

Isolation and Characterization of Motile *Escherichia coli* Mutants Resistant to Bacteriophage χ

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Four mutants of *Escherichia coli* that are resistant to the flagellotropic phage χ , but are motile, were isolated. When they were observed in liquid culture by light microscopy, one mutant exhibited circular movement and another tumbled at high frequency on the surface of a glass slide. The remaining two mutants moved normally. None of these mutants adsorbed the wild-type strain of χ . P1 transduction revealed that the mutation sites of these four mutants were more than 97% cotransducible with a site in *hag*, the structural gene for flagellin. When flagellins of these mutants were chromatographed on a diethylaminoethyl-cellulose column, two eluted slower and one eluted slightly faster than the flagellin of the parental strain. The other flagellin eluted at the same position as that of the parent. Host range mutants of phage χ , which could infect these bacterial mutants, were isolated.

Flagellotropic phage χ infects only motile cells of various species belonging to the genera *Salmonella* (10, 26), *Escherichia* (11, 24), *Serratia* (11), and *Proteus* (2). When these bacteria are infected with χ , their movement is rapidly interrupted (7, 21). The phage cannot infect paralyzed mutants whose flagella do not rotate (21). The first adsorption site of the phage is the flagellar filament. The phage then presumably moves down to the base of the flagellum and injects its DNA (25). *Salmonella* strains having flagellar antigens of specific serotypes such as *g*, *l*, and *y* are resistant to χ even though they are motile (21). On the other hand, χ attacks mutants which carry rotating, curly or straight flagellar filaments (12, 21). The filaments of these mutants seem to have altered their shapes without losing the ability to adsorb χ .

Phage χ has been used as a selective agent for the isolation of flagellar defective mutants (30). All of the bacterial mutants resistant to phage χ isolated so far have been nonmotile. In this paper, we describe the isolation of *Escherichia coli* mutants whose movement was not disturbed by phage χ . They had altered flagellins. Some of them exhibited abnormal movement as a result of the interaction of the flagellar filaments with the surface of a glass slide. Host range mutants of phage χ which can infect these mutants were isolated.

MATERIALS AND METHODS

Bacterial strains. The derivatives of *E. coli* K-12 used in this work are listed in Table 1. For the construction of flagellar defective mutants, derivatives of

strain MSF1338, carrying flagellar mutation *hag-726*, *mot-797*, *flaE234*, or *flaD876* on plasmid F1339, were mated with strain EJ502, and segregants acquiring flagellar defective alleles were selected.

Bacteriophages. As a parental strain of phage χ , strain XJ1 was used. Strain XJ1, a derivative of strain χ 1 (8), can grow at higher temperature and has host specificity different from that of strain χ 1 (Table 2).

Phage P1_{mc}, a derivative of P1_kc, was used for transduction. It was a gift from M. Abe, the National Institute of Health, Tokyo.

Media. L broth contained (per liter of distilled water): tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 5 g. The medium was adjusted to pH 7.2 with NaOH. L agar plates were prepared by adding 1.5% agar (Shoei) to L broth. Nutrient agar plates contained (per liter): nutrient broth (Difco), 8 g; NaCl, 5 g; agar, 15 g. The medium was adjusted to pH 7.2 with NaOH. Double-strength (2 \times) nutrient agar plates differed from nutrient agar plates in that 16 g of nutrient broth was added per liter. Motility plates (TGA) contained (per liter): tryptone, 10 g; NaCl, 5 g; agar, 2.5 g; gelatin (Nitta), 80 g. Gelatin-agar plates containing χ (χ -TGA) were prepared by adding phage χ suspensions (final concentration, 10⁹ plaque-forming units per ml) to TGA. Soft agar contained (per liter): peptone (Kyokuto), 10 g; NaCl, 5 g; agar, 3.75 g. The medium was adjusted to pH 7.2 with NaOH. Minimal medium contained (per liter): K₂HPO₄, 10.4 g; KH₂PO₄, 4.5 g; MgSO₄, 0.1 g; sodium citrate, 1.0 g; glucose, 4.0 g. Minimal glucose-gelatin-agar plates were prepared by adding 0.25% agar and 8% gelatin to minimal medium. Casamino Acids-glucose medium was prepared by adding 1% Casamino Acids (vitamin free, Difco) to minimal medium. Peptone-glucose medium was prepared by adding 1% polypeptide (Daigo) to minimal medium. SM buffer contained 0.1 M NaCl, 10⁻³ M MgSO₄, 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5), and 0.01% gelatin.

