild-type TolA produced from plasmid pSKL10. However, when the carboxyl-terminal half of domain II was deleted (TolAΔIIC), the rate of infection was reduced. Removal of the entire domain II region (TolAΔII) further reduced the rate of infection. This low rate of infection in the presence of the TolAΔII protein was still significantly above the background rate observed in K17DE3/F$^+$ containing plasmid pBR322 (Fig. 3C). As expected, little if any infection over background was observed when TolAΔIII lacking domain III was the only TolA species present.

There was always an extremely low but significant level of infection of K17DE3/F$^+$ carrying the null Tn*10* insertion mutation of *tolA* in the rate assays (Fig. 3C, pBR322). This suggested the possibility that an f1 bound to the F pilus can occasionally bypass the requirement for TolA and enter the *tolA* mutant bacterium. Alternatively, this low level of infection

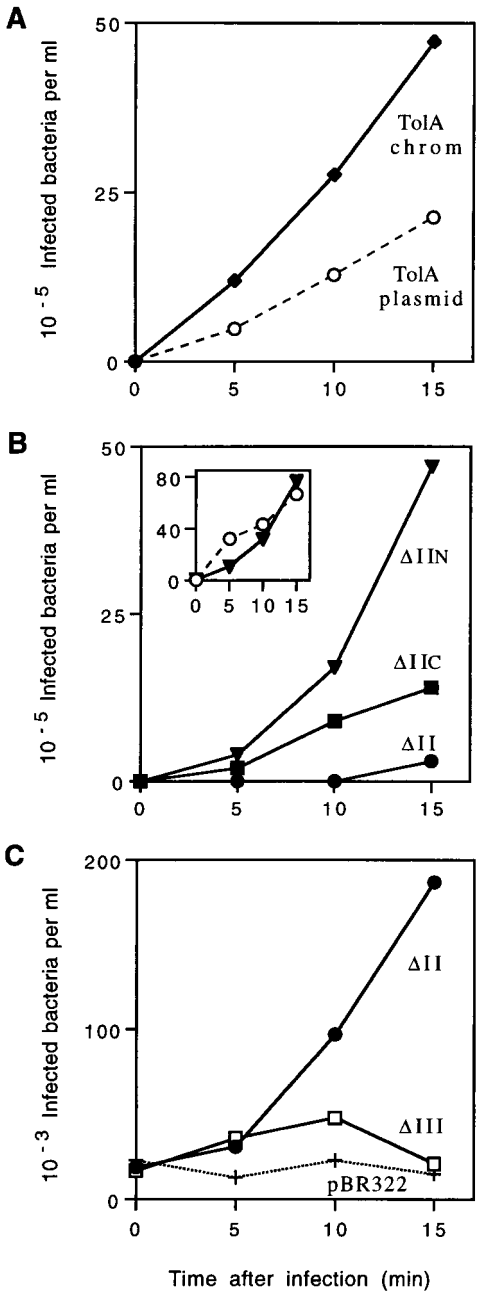

FIG. 3. Rate of f1 phage infection in *tolA* mutant expressing various TolA deletion proteins. Wild-type K17DE3/F$^+$ bacteria (◆) or *tolA*::Tn*10* mutant K17DE3*tolA*/F$^+$ bacteria containing plasmids expressing TolA (○), TolAΔIIN (▼), TolAΔIIC (■), TolAΔII (●), TolAΔIII (□), or pBR322 (+) were grown to 2×10^8 bacteria/ml and then infected with f1 phage at a multiplicity of infection of 1. At the times indicated after infection, aliquots were withdrawn and added to anti-M13 antiserum on ice; 0.1-ml volumes of appropriate dilutions were then plated on a lawn of K91, and the number of plaques was counted. Each panel or inset presents the results of one of several independent experiments.

may simply reflect the occasional phage bound to the F pilus which was not sequestered by the antibody before and during plating on K91, which is *tolA*$^+$. To test this, infection was repeated with the ampicillin-transducing f1 bacteriophage pD8 and resistance to ampicillin was scored, thus eliminating the need for plaque development on a lawn of K91. The results revealed no detectable infection of the *tolA* mutant strain, in

either the presence or absence of F pili (2.2 Amp^r bacteria in 10^2 for K17DE3/F^+, 6 in 10^7 for K17DE3/F^-, <0.5 in 10^7 for both K17DE3*tolA*/F^+ and K17DE3*tolA*/F^-). Therefore, TolA appears to be an absolute requirement for phage infection in F^+ bacteria, as was previously shown for F^- bacteria (36).

