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Detection of a Protein, Similar to the Sex Pilus Subunit, in the Outer Membrane of *Escherichia coli* Cells Carrying a Derepressed F-Like R Factor

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The outer membranes of *Escherichia coli* K-12 cells carrying a derepressed F-like R factor contain about 7×10^4 molecules per cell of a protein similar to the subunit of the sex pili specified by the R factor. This protein pool is absent in cells carrying the repressed variant of the R factor. The size of the pool is about one-half of the amount of protein incorporated into mature sex pili at the peak of production and is independent of the phase of growth of the culture. The molecular weight of the protein in the pool and of the subunit of the sex pili specified by the cells is 12,500 ± 600.

Sex pili produced by strains of *Escherichia coli* carrying F-like R factors resemble those specified by F factors in F^+ and Hfr cells (20), although some can be distinguished serologically (18) and by density (2). The presence of these structures is correlated with the ability of the host to act as a genetic donor (22), and strains carrying derepressed F-like R factors, like F^+ strains, produce large numbers of sex pili (20) and donate their plasmids during conjugation with high efficiency. Strains carrying repressed F-like R factors, on the other hand, produce few sex pili and are poor donors (20).

Sex pili produced by bacteria carrying both F and F-like R factors are antigenically mixed (18) and are of intermediate density in cesium chloride (2), suggesting that the individual pilus subunits are synthesized at a site remote from their point of assembly into mature sex pili. Evidence for the existence of a pool of preformed subunits has been provided by the observation that F pili can be regenerated, after mechanical removal, in the presence of growth-inhibiting concentrations of chloramphenicol (7) and by studies on the effect of temperature on pilus regeneration and synthesis (21). Efforts to locate this pool in the cytoplasm of F^+ cells have been unsuccessful (8, 21), and it has therefore been suggested that the site of assembly of mature sex pili from a pool of subunits is located in the cell envelope (2, 8), presumably in or on one of the membranous layers, since disruption of the mucopeptide with penicillin has no effect on F pilus production (7).

It is now generally accepted that the cell envelope of gram-negative bacteria is a basic three-layered structure (11) consisting of an inner (cytoplasmic) membrane, an outer membrane containing, or attached to, the lipopolysaccharide and lipoprotein components and, sandwiched between the two, the mucopeptide or murein layer (4, 5), thought to be responsible for maintaining the shape of the cell (6). Recently, techniques have become available for isolating and separating in relatively pure form the inner (cytoplasmic) and outer membranes (23) of *E. coli*, and these techniques, together with improved methods for analyzing the proteins contained in these membrane fractions, have facilitated the present investigation into the possible location of the sex pilus subunit pool in the surface layers of the cell.

MATERIALS AND METHODS

Bacterial strains and R factors. The strains and R factors used are listed in Table 1.

Media. Nutrient broth, described previously (3), was used.

Preparation of whole-cell envelopes. Whole-cell envelopes were prepared essentially by the method of Holland and Tuckett (15), except that all washing and resuspension steps were performed with deionized water and the cells were broken at −30 C in a Hughes press (16) at a concentration of 10^{11} cells per ml. Under these conditions, better than 99% breakage was obtained as determined by loss of viability, decrease in turbidity, and electron microscopy. After breakage, the suspension was subjected to a clearing spin at $10{,}000 \times g$ for 5 min and then treated for 30 min at 37 C with 1 mg each of ribonuclease (EC 2.7.7.16) and deoxyribonuclease I (EC 3.1.4.5) per ml. After further purification in the ultracentrifuge (15), the final pellet

TABLE 1. *Bacterial strains and plasmids*[a]

Strain/plasmid	Description	Origin or reference
UB1005	*E. coli* K-12 *met* Nal^r	Laboratory collection
UB1025	*E. coli* K-12 *lac leu* Fla$^-$ Str^r	3
R1	F-like *fi*$^+$ plasmid. Confers resistance to ampicillin, chloramphenicol, kanamycin, streptomycin (low level), and sulfonamide. Repressed for transfer	E. Meynell
R1*drd*19	Derepressed derivative of R1	E. Meynell
R1*drd*19.K1	Spontaneous segregant of R1*drd*19 carrying kanamycin resistance only	Laboratory collection
R1*drd*19.K$^-$1	Spontaneous segregant of R1*drd*19; confers all resistances of R1 except kanamycin resistance	Laboratory collection

[a] Phenotypic symbols: Nal^r, phenotypically resistant to 80 μg of nalidixic acid per ml; Fla$^-$, phenotypically nonflagellate; Str^r, phenotypically resistant to 200 μg of streptomycin per ml. Gene symbols follow standard conventions.

was suspended in a small volume of deionized water and lyophilized.

