***Escherichia coli tolQ* Mutants Are Resistant to Filamentous Bacteriophages That Adsorb to the Tips, not the Shafts, of Conjugative Pili**

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The *tolQ* (previously *fii*) mutation in *Escherichia coli* K12 inhibits infection by filamentous bacteriophages f1 and IKe but not by RNA-containing phage f2. This work extends these observations to other plasmid-specific bacteriophages including various filamentous, RNA-containing, and lipid-containing isolates. Only tip-adsorbing filamentous phages were affected by *tolQ* and not shaft-adsorbing ones. Electron microscopy showed that RP4-specific filamentous phage Pf3 was one of the latter kind. Several tip-adsorbing filamentous phages inhibited conjugation between *tolQ* strains carrying their specific plasmids, implicating the phage receptors (conjugative pili) as mating organelles. *tolQ* mutant strains were as proficient as their parents in conjugation mediated by a wide range of plasmids.

INTRODUCTION

Plasmid-specific filamentous and RNA-containing bacteriophages of *Escherichia coli* adsorb to conjugative pili (Bradley, 1967; Marvin & Hohn, 1969). Most filamentous phages attach to the pilus tips, but a few isolates, together with all RNA phages, adsorb to the pilus shafts (sides). Sun & Webster (1986) described a mutation, *fii*, which caused the inhibition of infection of F⁺ bacteria by filamentous bacteriophage f1, but not by RNA-containing phage f2. Similarly, the mutation inhibited the infection of bacteria harbouring plasmids of incompatibility group N (IncN) by filamentous phage IKe. The *fii* mutation also provided tolerance against E colicins, which are classified as low-*M_r* bacteriocins, as opposed to high-*M_r* bacteriocins of bacteriophage origin (Bradley, 1967). Sun & Webster (1987) sequenced the *fii* region, and renamed the mutation for filamentous phage inhibition *tolQ*. The entire gene cluster, *tolQRAB*, encodes proteins which form a transport system across the bacterial envelope for large molecules. The main purpose of this work has been to extend the observations of Sun & Webster (1986) to the additional plasmid-specific bacteriophages in our collection. Furthermore, the hypothesis that plasmid DNA transfer in conjugation (see Willetts & Wilkins, 1984) might be affected by *tolQ* has been tested.

The inhibition of F plasmid transfer by the presence in mating mixtures of inactivated pilus-specific filamentous bacteriophages demonstrated unequivocally that F pili were involved in conjugation (Ippen & Valentine, 1967; Novotny *et al.*, 1968). Similar experiments have been done here with filamentous phages specific for plasmids other than F. The presence of the *tolQ* mutation rather than phage inactivation was used to prevent infection of donor strains.

METHODS

Strains, bacteriophages and plasmids. The parent strain used by Sun & Webster (1986) was *E. coli* K12 P90C [*araΔ* (*lac pro*) *thi*]. Their strain GM1 was P90C/F'*lac pro*. The *tolQ* (= *fii-1*) derivative of GM1 was TPS13. For this work we cured GM1 and TPS13 of the F'*lac pro* plasmid by overnight growth in broth containing novobiocin (150 µg ml⁻¹); Lac⁻ colonies were then sought on MacConkey agar; plasmid loss was checked by agarose gel electrophoresis. Cured GM1 (= P90C) was designated P90C; cured TPS13 (= P90C*tolQ*) was designated TPS13.

Nalidixic acid- and rifampicin-resistant derivatives of both strains (Nal^R, Rif^R; suffix -1 and -2 respectively) were isolated. In addition to phage fd, tolerance to colicin E1 was used to check the *tolQ* phenotype. A loopful of *E. coli* K12 strain E8705(ColE1::Tn7) was suspended in 2 ml broth and about 10 µl of this suspension was spotted on the *tolQ* strain (see spot test for phages below); after incubation, lack of clearing around the bacterial overgrowth indicated tolerance. Strains JE2571 (*lac leu thr str fla pil*) and J53 (Lac⁺ *pro met*) or their Nal^R or Rif^R derivatives were used as plasmid hosts.