The above data are consistent with TolA being absolutely required for f1 phage infection and with the carboxyl-terminal domain III being essential for this process. However, domain III alone is not sufficient, since K17DE3*tolA*/F^+ containing pSKL21, which produces periplasmic soluble TolA domain III (Fig. 1), is unable to plaque f1 phage (data not shown). Domain II is not required, although the presence of the carboxyl-terminal portion of this domain enhances the rate of infection.

Role of TolA domain III in the infective process. Since TolA domain III, tethered to the membrane, is required for infection, there must be an essential interaction involving TolA domain III during the process of phage infection. If this were the case, the presence of soluble domain III of TolA (TolA-III) in the periplasm of a wild-type bacterium should interfere with the interactions of chromosomally expressed TolA that facilitate phage infection.

To test this, the wild-type strain K17DE3/F^+/pLysS was transformed with pSKL21 or pSKL19. Plasmid pSKL21 encodes TolA-III with a cleavable signal sequence attached to its N-terminal sequence (26), and plasmid pSKL19 encodes TolA-III without the signal sequence (25). Addition of IPTG induces the synthesis of enough T7 polymerase to overcome the inhibitory effect of plasmid-encoded lysozyme, resulting in the production of either the cytoplasmic or periplasmic TolA-III shown in Fig. 1. Bacteria containing each plasmid were incubated for 1.5 h with 0.04 mM IPTG and infected with f1 phage, and the rate of infection was measured. As shown in Fig. 4A, bacteria expressing cytoplasmic TolA-III from pSKL19 were normal for infection, with 8.6% of the bacteria being infected after 10 min. However, the rate of infection of bacteria expressing periplasmic soluble TolA-III from pSKL21 was greatly diminished. Only 0.001% of the bacteria were infected in 10 min.

Expression of periplasmic TolA-III in wild-type bacteria has been shown to result in other *tolA* mutant phenotypes such as leakage of periplasmic enzymes (26). Thus, bacteria expressing periplasmic TolA-III would probably leak TolA-III into the medium, where it could interact with the f1 phage before or during initiation of infection. Infection of the bacteria is initiated by the interaction of the gene III end of the phage particle with the tip of the conjugative pilus. If TolA-III is able to bind directly to the gene III protein of the phage, the presence of TolA-III in the medium would inhibit the rate of infection. To test this, the culture medium was collected from bacteria containing pSKL21 (periplasmic TolA-III) or pSKL19 (cytoplasmic TolA-III) after induction with IPTG, as in Fig. 4A. The medium from the pSKL21-containing bacteria contained approximately 1 to 3 μg of TolA-III per ml as judged by Coomassie staining of gels (data not shown). The wild-type K17DE3/F^+/pLysS containing pEMC18, a control plasmid conferring ampicillin resistance, was suspended in the medium, and the rate of infection was determined (Fig. 4B). The presence of medium obtained from bacteria containing either pSKL21 or pSK19 had no effect on the rate of phage infection of wild-type bacteria. There was also no effect when f1 phage was preincubated for 20 min with 60 μg of purified TolA-III per ml prior to infection (data not shown).