TABLE 1. *Bacterial strains used*

Strain	Pertinent genetic characters ^a	Origin or reference
EJ500	Prototrophic, Cfs ^b	From W3110, motility selection on MGA ^c
EJ501	<i>his</i> Cfs	From EJ500, penicillin counterselection for <i>his</i>
EJ502	<i>his rpsL</i> Cfs	From EJ501, streptomycin resistance
EJ510	<i>thyA</i> Cfs	From EJ500, aminopterin selection (22)
EJ511	<i>thyA deoB</i> or <i>deoC</i> Cfs	From EJ510
MSF1338	<i>Phis</i> ⁺ <i>uvr</i> ⁺ <i>zwf</i> ⁺ /JC1553	Silverman and Simon (27)
EJ521	<i>his rpsL hag-726</i>	MSF1338 <i>hag-726</i> → EJ502 for <i>his</i> ⁺
EJ522	<i>rpsL mot-797</i>	MSF1338 <i>mot-797</i> → EJ502 for <i>his</i> ⁺
EJ523	<i>rpsL flaE234</i>	MSF1338 <i>flaE234</i> → EJ502 for <i>his</i> ⁺
EJ234	<i>rpsL flaD867</i>	MSF1338 <i>flaD867</i> → EJ502 for <i>his</i> ⁺
EJ711	Polyauxotrophic, flagellar antigenic mutation	From EJ710 (15), Komeda

^a Gene symbols are those of Bachmann and co-workers (5).^b Constitutive flagellar synthesis (29, 38).^c MGA, Minimal glucose-gelatin-agar.TABLE 2. *Comparison of growth of χ 1 and XJ1^a*

Phage	Size of plaque	Growth at:			EOP ^b on:	
		36°C	38°C	40°C	SJ929	SJ25
χ 1	Small	+	—	—	<10 ⁻⁶	1
XJ1	Large	+	+	—	10 ⁻⁴	10 ⁻⁵

^a The growth of the wild type of phage χ , χ 1, and its derivative, XJ1, on *Salmonella abortus-equi* SJ241 (35), was compared at various temperatures. The efficiencies of plating these phages on flagellar antigen strains SJ929 (*fg*) (36) and SJ25 (1.2) were calculated, taking the value for strain SJ241 (*a*) as 1.

^b EOP, Efficiency of plating.

Preparation of lysates of phage χ . Lysates of phage χ were prepared by the confluent lysis method (1). As overlays and bases, soft agar and 2× nutrient agar were used, respectively.

For large-scale preparation of lysates, cells of strain EJ501, exponentially growing on peptone-glucose medium, were infected with phage χ at a multiplicity of infection of 1. The culture was gently shaken for 24 to 30 h at 33°C. Cells and cell debris of the partially lysed culture were removed by low-speed centrifugation. Phages were precipitated by adding polyethylene glycol to the supernatant (37), collected by low-speed centrifugation, and purified with a CsCl step gradient (20).

Titration of phage χ . The surface method and the agar layer method described by Meynell (21) were used for phage titration. The agar layer method was also used for plaque isolation.

In the surface method, 0.01 ml of appropriately diluted phage suspension was spotted on 2× nutrient agar previously spread with indicator bacteria. In the agar layer method, 0.1 ml of phage suspension and 0.1 ml of an indicator culture were mixed with 2.5 ml of melted soft agar on nutrient agar. These plates were incubated overnight at 33°C, and the number of plaques was counted.

Isolation of motile, χ -resistant mutants. Two methods were used for the isolation of the mutants. In the first, bacteria in peptone-glucose medium (1 ml, 2 × 10⁸ cells per ml) were infected with χ at a multiplicity

of infection of 1 and incubated at 33°C overnight. A drop of culture suspension was spotted with a capillary tube on χ -TGA. After overnight incubation of the plate at 33°C, mutants that migrated as swarms on χ -TGA were picked up. Single-colony isolation of the mutants was repeated on L agar to free them from the phages.

In the second method, bacterial cultures mutagenized with UV light were incubated in L broth for 2 h at 36°C. A 0.1-ml volume of culture and 2.5 ml of melted soft agar containing 10⁹ plaque-forming units of χ per ml were mixed and plated on nutrient agar. The plate was incubated overnight at 36°C. Motile clones that swarmed in the soft agar were picked up and repeatedly purified on L agar.

