Defective F Pili and Other Characteristics of Flac and Hfr Escherichia coli Mutants Resistant to Bacteriophage R17

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Mutants resistant to the donor-specific bacteriophage R17 were isolated from Hfr and Flac-containing strains of Escherichia coli K-12. Thirty-five mutants were examined for the presence of F pilus by electron microscopy. The pilus morphology was studied, as were the abilities of the cells to retract their pili and to synthesize new pilus. Measurements were made of the efficiency of the conjugal deoxyribonucleic acid transfer and of M13 and R17 phage infection. All mutants had noticeable defects in pilus production, structure, or function. Mutants were found which produced unusually long pili, displayed wide variations in the number of pili per cell, and were deficient in pilus retraction and synthesis. Evidence is presented that there may be two pathways of pilus retraction.

F pili have several known functions (for review, see 1, 19). They are necessary for bacterial conjugation, and they serve as the receptor sites for two classes of bacteriophage: isometric RNA phage (for example, R17), which binds to the pilus sides, and donor-specific DNA phage (for example, M13), which binds to the pilus tip. F pili are rapidly regenerated after they have been removed from the cell surface by mechanical means (blending) (1, 9), by heating (11), or by treatment with cyanide (10). F pili rapidly disappear from the surface of donor strains when the cells are infected with donor-specific DNA phage (3, 7), treated with cyanide (10) or arsenate (14), or heated to 50°C (11). This disappearance is thought to take place via pilus retraction (7), since the process is not accompanied by an increase in the number of free pili in the culture supernatant (10, 11). Alternatively, it is possible that this disappearance is due to disaggregation of the pili into subunits too small to be observed by electron microscopy (10, 11).

The biochemistry of F pilus synthesis and function may be quite complex. The isolation and study of mutants which produce structurally or functionally defective F pili should help to unravel these complexities.

There have been a number of reports of the isolation of mutants defective in pilus-related functions, but few of these involved the study of pilus functions other than donor-specific phage infection and the ability to transfer DNA.

Specifically, no mutants deficient in F pili retraction or regeneration have been reported. This paper is a report of the isolation and the study of a number of R17-resistant mutants of Escherichia coli with altered F pilus structure and function.

MATERIALS AND METHODS

Bacteria and phage. Strains of E. coli K-12 and their relevant genotypes are listed in Table 1. All mutants were derived from the donor strains PT3 and W1895. The donor-specific phages R17 and M13 were used in mutant selection and phage sensitivity tests. R17 was used to label F pili for electron microscopy.

Media. ZT medium (2) was used to grow bacteria. For phage sensitivity tests, 2,3,5-triphenyl-tetrazolium chloride was incorporated into ZT agar plates. Minimal lactose-salts medium supplemented with thymine, threonine, leucine, and methionine (M1 medium) was used for the purification of PT3 and its mutant derivatives. M1 medium contains, in gram per liter, MgSO4·7H2O, 0.025; (NH4)2SO4, 1.0; KH2PO4, 3.0; glucose, 2.0; thymine, 0.1; DL-threonine, 0.08; L-leucine, 0.04; and L-methionine, 0.04. Nalidixic acid, final concentration 30 mg/liter, was added to M1 medium for the selection of Lac⁺ nalidixic acid-resistant (Na⁺) transconjugants in PT3 × JBR4 matings (M1 nal medium). M2 medium was used for the selection of Lac⁺ streptomycin-resistant (Str⁺) recombinants in W1895 × 200u matings. The composition of M2 medium is the same as that of M1 medium, except that thymine and the amino acids were deleted and thiamine, final concentration 5 mg/liter, and streptomycin, final concentration 200 mg/liter, were added. Levine EMβ agar medium (Difco) supplemented with thymine, final concentration 100 mg/liter (EMβ thy medium), was used for the detection of R17⁺ mutants. EMβ thy medium supplemented with lactose, final concentration 5.0 g/liter, was used for the differentiation of Lac⁺ and Lac⁻.
were tested for acridine orange curing of Flac by previously published methods (2). All further studies of mutants derived from PT3 were done with the recombinants, if they could be isolated.

Four R17 mutans isolated from W1895 were available from a previous study. They are spontaneous mutants which were isolated by growing 10⁶ cells in each of 50 tubes containing 5 ml of ZT broth with 10¹² plaque-forming units of R17 overnight at 37°C. Survivors were purified, and one mutant from each culture tube was checked for R17 resistance. Mating efficiencies were measured by the same method that was used for PT3, except that 200 μl was used as the recipient and Lac⁺ Str⁺ recombinants were selected on M2 agar plates. Strain W1895 is an Hfr which transfers lac at about 10 min (9).