In the converse experiment, bacteria expressing periplasmic TolA-III encoded by pSKL21 were washed and resuspended in fresh medium before addition of f1 phage, so that the majority of the TolA-III would be located in the periplasm at the time

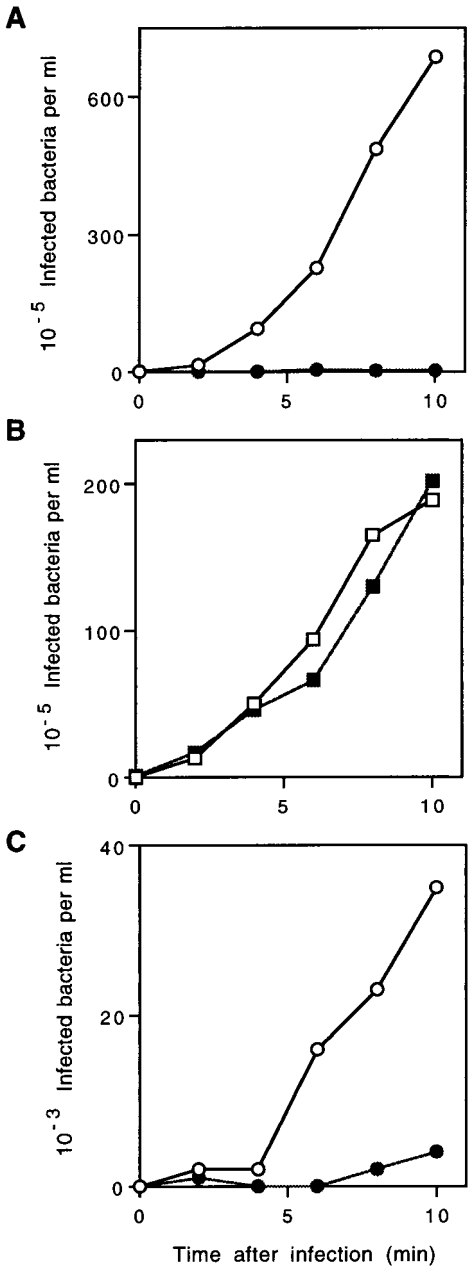

FIG. 4. Expression of periplasmic TolA-III in *tolA*$^+$ bacteria inhibits f1 phage infection. (A) Cultures of K17DE3/F^+/pLysS containing either pSKL19 (open circles) or pSKL21 (solid circles) were incubated for 1.5 h with 0.04 mM IPTG prior to infection with f1 phage. Infected bacteria were quantitated at various times as in Fig. 3. Soluble TolA-III is expressed in the cytoplasm from pSKL19 and in the periplasm from pSKL21. Both strains are approximately 10% infected after a 10-min incubation with phage. (B) K17DE3/F^+/pLysS bacteria containing either pSKL19 or pSKL21 were induced as in panel A, and the culture medium was collected and sterile filtered. Aliquots of K17DE3/F^+/pLysS bacteria (containing pEMC18 which expresses no protein) were suspended in the pSKL19- or pSKL21-derived culture medium and then infected with f1 phage as in panel A. Results for wild-type bacteria in pSKL19-derived medium (open squares) and wild-type bacteria in pSKL21-derived medium containing TolA-III (solid squares) are shown. (C) K17DE3/F^+/pLysS bacteria containing either pSKL19 (open circles) or pSKL21 (closed circles) were induced and infected as in panel A, except that before infection, the bacteria were washed and suspended in fresh medium.

of infection. Bacteria expressing cytoplasmic TolA-III encoded by pSKL19 were treated in the same manner and served as a control. As shown in Fig. 4C, f1 phage infection is dramatically reduced in the strain expressing periplasmic TolA-III relative to the level of infection observed in the control strain. These data show that the membrane-anchored domain III of TolA acts within the bacterial cell envelope to facilitate f1 phage infection and that expression of soluble domain III in the periplasm, but not in the cytoplasm or in the medium, interferes with the infection process.

DISCUSSION

Infection of *E. coli* by the Ff filamentous bacteriophage is minimally a two-step process (30, 44). The first involves the interaction of the phage and the F conjugative pilus. This interaction is initiated by the specific binding of the phage, via the phage gene III protein (pIII), to the tip of the F conjugative pilus. Retraction of the pilus is thought to bring the tip of the phage to the membrane surface (11). During the second step, the major capsid proteins integrate into the cytoplasmic membrane while the DNA genome is translocated into the cytoplasm. Whether the minor capsid proteins, including pIII, also integrate into the cytoplasmic membrane is unclear (27). This second process requires both pIII and the products of the bacterial *tolQRA* genes.