Chromatographic separation of flagellins. Bacteria, growing exponentially in 5 ml of minimal medium, were labeled with a ¹⁴C-amino acid mixture (1 μ Ci/ml of culture; New England Nuclear Corp.) for 30 min, collected by centrifugation, and suspended in 5 ml of 0.15 M NaCl–10 mM phosphate buffer (16). Purified cold flagella were added to the suspension as carrier. After depolymerization of flagella with acid at pH 3.0, cell debris was removed by centrifugation. The supernatant fraction was neutralized with 1 M K₂HPO₄. Purification and chromatography of flagellin were carried out as previously described (15, 31).

Radioactive assay for adsorption of phage χ . The adsorption of χ to bacterial flagella was assayed by a modification of the procedure of Thipaya-thasana and Valentine (34).

For the preparation of radioactive phages, 20 ml of a culture of strain EJ511 growing exponentially on Casamino Acids-glucose medium supplemented with 5 μ g of thymine per ml was infected with phages at a multiplicity of infection of 1. At the same time, 0.5 ml of tritiated thymine (1.0 mCi/ml; specific activity, 13.5 Ci/mmol; Daiichi Pure Chem. Co., Ltd.) was added to the culture. The phage-infected culture was incubated for 6 h at 36°C. The labeled phages were purified, as described above, using 10¹² plaque-forming units of cold phage as carrier. About 10⁶ cpm/1 ml of the purified phage suspension was obtained.

For the adsorption assay, cells were grown in L broth at 33°C to 8 × 10⁸/ml. A cell sample (1.5 ml) was mixed with 5 μ l of radioactive phages (ca. 5 × 10³

cpm), and the mixture was incubated for 10 min at 33°C. The whole volume was taken up with a syringe without a needle and filtered through a glass-filter pad (Whatman, GFF-24) fitted on a Millipore holder. The glass filter was washed with 5 ml of water, followed with 10% trichloroacetic acid and several drops of ethanol-ethyl ether (1:1, vol/vol). After the filter was dried, the radioactivity was determined in 5 ml of toluene scintillator (0.5% of 2,5-diphenyloxazole) with a Beckman liquid scintillation spectrometer.

RESULTS

Movement of the mutants in liquid cultures. The movement of bacteria in liquid cultures upon χ infection was observed by either phase-contrast or dark-field microscopy. When χ -sensitive strain EJ500 was infected with phage χ at a multiplicity of infection of about 10, almost all of the bacteria were immobilized, just as described by Meynell (21). However, a small fraction of the cells, tethered by their filaments to the surface of the glass slide, continued their rotational movements even after the addition of the phage lysate. When a drop of concentrated χ suspension (10^{13} plaque-forming units per ml) was added to a drop of the bacterial culture, individual flagellar shape, which is otherwise visible only by dark-field microscopy with high-intensity illumination (19), became visible even with a phase-contrast microscope with normal illumination (Fig. 1).

From strain EJ500 we isolated 23 mutants whose movement was not disturbed by phage χ . These mutants continued to move for more than 30 min after the addition of the concentrated suspension of χ , and their flagellar filaments were not visible by phase-contrast microscopy.

Among these mutants, four strains (EJ512, EJ513, EJ514, and EJ515) were chosen for further experiments. When a drop of an exponentially growing culture of each strain was put on a glass slide, covered with a cover slip, and observed with a microscope, the cells of EJ513 and EJ514 moved like their parent. However, cells of EJ512 tended to move circularly, and those of EJ514 tumbled at high frequency compared with their parent.

When the lower surface of the slide chamber was observed, all of the circularly swimming cells of EJ512 moved in a clockwise direction. On the contrary, when the upper surface was observed, the cells moved in a counterclockwise direction. The cells of EJ515 tended to adhere to the surface of glass slides. This phenomenon suggests the interaction of the altered flagellin with the glass surface (19, 33). The moving cells of EJ515 continually changed their direction of