Bacteriophages and references are listed in Table 1. Most known filamentous phages were used with the exception of SF (Coetzee *et al.*, 1986), which was an fd-like phage with an extended host range, but is no longer available. The two filamentous phages If1 (Meynell & Lawn, 1968) and PR64 (abbreviated from PR64FS; Coetzee *et al.*, 1980) adsorb to the thin flexible pili as opposed to the thick rigid pili of I complex plasmids (Bradley, 1984). Since they are serologically related (Coetzee *et al.*, 1982) we used only PR64. We could not test the only other filamentous phage known to adsorb to the pilus shaft, C-2 (Bradley *et al.*, 1982a), since it only infected *Salmonella typhimurium* strains carrying transfer-derepressed IncC plasmids, not similar *E. coli* K12 strains.

Plasmids were selected on the basis of availability and for good responses to plasmid-specific phages with host strain P90C; most are cited by Jacob *et al.* (1977) with the exceptions of EDP208 (transfer-derepressed *F_olac* mutant constructed by N. Willetts; see Worobec *et al.*, 1983), which is IncSI (Coetzee *et al.*, 1986), pMG110 (IncHII; Wolfson *et al.*, 1982), pIN25 (isolated by S. B. Levy; see Bradley & Whelan, 1985), pAr-32 (Bradley *et al.*, 1982b) and R687 (IncD; see Datta, 1979). Strain TPS-2(R687) gave poor clearing in spot tests with phage fd, so the plasmid could not be used in some experiments.

Media, bacteriophage techniques and mating methods. Brain Heart Infusion broth or agar (BBL) was used for most cultures. The minimal salts medium used was M9, and drug concentrations were as listed by Bradley (1984).

Methods for phage growth, and the spot test for phage activity, were as described or cited in Bradley (1967). Filamentous phages were grown using strain JE2571 harbouring the appropriate plasmid. Brain Heart Infusion broth was inoculated with bacteria and phage, then grown statically overnight at 37 °C (30 °C for phages tf-1, t, and pilHα). After removing bacteria by several cycles of centrifugation (filtration removes filamentous phage virions and is unsuitable), the suspension was titrated by spotting 10 µl samples of serial dilutions on a soft agar layer plate of the host strain, and counting plaques after incubation. Mating methods were as described previously (Bradley *et al.*, 1980; Bradley, 1984).

Electron microscopy. Techniques were those previously employed (Bradley *et al.*, 1981a, b, c).

RESULTS

Effect of tolQ mutation on infection by plasmid-specific bacteriophages

As many plasmid-specific bacteriophages as possible, including filamentous, RNA-containing, and lipid-containing types were tested for inhibition of infection by the *tolQ* mutation. Results obtained with spot tests for lytic activity, together with the locations of the phage adsorption sites on their conjugative pilus receptors, are given in Table 1. In agreement with the results of Sun & Webster (1986), it was found that filamentous phage fd infected the TolQ⁺ strain P90C-2(F::Tn5) but not its *tolQ* derivative TPS-2(F::Tn5), and that RNA-containing phage of Qβ infected both strains. Similar results were obtained with R1drd19 (IncFII) and EDP208 (IncS); the RNA phage *F_olac* (Bradley *et al.*, 1981c) replaced Qβ for the latter. The *tolQ* mutation in strain TPS-2(N3) also inhibited infection by the IncN-specific filamentous phage Ike (in agreement with Sun & Webster, 1986). Filamentous phages PR64 (specific for I complex plasmids) and X-2 (IncX-specific) were also inhibited. Three other filamentous phages, Pf3, tf-1, and X, together with all non-filamentous phages, were not.