Phage sensitivity tests were done by touching sterile toothpicks which had been dipped in phage lysates to the surface of ZT-2,3,5-triphenyl-tetrazolium chloride plates which had been overlaid with a ZT-2,3,5-triphenyl-tetrazolium chloride soft-agar lawn containing 10⁶ cells of the bacterial strain to be tested. The plates were incubated at 37°C. All mutants and recombinants were tested to determine whether they were sensitive or resistant to phage R17, M13, and T4. PT3 and JBR4 controls were always used. A strain was called "sensitive" (S) if the phage produced a clearing as large and as clear as the clearing on a lawn of sensitive PT3 control cells, "resistant" (R) if the phage produced no clearing and the area was indistinguishable from the corresponding area of a lawn of resistant JBR4 control cells, and "slightly sensitive" if a clearing was produced which was unmistakably smaller, more turbid, or both, than the PT3 control.

Techniques used for the preparation and examination of cultures by electron microscopy have been described in detail (13). Samples of cultures were rapidly chilled on ice and were simultaneously fixed with 3.4% (wt/vol) formaldehyde. F pilis were labeled with phage R17, and cells were allowed to settle on Formvar-coated copper grids. Samples were stained with 1% (wt/vol) uranyl acetate.

The number of F pilis per cell was determined by counting the number of attached pili in micrographs of 70 to 800 cells.

F pilus length was measured by comparing the mean length of at least 50 pilis with that of the appropriate PT3 or W1895 control. Measurements were made directly from electron micrographs of mutants and controls taken at the same magnification and on the same day (2).

F pilus retraction was studied by heating 1 ml of 37°C cultures of cells in the exponential phase of growth for 60 s in a 50°C water bath (11), and by exposing another 1-ml sample of the same culture to 10⁻² M NaCN for 60 s at 37°C (10). The samples were then fixed and examined by electron microscopy. The number of attached F pilis per cell was determined. In some instances, the lengths of these pili were also measured.

To measure pilus regeneration, pilis were removed from cells by mechanical agitation (blending) at 10,000 rpm for 2 min at 0°C in a Sorvall Omnimixer (9). The cells were centrifuged out of the medium at 4°C and suspended in fresh ZT broth at 37°C. Regeneration
was followed by making electron microscope samples and measuring the number of pili per cell as a function of time after resuspension (9). Regeneration was also measured by removing pili from the surface of cells by heating cells to 50°C for 1 min and following regeneration as a function of time after a temperature shift back to 37°C (11).

RESULTS

Isolation of R17" mutants. Approximately 41,000 mutagenized PT3 clones were screened by the EMB-R17 plate method, and 1,813 R17-resistant mutants were obtained. Twelve percent of the mutants had probably become resistant to the phage via loss of Flac, as indicated by their failure to grow in the M1 agar stabs. The frequency of Flac" R17" mutants was 0.039.

A search was made for mutants that were M13 resistant but R17 sensitive. About 7,600 mutagenized PT3 clones were screened on the EMB-R17 plate which had been seeded with M13, and 128 Flac" M13" mutants were found. All of the M13-resistant mutants had also become resistant to R17.

Characterization of mutants. Thirty-one Flac" R17" mutants obtained by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis of PT3 and four spontaneous R17" mutants derived from W1895 were randomly selected for further study.

The following tests were done to collect data for the mutant classification. The mutants were tested to determine whether they were sensitive or resistant to phage R17 and M13. Mating efficiencies were determined. Transconjugants were isolated from matings involving transfer-proficient PT3 mutants. The transconjugants were tested to determine whether they were sensitive or resistant to R17 and M13. Electron microscopic examination of all mutants and transconjugants revealed whether or not F pilus were present. All transconjugants derived from PT3 mutants were tested for acridine orange curing of Flac. Eighteen mutants had no pilus and no transfer ability and were R17" M13". These classical Tra" mutants were not studied further.

All mutants and transconjugants that had pilus were studied by electron microscopy to determine the morphology of the pilus, their ability to bind R17, and the number of pilus per cell. All mutants and transconjugants with an average of more than 0.2 pilus per cell, and some with fewer, were tested to determine whether or not the pilus retracted in cyanide, retracted at 50°C, and regenerated normally after removal by blending. Mutants and transconjugants which retracted their pilus at 50°C were tested to determine whether or not they regenerated them after the retraction.