Preparation of inner and outer membranes. Inner and outer membranes were prepared by the sonic treatment method of Osborn et al. (23), except that the final sucrose gradient for the isopycnic separation of the membrane fractions was composed as follows: sucrose, 1 ml of 65% (wt/wt), 3.6 ml of each of 60, 55, 50, 45, and 40% (wt/wt) layered in turn, followed by 1 ml of 35% (wt/wt) and 2 ml of 30% (wt/wt) containing resuspended membranes. All solutions were in 5 mM ethylenediaminetetraacetic acid (pH 7.5). The rotor was a 3 × 25 SW on an MSE Superspeed 50 centrifuge at 5 C for 72 h at 90,000 × *g*. Cultures were routinely grown in nutrient broth containing 0.5 mCi of [2-^{3}H]glycerol (Radiochemical Centre, Amersham) per liter. After fractionation and analysis, the fractions containing the inner and outer membrane components, respectively, were pooled and washed three times in deionized water before lyophilization. Fractions from the gradients were assayed for radioactivity by addition of 20-μl samples to vials containing 10 ml of 30% (vol/vol) Triton X-100 in toluene containing 6 g of 2-(4′-*t*-butylphenyl)-5-(4″-biphenylyl)-1,3,4,-oxadiazole per liter as described previously (3). Quench corrections were performed by the channels-ratio method.

The degree of spheroplasting was estimated by phase-contrast microscopy and by two kinds of measurement on the treated culture after addition of ethylenediaminetetraacetic acid. A small volume was removed, its absorbancy at 675 nm was determined, and the spheroplasts were lysed osmotically by the method of Osborn et al. (23). The loss of absorbancy at 675 nm was determined and converted to percent loss of whole bacterial cells by use of a previously determined calibration curve. Alternatively, after osmotic lysis the suspension was centrifuged at 10,000 × *g* for 15 min, and the amount of radioactivity in the pellet (unlysed cells) and in the supernatant (membrane fragments) was compared with that in a similarly treated but nonspheroplasted control cell suspension.

The remainder of the spheroplast suspension was lysed by sonic treatment for ease of handling.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was achieved by a combination of the major aspects of the methods of Laemmli (17) and Fairbanks et al. (12), except that 11-cm 12.5% running gels and 1-cm 3% stacking gels were employed. Membrane samples were dissolved in the sample buffer at a concentration of 4 mg/ml and were denatured by heating for 3 min at 100 C followed by 30 min at 70 C (24). Small volumes (25 μl; i.e., 100 μg) were layered on top of each gel, and electrophoresis was performed at a current of 3 mA per gel until the marker dye had traveled to within 5 mm of the bottom of the gel (about 3 h). After staining and destaining, the gels were scanned in a Unicam SP1809 scanning unit in an SP1800 spectrophotometer at a wavelength of 540 nm and a chart speed of 5 s/cm, giving a linear expansion of 10:1 relative to the length of the gel. All mobility measurements were carried out on the traces, and the molecular weights were estimated to within 5% of the mean from the mobilities of a range of standard proteins that were electrophoresed at the same time. The relative protein content of the various bands was estimated from the areas under the peaks.

Assay for sex pili. Whole sex pili were assayed by the radioactive male-specific phage MS2 filtration assay (3) (capable of detecting less than 0.1 μg of sex pili per ml) and by electron microscopy (both platinum shadow casting and uranyl acetate negative staining—capable of detecting sex pilus stumps less than 0.1 μm long) (3).

Materials. All chemicals were BDH AnalaR grade wherever possible. Tris(hydroxymethyl)aminomethane (reagent grade) was from Sigma Chemical Co., St. Louis. Crystalline Coomassie brilliant blue R-250 was obtained from Serva Feinbiochemica, Heidelberg. Sodium dodecyl sulfate was the "specially pure" grade from BDH Chemicals, Poole, and was used without recrystallization. Standard proteins for molecular weight calibration were all high-purity reagents obtained from Sigma Chemical Company.