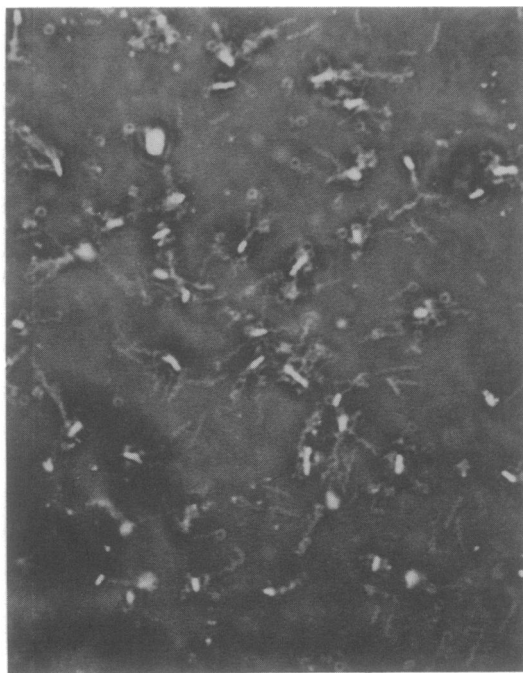


FIG. 1. Flagella of *E. coli* that have adsorbed phage χ . A drop of an exponentially growing culture of strain EJ500 was mixed with a drop of phage χ suspension (10^{13} plaque-forming units per ml) on a glass slide. The sample was observed by negative phase-contrast microscopy (Nikon, Apo.B.M. $\times 40$ objective and H.K.W. $\times 15$ eyepiece). The photograph was taken on Kodak Tri-X film. Flagella manifest polymorphic shapes such as normal, curly, or coiled (13, 14). The shape of filaments of strain EJ500, as shown by electron microscopy, is known to be susceptible to a change of pH or ionic strength (Icho, unpublished data). However, we cannot exclude the possibility that the attachment of χ tail fiber changes the local environment of the filaments, which, in turn, may affect filament shape.

swimming. The fraction of the cells tethered by their flagellar filament to the slide and thus rotating was remarkably larger than that of the parental cells. When the upper surface of the bacterial suspension not sealed by a cover slip was observed, cells of both EJ512 and EJ515 moved normally. With electron microscopy, no difference in the flagellar shape could be detected between these mutants and their parental strain. They had 5 to 10 normally shaped flagella per bacterium, like their parent.

Transduction of χ resistance. To identify the genetic locus responsible for χ resistance of the isolated mutants, P1 transduction was carried out (Table 3).

Lysates of P1mc propagated on the four motile χ -resistant strains were infected into five non-

TABLE 3. Cotransduction frequency of motility and χ resistance genes^a

Donor	Cotransduction frequency			
	EJ522 ^b (<i>mot</i>)	EJ524 (<i>flaD</i>)	EJ521 (<i>hag</i>)	EJ523 (<i>flaE</i>)
EJ512	45	92	98	57
EJ513	45	88	97	67
EJ514	46	94	100	74
EJ515	36	94	100	53

^a P1 transduction was carried out as described by Miller (20). Four motile, χ -resistant mutants were used as donors, and four representative nonmotile strains were used as recipients. All recipient strains used were point mutants and reverted at low frequency. Motile transductants grown as swarms on TGA were purified on L agar and tested for χ resistance. The frequency of χ -resistant clones among motile transductants was taken as the cotransduction frequency. More than 50 transductant swarms were tested in each experiment.

^b Recipient.

motile strains. Motile transductants which appeared as swimmers on TGA were picked up and spotted on both TGA and χ -TGA to test for χ resistance. Mutant sites of χ resistance in these strains were more than 97% cotransducible with the allelic site *hag*-726. It was ascertained microscopically that all transductants which acquired the χ -resistant character from EJ512 or EJ515 simultaneously acquired the abnormal motion character of the corresponding donor.

Chromatographic separation of normal and mutant flagellins. The results of transduction suggested that mutation sites of four motile, χ -resistant mutants were within the structural gene of flagellin, *hag*. Therefore, flagellins of these strains were compared chromatographically with the flagellin of their parent, EJ500. Flagellins of mutants EJ514 and EJ515 eluted, in diethylaminoethyl-cellulose columns, one fraction behind the flagellin of EJ500 (Fig. 2). The flagellin of EJ512 eluted slightly ahead of the flagellin of EJ500. The flagellin of EJ513 eluted at the same position as that of the parent.

Isolation of host range mutants of phage χ . Spontaneous mutants of phage χ which could infect the four motile, χ -resistant strains were isolated. Two spontaneous mutants, XJ2 and XJ3, were isolated from XJ1 on EJ512 and EJ513, respectively. Mutant XJ4 was isolated from XJ1 in continuous culture with EJ514. Mutant XJ5 was isolated from XJ3 on EJ515.