Table 2 summarizes the phenotypes of the mutants that do not have the classical Tra" phenotype (Tra", no pilus, R17" M13"").

Mutants producing long F pilus. Four mutants, JB134, JB150, JB160, and JBC8, produced

\[ \text{Table 2. Mutant phenotypes} \]

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Trab</th>
<th>R17c</th>
<th>M13c</th>
<th>Structural defects</th>
<th>Functional defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB134</td>
<td>-1</td>
<td>R</td>
<td>R</td>
<td>Long pili</td>
<td></td>
</tr>
<tr>
<td>JB136</td>
<td>-2</td>
<td>R</td>
<td>R</td>
<td>Few pili (0.02)*</td>
<td></td>
</tr>
<tr>
<td>JB137</td>
<td>-4</td>
<td>R</td>
<td>R</td>
<td>Few pili (&lt;0.003), altered cell shape</td>
<td></td>
</tr>
<tr>
<td>JB146</td>
<td>-3</td>
<td>R</td>
<td>al.S</td>
<td>Few pili (0.014)</td>
<td></td>
</tr>
<tr>
<td>JB147</td>
<td>-3</td>
<td>R</td>
<td>R</td>
<td>Few pili (0.08)</td>
<td></td>
</tr>
<tr>
<td>JB148</td>
<td>-6</td>
<td>R</td>
<td>S</td>
<td>Many pili (2.4)</td>
<td>Ret^-</td>
</tr>
<tr>
<td>JB149</td>
<td>-1</td>
<td>al.S</td>
<td>al.S</td>
<td>Few pili (0.4)</td>
<td></td>
</tr>
<tr>
<td>JB150</td>
<td>-1</td>
<td>R</td>
<td>S</td>
<td>Few pili (0.4), long pili</td>
<td>Reg^-</td>
</tr>
<tr>
<td>JB153</td>
<td>-3</td>
<td>R</td>
<td>R</td>
<td>Few pili (0.01)</td>
<td></td>
</tr>
<tr>
<td>JB164</td>
<td>-3</td>
<td>R</td>
<td>R</td>
<td>Few pili (0.01)</td>
<td></td>
</tr>
<tr>
<td>JB160</td>
<td>-1</td>
<td>al.S</td>
<td>al.S</td>
<td>Few pili (0.4), long pili</td>
<td>Ret^- Reg^-</td>
</tr>
<tr>
<td>JB163</td>
<td>-2</td>
<td>R</td>
<td>al.S</td>
<td>Few pili (0.1)</td>
<td>Ret^-</td>
</tr>
<tr>
<td>JBC7</td>
<td>-5</td>
<td>R</td>
<td>R</td>
<td>Few pili (0.002)</td>
<td></td>
</tr>
<tr>
<td>JBC8</td>
<td>-1</td>
<td>al.S</td>
<td>R</td>
<td>Few pili (0.3), long pili</td>
<td>Ret^-</td>
</tr>
<tr>
<td>JBC34</td>
<td>-1</td>
<td>R</td>
<td>R</td>
<td></td>
<td>Ret^- Reg^-</td>
</tr>
</tbody>
</table>

* Mutants with a "C" in their strain number are derived from strain W1895; others are from strain PT3.
* Values are the logarithm of the mating efficiencies.
* Abbreviations: R, resistant; S, sensitive; al.S, slightly sensitive.
* Mean number of F pilus per cell.
* Retraction deficient at 50°C, Ret+ in CN-.
* Regeneration deficient after blending and retraction.
* Retraction deficient in CN-, Ret+ at 50°C.
* Regeneration deficient after blending, Reg- after retraction.
* Retraction deficient at 50°C and in CN-.

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unusually long pili. Figure 1 shows the distribution of pili lengths for a representative mutant, JB150, and the control strain PT3. Two features are noteworthy. Eighteen percent of the JB150 pili were longer than any of the PT3 pili. The modal length of JB150 pili was the same, approximately 2.5 µm. All four mutants produced pili whose mean lengths were about twice the mean lengths of controls. In three cases the numbers of pili per cell were about half those of controls.

**Mutant producing many F pili per cell.** One mutant, JB148, produced an average of 2.4 pili per cell (Table 2), over twice as many as were produced by a PT3 control. The JB148 pili had the same mean length as pili on a PT3 control. JB148 was unable to transfer Flac at detectable levels and was R17" M13" QB'. The traD60 mutation of Willetts and Achtman (20) has an identical phenotype (Tra-, many pili, MS2'. f' Q'.). JB148, then, may also be a traD mutant.