Miscellaneous. Protein (13, 19), ribonucleic acid (10), deoxyribonucleic acid (9), cytochrome (23), and succinate dehydrogenase (1) assays were all performed as described. Trichloroacetic acid precipita-

tion was carried out at 0 C as described previously (14). Density determinations on fractions from the sucrose gradients were made from measurements of the refractive index of the samples at 20 C on an Abbe model 60/ED high-accuracy refractometer by using the sodium D_1 line. The densities at 2 C were then calculated from standard curves derived from reference tables.

RESULTS

Choice of strains and growth conditions. To investigate the effect of plasmid transfer genes on the protein composition of the cell envelope, we imposed two experimental constraints on our system. First, we employed two spontaneous derivatives (Table 1) of the derepressed R factor R1*drd*19 which specified mutually exclusive resistances to antibiotics while retaining, as far as we could tell, identical transfer properties and sex pilus production abilities, and we examined the protein profiles specified by these plasmids in two different host strains (UB1005 and UB1025). Second, to eliminate the possibility of being misled during these studies by the presence of mature (assembled) sex pili on the surface of the cells, we prepared our envelopes from cultures of these strains that had been grown for 20 h in shaken culture, i.e., some 4 h into stationary phase, and which could be shown to be devoid of sex pili by electron microscopy and by radioactive male-specific phage MS2 filtration assay (3). We could thus be confident that any changes in membrane protein profiles noted in all four combinations of host and plasmid would be attributable to those plasmid genes not concerned with the expression of antibiotic resistance, the most obvious examples of which would be the transfer genes.

Analysis of membrane proteins in whole envelope preparations. Whole envelopes were prepared from the two host strains UB1005 and UB1025 and from the derivatives of these strains carrying R1*drd*19.K1 or R1*drd*19.K$^-$1. Analysis of these preparations showed that less than 0.1% of the dry weight was ribonucleic acid and less than 0.05% deoxyribonucleic acid, where 60% was protein and 20% was lipid. On sodium dodecyl sulfate electrophoresis, it was found that envelopes prepared from plasmid-containing strains contained an extra protein compared with envelopes from plasmidless strains, and the mobility of this band in acrylamide gels indicated that the protein had a molecular weight of about 12,500.

Similar experiments were performed with envelopes prepared from both host strains carrying the original derepressed R1*drd*19 factor and the repressed version R1 to ascertain the effect of transfer repression on membrane protein profiles and to check the effect of the segregation events that gave rise to R1*drd*19.K1 and R1*drd*19.K$^-$1. It was found that the additional protein of molecular weight 12,500 was again present in envelopes prepared from the derepressed strain (Fig. 1B and D) and that it was absent in the repressed strain (Fig. 1A and C).

Effect of growth phase on membrane proteins in whole-envelope preparations. In view of the observations (3, 7) that production of sex pili varies with the stage of growth of a culture, reaching a peak in late logarithmic phase, we compared the protein profiles of envelopes prepared from cells taken in early logarithmic, late logarithmic, and stationary phases. Cultures were chilled, and then blended and washed three times at 4 C (7) with an M.S.E. Homogeniser (Measuring & Scientific Equipment Ltd., Crawley, Sussex) fitted with 3-cm blades and running at 7,000 rpm (3). After blending and washing, the final suspension was monitored carefully by electron microscopy and male-specific phage MS2 assay to ensure complete removal of the sex pili. Envelopes were then prepared, and their protein compositions were assayed by electrophoresis. It was found that the amount of the protein with molecular weight 12,500 present in these envelopes was unaffected by the stage of growth of the cultures, although some minor variations in other proteins were observed.

Preparation of inner and outer membranes. Inner and outer membranes were prepared by the method of Osborn et al. (23) with the modifications described in Materials and Methods and with the further exception that cultures were grown into late stationary phase, as for the whole-envelope work, and then chilled and diluted to the concentration employed by Osborn et al. (optical density at 600 nm of 0.6 to 0.8) prior to spheroplasting. Centrifugation of the membranes to equilibrium in sucrose gradients resulted in the separation of three characteristic bands, two of which comprised the outer membrane or H band (densities 1.264 and 1.274), and one of which consisted of inner membrane, or L_1, material (density 1.197). The L_2 material of Osborn et al. (23) was never observed, and very little material was present at the M band position (unseparated material).