The efficiencies of plating of these mutant phages on the motile χ -resistant strains were compared (Table 4). All host range mutants of χ so far isolated have a wider host range than their parents (21, 33, 35). However, in the case of XJ5, increased infectivity on EJ515 was ac-

companied by decreased infectivity on EJ512.

These mutant phages were further tested for their infectivity on the flagellar mutants listed in Table 5. High-titer lysates of each phage (10^{10} plaque-forming units per ml) were spotted on a layer of each bacterial strain to be tested. Although phages XJ1, XJ2, and XJ3 did not lyse filamentless mutant EJ521, phage XJ5 could

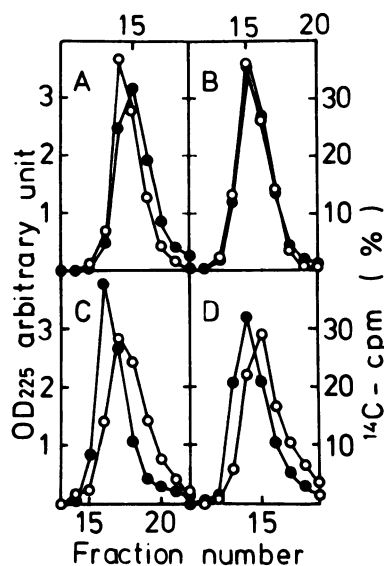


FIG. 2. Chromatographic separation of flagellins. Symbols: ●, EJ500 flagellin; ○, mutant flagellins. (A) EJ512; (B) EJ513; (C) EJ514; (D) EJ515. In experiments (A), (B), and (D), mutant flagellins were labeled with ¹⁴C-amino acids, and EJ500 flagellin was used as carrier. In experiment (C), EJ500 flagellin was labeled, and EJ514 flagellin was used as carrier. More than 1,000 cpm and an absorbance of more than 0.5 per peak fraction were obtained in each experiment.

TABLE 4. EOP of host range mutants of χ on motile χ -resistant mutants^a

Bacterial host	Log EOP ^b				
	XJ1 ^c	XJ2	XJ3	XJ4	XJ5
EJ500	0	0	0	0	0
EJ512	-8	0	0	-6	-3
EJ513	-7	-4	0	-1	0
EJ514	-9	-4	-4	0	-1
EJ515	-9	-4	-2	0	0

^a Host range mutants of χ which could infect motile, χ -resistant strains EJ512, EJ513, EJ514, and EJ515 were named XJ2, XJ3, XJ4, and XJ5, respectively. Phages were titrated by the surface method. Efficiency of plating was calculated by taking the value for χ -sensitive strain EJ500 as 1.

^b EOP, Efficiency of plating.

^c Phage.

TABLE 5. Lysis of flagellar mutants by host range mutants of χ^a

Bacterial strain	Flagellar character	Lysis		
		XJ1 ^b	XJ4	XJ5
EJ522	Polyhook	+	c	c
EJ521	No filament	—	+	c
EJ711	Altered flagellar antigen	—	—	c
EJ523	Paralyzed flagella	—	—	—

^a Phage lysate (10^{10} plaque-forming units per ml) was spotted on a lawn of each flagellar mutant. Plates were incubated at 36°C overnight, and lysis was recorded. Host range mutants XJ2 and XJ3 showed the same spectrum of lysis on the flagellar mutants as did XJ1. Symbols: c, complete lysis; +, turbid lysis; —, no lysis.

^b Phage.

lyse it completely and XJ4 could lyse it partially. Moreover, XJ5 could lyse flagellar antigen mutant EJ711, which was not lysed by other mutants of χ . None of these mutants could lyse paralyzed mutant EJ522.

Adsorption of radioactive phages to motile χ -resistant mutants. The adsorption of host range mutants XJ4 and XJ5 to the bacterial strains EJ500, EJ514, and EJ515 was compared with that of their parent, XJ1, using radioactive phages (Table 6).

When labeled phages of the parent, XJ1, were mixed with χ -resistant strains EJ514 and EJ515, adsorption was not detected. However, the radioactive counts of the host range mutants were well retained on EJ514 and EJ515 cells. Moreover, a low level of adsorption of XJ5 to EJ514 cells was observed. These results were consistent with the difference in efficiency of plating among these phages (Table 4).