**Mutants producing few F pili per cell.** Examination of electron micrographs of 100 to 430 cells of each mutant strain showed that 12 pilated mutants produced fewer than half as many pili per cell as did control cultures (Table 2). For example, JB153 produced an average of only 0.012 F pili per cell. Nineteen mutants seemed to have no F pili. However, it is possible that pili would have been found if micrographs of more cells had been examined. All of these mutants had very low mating efficiencies, and most were resistant to both R17 and M13.

Mutant strain JB146 is unique in that it is sensitive to M13 in a spot test in spite of having an average of only one pilus on every 73 cells. Under conditions where 100% of PT3 control cells could be productively infected by M13 (multiplicity of infection of 20; 5-min adsorption), only 0.62% of JB146 cells were infected. The way in which this mutant becomes infected by M13 is unknown.

**Mutant with deformed cells.** One mutant, JB137, had deformed cells. The cells often had nonparallel sides and asymmetrical ends (Fig. 2), although cells of normal shape were also seen. No F pili were observed on JB137, although common pili were occasionally seen. JB137 grew more slowly than did PT3 control cultures. The doubling time of aerated cultures of JB137 in the exponential phase of growth in ZT broth at 37°C was 39 min, compared to 30 min for PT3. JB137 also formed colonies smaller than did PT3 on ZT, M1, and EMB thy lac agar plates.

**Mutants deficient in retraction of F pili.** Five mutants were deficient in the retraction of their F pili (Ret-, Table 2). Representative data for one mutant of each type and control strains are shown in Fig. 3. The number of pili per cell of PT3 and WI895 control cultures decreased to 10 to 25% of their normal values when the cultures were heated to 50°C for 1 min and when they were exposed to 10⁻² M NaCN for 1 min. The pili of one mutant, JBC8, did not retract when the culture was heated to 50°C or when it was exposed to cyanide. Three mutants, JB148, JB163 and JBC34, produced pili which did not retract when the culture was heated to 50°C, but which did retract when it was exposed to cyanide. A fifth mutant, JB160, produced pili which retracted when the culture was heated to 50°C, but which did not retract when it was exposed to cyanide.

**Mutants deficient in the regeneration of F pili.** Three mutants, JB150, JB160, and JBC34, showed marked deficiencies in their ability to produce new F pili after existing pili had been removed from cells by blending or by heat-
ing cultures to 50°C for 1 min (Reg−, Table 2). Typical experiments are shown in Table 3. After the removal of the pili of a PT3 control culture by blending, 64% of the pili had returned by 3 min, and all had returned by 30 min (one cell generation). After the retraction of PT3 pili at 50°C, all of the pili had returned by 3 min. These return kinetics are consistent with previously reported results (9, 11). JB150 and JB234 were deficient in regeneration after the pili had been removed by either method. For example, none of the pili of mutant strain JB150 had returned by 3 min after they had been induced to retract at 50°C. Only 4% of the JB150 pili had returned by 3 min after they had been removed by blending, but all had returned by 30 min. Another mutant, JB160, was deficient in pilus regeneration after removal by blending, but regenerated its pili at a near-normal rate after removal by retraction at 50°C. Seven percent of the pili had returned by 3 min after blending, and all had returned by 30 min. In contrast, 80% of the pili had returned by 3 min after they had been removed by retraction at 50°C.

**Mutants deficient in conjugal DNA transfer.** Thirty of the thirty-five mutants that were selected for R17 resistance had mating efficiencies of less than or equal to 10−3 of the control strains. Thirteen of these had mating efficiencies below 10−6, which is the system's threshold of detection. With the exception of strain JB148, all mutants with mating efficiencies below 10−6 had no F pili.

**Sensitivity of mutants of phages M13 and R17.** All mutants were tested for sensitivity to phages M13 and R17. Twenty-seven of the mutants were resistant to both R17 and M13. Four of the mutants were resistant to R17, but sensitive to M13. Three mutants were slightly sensitive to both R17 and M13, and one mutant was slightly sensitive to R17, but resistant to M13. (Four mutants that had scored as R17+ in the mutant screening proved to be slightly sensitive in the spot lysis test.)

**Location of the mutations.** The plasmids of all of the mutants of PT3 in Table 2, with the exception of that of JB148, could be transferred by conjugation to the isogenic recipient strain, JBR4. All transconjugants had the same pattern of R17 and M13 sensitivity as did the original mutants. The pilus morphology and number of pili per cell were always similar. Pili retraction and regeneration were only measured in transconjugants, except for JB148. Therefore, all of the above phenotypes are due to plasmid mutations.