The identity of the inner and outer membrane fractions was confirmed further by assays for cytochrome and for succinate dehydrogenase contents. Outer membrane contained less than 0.001 U of succinate dehydrogenase per mg (dry

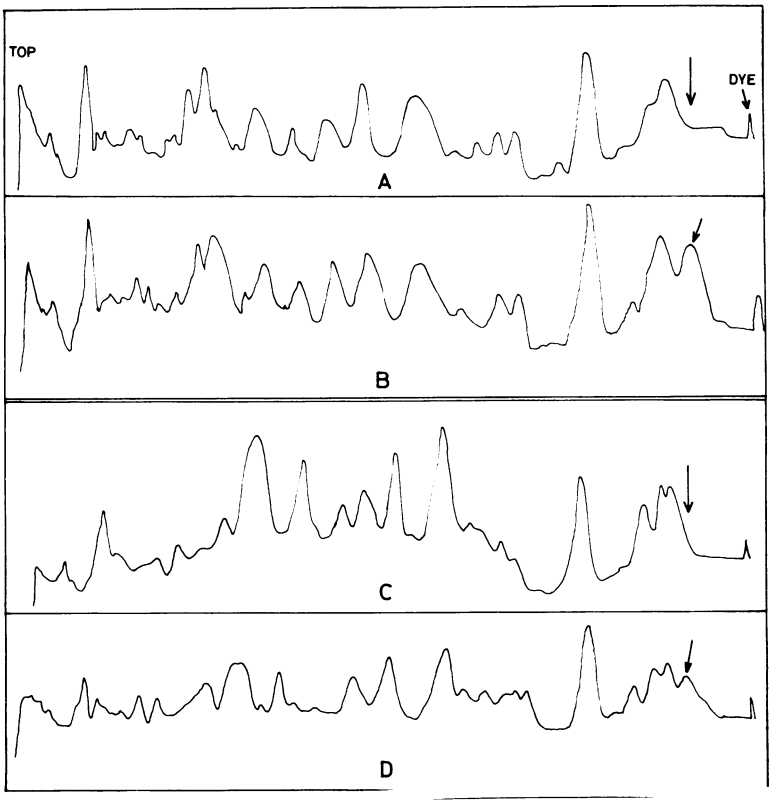

FIG. 1. *Membrane protein profiles of whole-envelope preparations from strains (A) UB1025 (R1), (B) UB1025 (R1drd19), (C) UB1005 (R1), and (D) UB1005 (R1drd19). The arrow marks the position of the 12,500-molecular weight protein.*

weight) and had no detectable cytochromes, whereas inner membrane contained between 6 and 7 U of succinate dehydrogenase per mg and 0.05 to 0.06 mg of cytochrome (calculated as cytochrome b_1 [23]) per mg (dry weight). Inner membrane contained 30 to 35% protein (dry weight), less than 0.01% deoxyribonucleic acid, and no detectable ribonucleic acid, whereas the outer membrane consisted of 30 to 40% protein (dry weight) with no ribonucleic acid or deoxyribonucleic acid. Phospholipids (estimated from the [^{3}H]glycerol disintegrations/min per mg [23]) were present in the inner and outer membrane fractions in the ratio of 5:3 as reported for *Salmonella typhimurium* (23).

Analysis of protein composition of inner and outer membranes. Inner and outer membranes prepared from UB1005 and UB1005 (R1*drd*19.K1) were analyzed by electrophoresis. Figure 2 shows that the presence of the plasmid causes no significant change in the protein profile of the inner membrane of this strain, whereas Fig. 3 shows that the outer membranes prepared from the plasmid-carrying strain contain an extra protein of molecular weight 12,500 when compared to the plasmidless strain, similar to that seen in the whole-envelope preparations.

Calculations of the areas under the peaks on traces of the membrane protein profiles of UB1005 (R1*drd*19.K1) indicate that the 12,500-molecular weight protein represents between 2 and 4% of the total protein applied to the gels in the case of the whole-envelope preparations (Fig. 1) and between 7 and 9% of the total protein in the outer envelope (Fig. 3). Both of these figures imply a total pool size for this protein of 6×10^4 to 8×10^4 molecules per cell, taking account of the dry weight of a known number of cells and the fact that about 10% of this dry weight consists of membrane protein (unpublished observations). That is, the 12,500-molecular weight protein represents about 0.3% of the dry weight of the cells.