The presence of the three Tol proteins appears to be absolutely required for infection. F^- bacteria, missing the F pilus, can be infected with f1 bacteriophage at frequencies of approximately 10^{-6}, but only if TolQ, TolR, and TolA are present (36). Conversely, F^+ bacteria lacking TolA are unable to be infected with f1 phage, even at this low frequency (see above). Therefore, the pilus appears to function as a mechanism to bring the phage particle close to the Tol proteins which are anchored in the cytoplasmic membrane. The central portion of pIII is responsible for the pilus-binding step and can be deleted, allowing low-frequency infection (38). However, no infection can be detected when the phage gene III protein lacks the amino-terminal translocation domain (35, 36). Thus the N-terminal portion of pIII is predicted to interact with the Tol proteins during the translocation step.

Since the pilus is thought to be elaborated from the cytoplasmic membrane by polymerization of pilin subunits and protrudes through the outer membrane (11, 12, 30), retraction of the pilus could potentially bring the pIII end of the phage particle through the outer membrane and into proximity to the periplasmic face of the cytoplasmic membrane. This position would allow pIII to interact with TolA and TolR, the two membrane-bound Tol proteins which have large domains exposed in the periplasm. Since TolA has the structural potential to extend the farthest from the cytoplasmic membrane, the observation that the complete removal of domain II of TolA still allows infection to occur is consistent with the hypothesis that pilus retraction allows pIII to interact with the Tol proteins near the surface of the cytoplasmic membrane.

We propose that once the retracting pilus has brought the pIII end of the phage particle close to the bacterial membrane, pIII interacts with TolA. The observation that increasing the amount of wild-type TolA diminishes the rate of infection is consistent with this hypothesis. Interactions between the transmembrane regions of TolA, TolQ, and TolR have been shown to be required for the function of the Tol complex (8, 23). Expression of TolA from plasmid pSKL10 results in a fourfold increase over the normal amount of TolA (Fig. 2, compare lanes 1 and 2) and a twofold decrease in the rate of infection (Fig. 3A). Under these conditions, some TolA may interact with the phage but may not be able to find free TolQ and TolR to form a productive complex for infection. Therefore, uncomplexed and consequently nonfunctional TolA may compete with complexed, functional TolA for interactions with the phage.

The following observations suggest that the carboxyl-terminal domain III of TolA (TolA-III) is that portion of TolA which interacts with the phage (and, by implication, with the N terminus of pIII). First, membrane-associated TolA-III is absolutely required for infection. Removal of TolA-III results in complete loss of infection (TolAΔIII in Table 1 and Fig. 3). Also, the presence of soluble TolA-III in the periplasm dramatically lowers the rate of infection, suggesting the occurrence of a competing and nonfunctional interaction between soluble TolA-III and phage pIII (Fig. 4C).

Second, the presence of domain II of TolA is not required, although it contributes to the efficiency of TolA function in infection (Fig. 3B). Presumably, the phage have evolved to use domain III of TolA positioned at the end of the long helical domain II. The deletion studies presented in this paper show that the full length of domain II is not needed for infection, since there is little effect on the efficiency of infection when the amino-terminal half of the domain is removed (TolAΔII$_N$ in Table 1 and Fig. 3B). However, the absolute length of the domain may be less important than the secondary structure it adopts. Deletion II$_C$ has a much greater effect on the rate of infection than does deletion II$_N$, even though the sizes of the deleted segments are approximately the same (Fig. 1). The major difference between the amino- and carboxyl-terminal portions of domain II is the number of $i \ldots i + 4$ arrangements of positively charged lysine and negatively charged glutamic and aspartic acid residues (K-A-A-A-D or E). Domain II$_C$ contains all but 2 of the 13 K-A_3-D/E repeats found in the complete domain II (25). These repeats have been shown to allow the formation of salt bridges, which would provide stability to α-helical regions of proteins (28). Therefore, a tether of relatively stable α-helical structure may contribute to the proper presentation of TolA-III to the incoming phage particle.