DISCUSSION

We isolated *E. coli* mutants resistant to phage χ whose movement was not disturbed by phage χ . Detailed studies were carried out on four of these mutants. At least two of these mutants were clearly shown to have altered flagellin by diethylaminoethyl-cellulose column chromatography. The mutation sites of the four mutants were more than 97% cotransducible with the allelic site of a *hag* mutation by P1 transduction. No gene is known to occur near the *hag* gene which modifies flagellin in *E. coli* (17, 27). Therefore, it may be reasonable to conclude that these mutants have altered flagellin molecules with their mutation sites within *hag* gene.

Since a flagellum rotates at its base (6, 18, 28), contact of a flagellum with the surface of a glass slide results in a frictional force lateral to the direction of bacterial progress. It may be this

TABLE 6. Adsorption assay of host range mutants of χ on motile, χ -resistant mutants^a

Bacterial strain	Adsorption efficiency of phage:		
	XJ1	XJ4	XJ5
EJ500	100	100	100
EJ514	0	55	8
EJ515	0	0	55

^a The adsorption efficiencies of phages XJ1, XJ4, and XJ5 to bacterial strains EJ500, EJ514, and EJ515 were assayed by using radioactive phages. The radioactivity retained with cells of strain EJ500 on the glass filter was taken as 100%.

centripetal force which makes the bacterium move circularly close to a glass surface (19, 33). It is generally known that bacteria exhibit circular movement when the number of flagella per bacterium is reduced in the early exponential phase. Enomoto (personal communication) found mutants having short flagella, which showed circular movement. Iino and co-workers (manuscript in preparation) obtained short-flagella mutants among revertants of *flaL* mutants in *Salmonella*, which formed smaller swarms and exhibited circular movement. Since the motile χ -resistant mutant EJ512 has about the same number (and length) of flagella as the parent, the circular movement of the mutant may be explained by an increased frictional force without a decrease in the motive force, which results from an alteration in the molecular surface of the flagellar filament. Similar circular movement was observed among some of the motile revertants from *hag* mutant EJ521 (Icho, unpublished data).

The EJ515 cells tended to adhere to the surface of glass and tumbled frequently. The behavior was not due to a defect in chemotactic machinery, because they formed normal swarms (3). The interaction between the flagella of strain EJ515 and the glass surface may be so strong as to cause the filaments to adhere to the glass surface. Consequently, the flagellar filament may be stressed and its rotation may be interrupted. The frequent tumbling of the cells may result from the relaxation of such continual stress.

Like other host range mutants of χ that had been previously isolated (21, 23), the mutants described here show different efficiencies of plating on various bacterial mutants. The adsorption assay with labeled phages and χ -resistant mutants showed that these mutants did not adsorb to the wild-type strain of χ but that the host range mutants of χ could attack the flagella of these χ -resistant mutants. These facts indicate that a defined configuration of χ tail fiber can recognize a restricted configuration of flagellar filament.

We do not know why the shape of flagellar filaments could be visualized with the concentrated suspension of phage χ . One possibility is that the phage adsorption thickens filaments like the adsorption of antibody. A difficulty of this explanation is that even distribution of the phage particles along the filaments could not be observed by electron microscopy.

Recently, Komeda and co-workers (15a), using our isolation methods, isolated motile, χ -resistant mutants with altered hooks among revertants of a *flaK* mutant. These mutants continued to move even after the addition of a concentrated phage χ lysate, just like our motile, χ -resistant mutants (Icho and Komeda, unpublished data). Since these mutants have normal flagellar filaments, this fact suggests that the bacteria are immobilized, not by adsorption of phage χ to the flagellar filament, but by direct interaction of the phage to some component of its basal structure.

When the high-titer lysate of phage χ was added to a suspension of strain EJ500, only cells tethered to the glass surface could rotate. Also, cells artificially tethered to a glass surface with anti-flagellin serum continued their rotation even after the addition of the phage χ lysate (Icho, unpublished data). In such a tethered cell, the cell body is rotating, but the filament is fixed. This means that rotation of not only the flagellar basal portion but also the filament is necessary for the adsorption of the phage to the base (7, 25).

We could not isolate motile mutants which do not permit the growth of phage χ , such as *gro* (9) or *tab* (32) mutants in λ or T4. Such mutants might have become immobilized by the adsorption of phage χ and escaped selection.

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