The most obvious explanation for the observations reported above is that the 12,500-molecular weight protein is the sex pilus subunit (pilin) specified by the transfer region of

the R factor. Accordingly, the sex pili specified by UB1005 (R1*drd*19.K1) were purified by a previously reported procedure (3), and the molecular weight of the sex pilin was determined in the electrophoresis system. Sex pilin prepared in this way has a molecular weight of 12,500 ± 5%, both in the presence and absence of membrane proteins derived from the plasmidless strain UB1005.

A further check was made to ensure that the appearance of the 12,500-molecular weight protein in the electrophoretic profiles of the outer membrane was not the result of contamination of the outer membrane fractions with whole sex pili which cosedimented with them, as has been found with bacteriophage lambda (B. R. Booth and B. A. Fry, Proc. Soc. Microbiol. 1:40, 1974) and chromosomal deoxyribonucleic acids, which cosedimented with the inner membrane (Beard, unpublished data). Radioactive MS2 phage assay of the fractions from a sucrose gradient of UB1005 (R1*drd*19.K1) inner and outer membrane material and examination of the fractions in an electron microscope showed that there were neither detectable sex pili nor any other adsorption sites for MS2 phage in the preparation. Also, whole sex pili and denatured sex pilin were centrifuged to equilibrium in a sucrose gradient identical to that used for the inner and outer membrane fractionation. Both types of preparation remained close to the top of the gradient. Furthermore, if isolated sex pili or denatured sex pilin were mixed with inner and outer membrane preparations from UB1005 (the plasmidless strain) prior to centrifugation on sucrose, no association of either membrane fraction with either form of sex pilus material could be detected in the gradient fractions, and

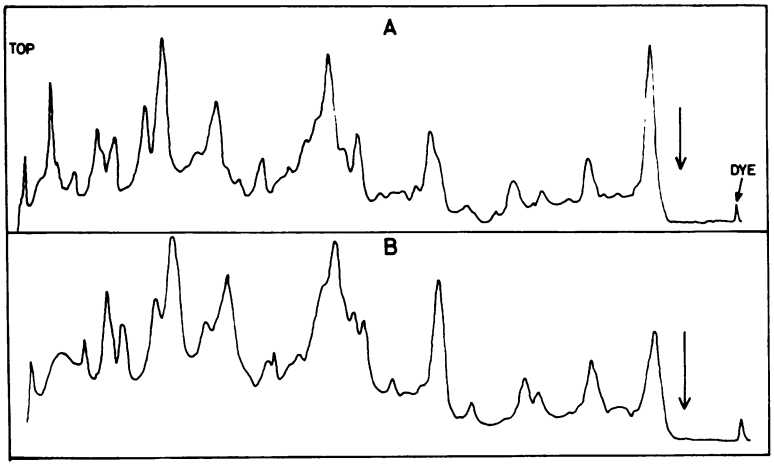

FIG. 2. *Membrane protein profiles of inner membrane material from (A) UB1005 and (B) UB1005 (R1drd19.K1). The arrow indicates the position for a protein of molecular weight 12,500. Minor differences in the positions or amounts of some of the proteins in these traces were not reproducible.*

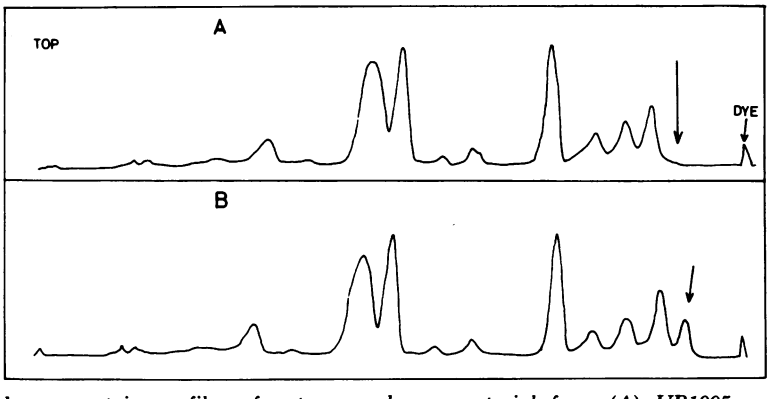

FIG. 3. *Membrane protein profiles of outer membrane material from (A) UB1005 and (B) UB1005 (R1drd19.K1). The arrow marks the position of the 12,500-molecular weight protein.*

there were no changes in the membrane protein profiles of the inner and outer preparations obtained from these gradients; that is, no extra band of molecular weight 12,500 appeared in the gels.