Plasmid expression of the amino-terminal fragment of phage pIII (residues 1 to 98) in the periplasm causes a wild-type cell to become leaky and detergent sensitive and to become more resistant (tolerant) to colicins and phage infection (4). Since these are the same phenotypes observed in *tol* mutants, it suggests that the periplasmic pIII amino-terminal fragment is in a conformation competent for interaction with the Tol system. The same phenotypes are observed when soluble TolA-III is expressed in the periplasm (26; also see above). Together, these data suggest that in the periplasm, the N-terminal region of pIII interacts with TolA-III, disrupting the normal interaction of TolA-III with its cellular substrate and also preventing TolA-III from interacting with incoming phage or colicins. It is possible, however, that these phenotypes are the result of interactions of the pIII fragment with other periplasmic components such as TolR.

Regarding the role of pIII in the infection process, analysis of deletion mutants with various mutations of phage gene III suggests that different regions of pIII are involved in recognition of the pilus tip and in the Tol-dependent translocation of the DNA into the bacteria (35, 38). The pilus recognition site appears to be present in residues 99 to 196, whereas amino acids 53 to 107 are required for interaction with the TolQRA proteins. Soluble TolA-III is able to interfere with phage infection only when it is present in the periplasm (Fig. 4). The presence of soluble TolA-III in the medium has no effect on the rate of infection. Taken together, these data would suggest

that intact pIII, when present in the phage particle, is not a good substrate for binding to TolA-III. Perhaps binding of the phage to the tip of the pilus is required to alter the structure of capsid-bound pIII, making the amino-terminal portion of pIII available for interaction with TolA-III within the cell envelope. The observation that expression of the amino-terminal fragment of pIII in the periplasm results in *tol* mutant phenotypes is consistent with this interpretation (4). The low level of infection observed in F^- strains (36) may reflect the small proportion of pIII in phage that, in the absence of pilus interaction, is in the proper conformation to interact with TolA domain III. However, the possibility that the phage pIII-pilus tip complex will have to interact with another periplasmic component along with TolA domain III for infection to occur cannot be ruled out.

The group A colicins also require the Tol proteins to facilitate import to their sites of action in the bacteria. It is proposed that the colicins first bind to a specific outer membrane receptor, translocate through the outer membrane, and then insert into the membrane to form a pore or send a nuclease activity into the cytoplasm (for reviews, see references 19 and 21). All of the group A colicins, except colicin E1, require the presence of the periplasmic TolB protein in addition to TolQ, TolR, and TolA. In this respect, colicin E1 is like f1 phage, which also requires only TolQ, TolR, and TolA for translocation to its target. Additionally, colicin E1 is similar to f1 phage in that it does not need domain II of TolA to exert its action, whereas colicins A, E3 and N require at least half of domain II to kill the bacteria (37).

These observations suggest that both colicin E1 and f1 phage, following translocation across the outer membrane, can interact directly with the cytoplasmic membrane. This is in contrast to the other colicins, for which arrival at the cytoplasmic membrane appears to be a two-stage process involving translocation across the outer membrane and then the periplasm, with the latter step presumably requiring TolB. It may be the retraction of the pilus which allows the phage to directly interact with the cytoplasmic membrane. In the case of colicin E1, it may be its unique requirement for TolC (31, 34). TolC is a minor outer membrane protein, which is involved in the Sec-independent export of alpha-hemolysin and colicin V (41, 44). TolC is proposed to help form a bridge across both membranes for the export of these molecules. In turn, it may function to form a direct bridge to the periplasmic face of the cytoplasmic membrane for colicin E1. It is probably the use of pilus retraction by phage and the use of TolC by colicin E1 that relieves the requirement for the longer TolA molecule to bridge the periplasm in the translocation of these entities to their respective destinations.

ACKNOWLEDGMENTS

We thank S. Schendel for providing plasmids.

This work was supported by Public Health Service grant GM 18306 from the National Institute of General Medical Sciences.

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