It was shown that the JB148 mutation to R17+ was located on the plasmid by curing JB148 with acridine orange and reintroducing the original.

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**Table 3. Mutants deficient in the regeneration of new pili after pilus removal**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Methods of pilus removal</th>
<th>F pili per cell</th>
<th>At time after pilus removal:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>0 min</td>
</tr>
<tr>
<td>PT3 (control)</td>
<td>Blending</td>
<td>0.94 ± 0.13</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>50°C retn.</td>
<td>0.94 ± 0.13</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>JB150</td>
<td>Blending</td>
<td>0.51 ± 0.03</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>50°C retn.</td>
<td>0.50</td>
<td>0</td>
</tr>
<tr>
<td>JB160</td>
<td>Blending</td>
<td>0.45 ± 0.04</td>
<td>0.08 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>50°C retn.</td>
<td>0.45 ± 0.04</td>
<td>0.08 ± 0.06</td>
</tr>
</tbody>
</table>

- Values given are the total number of pili per total number of cells, plus or minus one standard deviation.
- 50°C retn., 50°C retraction.
- ND, Not determined.
Flac plasmid from PT3 by mating. Transconjugants were R17.

The phenotype of strain JB137, a distorted cell shape and low growth rate, was also shown to be due to a plasmid mutation. The entire phenotype was always transferred along with the plasmid. After acridine orange curing, the cell shape and growth rate reverted to normal.

DISCUSSION

The selection of R17-resistant mutants proved to be a plentiful source of strains with defective F pili. All mutants had noticeable defects in pilus production, structure, or function. Since the mutants were selected for R17 resistance, it may be that the only way in which a cell can become resistant to R17 is by producing either defective pili or no pili at all.

It has been proposed that the retraction of F pili is necessary for infection by donor-specific DNA and RNA phage (7). The fact that five mutants that were selected for R17 resistance produce pili which bind R17 normally but are retraction deficient (JB148, JB160, JB163, JBC8, and JBC34) suggests that pilus retraction may well play some role in phage infection. The nature of that role is not clear, since three of these mutants (JB148, JB160, and JB163) are slightly sensitive to R17. This R17 sensitivity can be reconciled with the theory that retraction is necessary for R17 infection since, in the spot lysis test for phage sensitivity, a large number of phage are placed on the bacterial lawn, so that R17 phage probably bind to the entire length of the pilus. Phages which bind near the base of the pilus would not require pilus retraction to transport them to the cell surface. The corresponding argument cannot be made for M13 infection, since M13 binds to the pilus tip (7).

The fact that some retraction-deficient mutants are sensitive to M13 indicates that a pilus which retracts either at 50°C or in cyanide is not an absolute requirement for M13 infection.

Mutants were found which could retract their pili at 50°C, but not in cyanide. Others could retract their pili in cyanide, but not at 50°C. Since they can be separated, it appears that the mechanisms of F pilus retraction at 50°C and in cyanide are, in some way, different.

Regeneration-deficient mutants may be defective in either (i) the initiation of pilus elongation, possibly in the establishment of a site for pilus synthesis, or (ii) the elongation process itself, possibly in the rate at which the pilus subunits are assembled. It is not possible to determine, from the data currently available, which of the alternative explanations is true. Strain JB160, which is defective in pilus regeneration only after pili are removed by blending, might have a structural component which is necessary for pilus elongation and which is unusually susceptible to physical damage.

Mutant strain JB137 contains a plasmid which alters the shape of the cells and lowers the rate of cell growth in enriched broth. Several groups have isolated temperature-sensitive replication mutants of other plasmids (4-6, 18) which cause their host cells to grow slowly at the nonpermissive temperature. Cultures of E. coli harboring some of these plasmids contained symmetrically elongated cells at the nonpermissive temperature (4, 6). The cell shape deformity of JB137 is distinct from this elongation, since JB137 cells have nonparallel sides and asymmetrical ends and are not elongated. There is no indication that the mutant plasmid of JB137 is defective in replication.

Biochemical analyses of the mutants described in this study should prove to be most useful in determining how F pili retract and regenerate, and may shed some light on the role of F pili in donor-specific phage infection and conjugation. Since several of the mutant phenotypes have not previously been described, it is possible that some are located in Flac genes which have not yet been identified. These genes could be located either within or outside the tra operon.

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LITERATURE CITED