Effect of tolQ mutation on plasmid transfer

A series of plasmids selected by incompatibility grouping and pilus type was tested for inhibition of transfer from JE2571 or J53 to *tolQ* strains TPS-1 or -2. The following transfer-derepressed plasmids with surface-obligatory conjugation systems (exceptions R1drd19 and pIN25) were tested by cross-streak mating (Bradley *et al.*, 1980): R1drd19 (IncFII), N3 (IncN), RP4 (IncP), pIN25 (IncT), pAr-32 (IncU), R905 (IncV), Sa (IncW). Quantitative broth matings were used for R453 (IncFI), R478 (IncHI2), MIP233 (HI3), R144 (IncI₁ + B), R391 (IncJ), *F_olac* (IncSI) and quantitative plate matings for RIP64 (IncC), R687 (IncD), pMG110 (IncHII), RIP69 (IncM) and TP228 (IncX). A significantly lower transfer frequency with recipients TPS-1 or -2 compared with recipients P90C-1 or -2 was not detected with any plasmid. The possibility

Table 1. Inhibition of plasmid-specific bacteriophage infection by *tolQ* mutation

Phage	Type	Adsorption site on pili	Plasmid	Inc	Phage sensitivity of plasmid host strain*		Reference for phage
					P90C-2	TPS-2	
fd	Filamentous	Tip	F::Tn5	FI	+	—	Marvin & Hohn (1969)
Q β	RNA	Shaft	F::Tn5	FI	+	+	Watanabe (1964)
fd	Filamentous	Tip	R163drd19	FII	+	—	Marvin & Hohn (1969)
Q β	RNA	Shaft	R163drd19	FII	+	+	Watanabe (1964)
fd	Filamentous	Tip	EDP208	S	+	—	Marvin & Hohn (1969)
F _o lac	RNA	Shaft†	EDP208	S	+	(+)	Bradley <i>et al.</i> (1981c)
PR64	Filamentous	Tip‡	R163drd7	I ₁ + B	+	—	Coetzee <i>et al.</i> (1980)
I α	RNA	Shaft‡	R163drd7	I ₁ + B	+	+	Coetzee <i>et al.</i> (1982)
IKe	Filamentous	Tip	N3	N	+	—	Khatoon <i>et al.</i> (1972)
PR4	Lipid	Tip	N3	N	+	+	Stanisich (1974)
Pf3	Filamentous	Shaft§	RP4	P	+	+	Stanisich (1974)
PR4	Lipid	Tip	RP4	P	+	+	Stanisich (1974)
tf-1	Filamentous	Shaft†	pIN25	T	+	+	Coetzee <i>et al.</i> (1987)
t	RNA	Shaft†	pIN25	T	+	(+)	Bradley <i>et al.</i> (1981b)
PR4	Lipid	Tip	Sa	W	+	+	Stanisich (1974)
X	Filamentous	Tip	R6K	X	(+)	(+)	Bradley <i>et al.</i> (1981a)
X-2	Filamentous	Unknown§	R6K	X	+	—	Coetzee <i>et al.</i> (1988)
pilH α	RNA	Shaft	pMG110	HII	(+)	(+)	Coetzee <i>et al.</i> (1985)

* Using the spot test for sensitivity to bacteriophages (see Methods): +, complete or slightly hazy clearing; (+), hazy but definite clearing; —, no visible clearing.

† Adsorption limited to the tapered region of the pilus shaft near the pointed tip; virions do not attach to the point itself.

‡ Incl₁ + B plasmids encode thin flexible and thick rigid pili (Bradley, 1984). Phages PR64 (= PR64FS) and I α adsorb to the thin pili, which stabilize mating aggregates, thick pili mediating DNA transfer.

§ The adsorption site of Pf3 on the P pilus was not known so was determined for this study as being on the shaft (sides) of the pilus (see Results). Attempts to visualize phage X-2 virions attached to X pili were unsuccessful (Coetzee *et al.*, 1988).

that *tolQ* prevented plasmid entry was therefore eliminated. In view of these results, and because of the nature of the conjugation mechanism (Willets & Wilkins, 1984), it seemed highly unlikely that plasmid exit from *tolQ* donor strains would be prevented by the mutation. Sun & Webster (1986) found that TPS13 functioned normally as a donor for plasmid F, and their equivalent of TPS-2(N3) for N3. While a complete series of transfer tests from TPS strains was not carried out, a few examples are given in Table 2, where matings were done for another purpose.