There remained the formal possibility that lysozyme, which was used in the inner and outer membrane preparations—although not in the whole-envelope preparations—and which has a mobility in acrylamide gels close to that of the 12,500-molecular weight protein, could be cosedimenting in association with the outer membrane, thus giving rise to the extra band on electrophoresis. Excess lysozyme (20 mg/tube) was therefore centrifuged in the sucrose gradient system in the presence and absence of membrane preparations. As with the sex pilin, lysozyme remained at the top of the gradients in both cases, and no association of the enzyme with either inner or outer membrane fractions could be demonstrated. Furthermore, it was easily shown that lysozyme and sex pilin were clearly separated on electrophoresis in the same gel even in the presence of fivefold excess of either protein.

DISCUSSION

Our experiments have demonstrated that the outer membranes of cells of *E. coli* that are carrying a derepressed F-like R factor contain about 7×10^4 molecules per cell of a protein with a molecular weight of 12,500 in sodium dodecyl sulfate-acrylamide gels. The presence of this outer membrane protein in cells carrying derivatives of the R factor with mutually exclusive resistance determinants implies that the protein is specified by one of the transfer genes, and this notion is strengthened further by the fact that membranes of cells carrying the repressed variant of the R factor do not contain detectable levels of the protein. Our observations that the size of the pool of the 12,500-molecular weight protein does not vary with the phase of growth of the culture and that it is maintained at the same level up to 4 h after the culture has entered stationary phase suggest that the concomitant loss of fertility and absence of mature sex pili on the surface of the cells at this time are not consequences of the unavailability of sex pilus subunits for assembly.

We have been able to show that the subunit protein of sex pili isolated from the strains we have used has identical behavior in our electrophoretic system to that of the outer membrane protein we detected. This correspondence, together with the compelling evidence for the existence of a pool of preformed subunits synthesized at a site remote from their point of assembly into mature sex pili (2, 7, 18, 21), makes the outer membrane protein a likely candidate for the sex pilus subunit. This is especially so in the light of the unsuccessful attempts to detect such a pool in the cytoplasm (8, 21); we also have been unable to detect a cytoplasmic pool of sex pilin with sodium dodecyl sulfate-acrylamide gels (unpublished results). Preliminary experiments with transfer-defective mutants of F *lac* (kindly supplied by Neil Willetts) lend further support to this notion; we have been able to show that the extra protein is absent in membranes derived from cells carrying F *lac traA1* (identified as the sex pilus subunit gene by Brinton [see D. Schlessinger (ed.), *Microbiology-1974*, American Society for Microbiology, Washington, D.C., in press]), although it is present in cells carrying the wild-type F *lac* (Beard, unpublished results). Formal identification of this band as sex pilin and determination of its state of phosphorylation and glucosylation (R1*drd*19-determined sex pili, like F pili [8], contain 1.56 mol of phosphates and 1.04 mol of glucose per mol of sex pilin [Beard, unpublished data]) await the large-scale isolation of this protein from the outer membrane, work that is currently in progress.

It is of interest to note that at the point of peak sex pilus production by cells of UB1005 (R1*drd*19.K1) it can be calculated that about 2×10^{-5} to 4×10^{-5} g of sex pili is produced per cell (3). This represents between one and two times the number of sex pilus subunit molecules per cell in the outer membrane. It is tempting to speculate that the pool size is maintained in the outer membrane by some sort of feedback inhibition system (see reference 21) and that the removal of subunits during assembly of whole sex pili allows further synthesis of pilin protein to "top up" the pool. If this is so, we can predict that the continuous removal of sex pili at the time of peak production by blending in the presence of chloramphenicol (7) should allow the regeneration of no more than 50 to 100% the amount of mature pili present on the cells at the peak before the pilin pool is completely depleted. Brinton (7) reported that after one cycle of blending and regeneration in the presence of chloramphenicol sex pili returned to a maximum of about 50% of the unblended value.

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