Inhibition of plasmid transfer between tolQ strains by filamentous phages

The effect of various filamentous phages on plasmid transfer frequencies (see Introduction) was assessed by determining the percentage reduction obtained (inhibition) using the plasmid-specific phage with reference to a non-specific filamentous phage control (Table 2). Repressed plasmids (determining few pili) were employed to avoid having too many receptors which might have used up all the phage virions, exceptions being F::Tn5 (only determined about one pilus per cell) and N3 (pili detach in a liquid; Bradley *et al.*, 1980). In all cases with over 90% inhibition, the pilus receptors were clearly implicated as mating organelles. The 45% inhibition obtained with phage IKe and thick I₂ pili was considered inconclusive and probably reflected a poor adsorption efficiency. Frequencies of $<1.0 \times 10^{-4}$ transconjugants per donor h⁻¹ were approximate, an example being the negative inhibition obtained with phage X-2 and IncX plasmid TP231. These frequencies were a mean of three matings (two with Tc, one with Cm selection) and indicate no effect on plasmid transfer by phage X-2. The value of -19% reflects the inaccuracy of mating techniques at low frequencies since it is clear that X-2 could not enhance mating.

Table 2. *Plasmid transfer from TPS-2 in the presence of tip-adsorbing filamentous phages*

All matings were done in liquid nutrient using strain TPS-1 as recipient and nalidixic acid counterselection against TPS-2 donor strains.

Phages in mating mixture (titre p.f.u. ml ⁻¹)		Pilus type*	Donor plasmid	Selection†	Transfer frequency‡ with		Inhibition§ (%)
Test phage	Control phage				Test phage	Control phage	
fd (10 ¹²)	IKe (10 ⁹)	F	F::Tn5	Km	1.0 × 10 ⁻³	6.1 × 10 ⁻²	98
fd (10 ¹²)	IKe (10 ⁹)	D	R687	Km	2.2 × 10 ⁻⁶	2.6 × 10 ⁻⁵	92
fd (10 ¹²)	IKe (10 ⁹)	S	F ₀ lac	Lac	4.9 × 10 ⁻⁸	1.4 × 10 ⁻⁶	97
PR64 (10 ⁸)	fd (10 ¹²)	I ₁ f	R144	Km	< 6.9 × 10 ⁻⁸	1.3 × 10 ⁻⁵	> 100
PR64 (10 ⁸)	fd (10 ¹²)	I ₂ f	R721	Tp	< 4.3 × 10 ⁻⁸	8.3 × 10 ⁻⁵	> 100
IKe (10 ⁹)	fd (10 ¹²)	I ₂ r	R721	Tp	6.1 × 10 ⁻⁵	1.1 × 10 ⁻⁴	45
IKe (10 ⁹)	fd (10 ¹²)	N	N3¶	Tc	1.1 × 10 ⁻⁵	3.6 × 10 ⁻⁴	97
X-2 (10 ¹⁰)	fd (10 ¹²)	X	TP231	Tc, Cm	3.1 × 10 ⁻⁵	2.6 × 10 ⁻⁵	-19

* Correspond to incompatibility groups F, D, S, N, X and I complex. The latter plasmids determine both thin flexible (f) and rigid (r) pili (Bradley, 1984).

† Plasmid selection: Cm, chloramphenicol; Km, kanamycin; Lac, lactose (M9 medium); Tc, tetracycline; Tp, trimethoprim. For concentrations see Bradley (1984).

‡ Transconjugants per donor h⁻¹. The test phage was specific for the pilus type indicated, whereas the control phage was not. Both were made similarly from strain JE2571 carrying the appropriate plasmids. Where no transfer could be detected, a '<' frequency was calculated assuming one colony on plates at the lowest dilution used (10⁻¹).

§ Frequency with the test phage expressed as a percentage of that with the control phage and subtracted from 100%. For an explanation of the single negative inhibition found, see text.

|| TPS-2(R687) was resistant to phage fd although R687 normally confers sensitivity to K12 strains (D. E. Bradley, unpublished). Transfer frequencies were approximate due to the instability of IncD plasmids in *E. coli* K12.

¶ N3 is a derepressed surface-mating plasmid (Bradley *et al.*, 1980) not inhibited for transfer by nalidixic acid. Surface mating occurred here on transconjugant-selecting plates at the low dilutions required to detect transfer frequencies in a liquid environment. Transconjugant colonies from a liquid mating are much larger than those from subsequent plate mating; the latter originate from bacteria having a shorter time to express drug resistance, and then to grow into colonies (D. E. Bradley, unpublished). Frequencies were obtained by counting the distinctive larger colonies.

Adsorption site of filamentous phage Pf3

The only filamentous phage whose adsorption site on conjugative pili had not been previously studied was Pf3 (specific for IncP plasmids; Stanisich, 1974). Its infection was not inhibited by the mutation in *tolQ*. Electron microscopy showed that it attached along the pilus shaft (Fig. 1) and was not specific for the tip or any area close to it, as was the case for tf-1 (Coetzee *et al.*, 1987). This explained why the *tolQ* mutation did not affect it.

DISCUSSION

It is clear that the *tolQ* mutation only inhibits infection by tip-adsorbing, not shaft-adsorbing filamentous phages, an exception being phage X. This virus appears to adsorb precisely at the pilus tip (Bradley *et al.*, 1981*a*) and yet is not inhibited; only single virions are attached at this locus, multiple adsorption being expected if attachment is proximal from the tip. Phage X has a very wide plasmid specificity range, but it is not known whether this is connected with its insensitivity to the *tolQ* effect. Filamentous phage X-2 (IncX-specific), whose receptor was not identifiable due to poor adsorption (Coetzee *et al.*, 1988), was also inhibited by *tolQ*; because of this, it seems very likely that it attaches to the tips of X pili. The inhibition of phage PR64 infection by *tolQ* is noteworthy. PR64 adsorbs to the tips of thin I₁ or thin I₂ pili, which act as 'grappling hooks' in conjugation, forming stable mating aggregates in a liquid. Cell-to-cell contact between outer membranes together with plasmid DNA transfer is believed to be mediated by rigid pili, the I₂ type being phage IKe receptors (see Bradley, 1984). Inhibition of

Fig. 1. IncP-specific filamentous phage Pf3 adsorbed to P pili from strain JE2571(RP4) after 1 h. Points of attachment along the pilus shaft can be clearly seen (arrowed). Negatively stained; bar, 100 nm.

phage PR64 infection suggests that these thin flexible pili may retract like thick flexible pili (see below).

The morphological type of pilus receptor, whether thick flexible (F, S, X), thin flexible (I complex), or rigid (N), does not affect filamentous phage inhibition. Possibly, after a filamentous phage has adsorbed precisely to the conjugative pilus tip and the pilus has retracted, the virion is pulled through a molecular assembly in the outer membrane as if it was an additional part of the pilus. After the capsid is removed, probably in the periplasm, the DNA is further processed through the inner membrane by the *tolQRAB* gene products. We suggest that, unless the phage attaches exactly at the pilus tip, it cannot be pulled through the outer membrane, so that a different transport mechanism would be required for the DNA of shaft-adsorbing filamentous phages. Clearly phages with a larger profile like those containing RNA or lipid could not be pulled through the outer membrane either; indeed their modes of infection are quite different (Bradley, 1972, 1978; Silverman & Valentine, 1969). The function of the *tolQRAB* gene products seems to be limited at present to the transport of tip-adsorbing filamentous phage DNA and E colicins across the bacterial envelope. The transport mechanism and natural function of the gene cluster might be easier to elucidate if other affected macromolecules could be identified.

We have used *tolQ* TPS strains as plasmid hosts to demonstrate mating inhibition by filamentous phages that adsorb to conjugative pilus tips. The pilus types that we have implicated as mating organelles in this way are D, S, thin I₁, thin I₂, and N. The F pilus acted as a control since it has been tested in similar experiments (Ippen & Valentine, 1967; Novotny *et al.*, 1968). Other pili have been shown to be involved in conjugation using several different agents for specifically inhibiting mating. They are thin I₁ (anti-pilus serum; Harden & Meynell, 1972), W (inactivated lipid-phage; Bradley, 1978), the HI3 pili of plasmid MIP233 (anti-pilus serum; Bradley, 1986) and N and P pili (concentrated pilus preparations blocking receptors on recipient cells; Bradley & Chaudhuri, 1980). We have thus confirmed the roles of thin I₁ and N pili, and added D, S, and thin I₂ pili.

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