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Isolation and Characterization of *Escherichia coli* Phase Variants and Mutants Deficient in Type 1 Pilus Production

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Type 1 pili of *Escherichia coli* are the prototype of the somatic class of pili found on many strains of bacteria. As a first step in the genetic analysis of type 1 piliation, an extensive series of nonpiliated derivatives of *E. coli* K-12, strain AW405, was characterized. None of the derivatives reacted with type 1 pilus antiserum or appeared to produce attached or free pili when examined in the electron microscope. The derivatives fell into two classes: phase variants and mutants. Phase variants that formed colonies of two distinctive types, one associated with a predominantly piliated (P^+), and the other associated with a nonpiliated (P^-) phase, were obtained. Each phase could give rise to the other at a relatively high rate, which was greater in the P^+ to P^- direction during nonselective culture on solid medium and greater in the P^- to P^+ direction during culture in unshaken liquid medium. In addition, 77 Pil^- mutants were selected on the basis of a subtle difference in colonial morphology. The mutants reverted, if at all, at a much lower rate than that of the P^- to P^+ change. The stability of Pil^- derivatives grown in unshaken liquid medium was used as a criterion for distinguishing between phase variants and mutants. Phase variation also affected colonial morphology and chemotactic swarming. These properties did not directly depend upon piliation since Pil^- mutants were only slightly altered in colonial form and unaltered in chemotactic swarming. Piliation of Pil^+ bacteria was quantitatively affected by growth conditions.

Pili can be defined as rod-like nonflagellar bacterial appendages assembled from protein subunits (7-9). They are ubiquitous in the bacterial world but vary in the details of their structure and function. We find it convenient to divide them into two broad classes: conjugal pili and somatic pili. Conjugal pili are coded by plasmids; they are organelles of conjugal deoxyribonucleic acid transfer; and, often, they are the penetration organelles for specific pilus phages (13, 15). There are usually only a few conjugal pili per cell (13). Somatic pili are coded for by chromosomal genes although these may be among chromosomal genes carried by plasmids. Their numbers per cell are usually relatively large (8, 9, 12). Somatic pili have no known direct function in conjugal fertility (7, 10) but have a variety of other important functions including adhesiveness (17, 18), surface translocation (22), growth enhancement at limiting oxygen concentrations (7, 17, 18; P. Gemski, Jr., Ph.D. thesis, University of Pittsburgh, Pittsburgh, Pa., 1964), genetic transformation competence (6), pitting of agar surfaces (20), and phage penetration (27). They also affect the form of colonies on solid medium (7).

The type 1 pili (formerly called type I pili [8, 9]; we adopted the arabic numeral to avoid confusion with "I" ["eye"] conjugal pili [25]) of *Escherichia coli* are the prototype of somatic pili. They were first characterized from strains of *E. coli* B but are widespread among laboratory strains of *E. coli* B and *E. coli* K-12 and among *E. coli* strains isolated from clinical and other natural sources (7-9, 12). Type 1 pili have a diameter of about 7 nm and typical lengths of 0.5 to 2 μm, and they usually occur in large numbers over the entire surface of the cell. They are self-assembling helices of identical protein subunits with a molecular weight of about 17,000. Pili may be concentrated and purified by crystallization in the presence of magnesium ion (8, 9). One of their functions is adhesion to mammalian cells and to other surfaces where attachment is usually endwise (9, 17, 18).

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The discovery that cellular piliation is a colonization factor necessary for the virulence of enteropathogenic strains of *E. coli*, which cause serious diseases in man and animals (C. C. Brinton, Jr., C. C. To, C. M. To, B. Nagy, and R. E. Moon, in preparation), has made research on these pili of increasing medical importance.

Purified *E. coli* type 1 pili are good antigens, and they elicit the formation of serum antibodies that agglutinate homologous or cross-reacting piliated bacteria (7, 11). Pilus antibodies do not agglutinate the nonpiliated phase of *E. coli* or its nonpiliated mutants (7, 26). Thus, antisera prepared against purified pili can be used to determine relationships among members of the *E. coli* type 1 pilus class.

As part of our laboratory program to achieve an understanding of the several mechanisms affecting pilus production, and to study the structure-function relationships in somatic pili, we investigated in detail the control of *E. coli* type 1 piliation. The expression of type 1 pili appears to be regulated by at least two kinds of mechanisms. One mechanism, which we term phase variation, is manifested as an alternation between two metastable states: piliated and nonpiliated (7, 12). All cells contain the genetic information to produce pili but do not produce pili in the nonpiliated phase. Each phase can give rise to the other suddenly and at random (7). The rate of change from one phase to another varies from strain to strain and can be influenced by environmental conditions (7). In general, the nonpiliated phase predominates under the usual conditions of laboratory growth on solid medium. A second kind of mechanism appears to govern the number of type 1 pili per cell according to the phase of the growth cycle, environmental conditions, or both. In this paper, we describe the isolation and characterization of both phase variants and nonpiliated (*pil*) mutants of a type 1 piliated strain of *E. coli* K-12 and the criteria which may be used to distinguish between them. The *pil* mutants were studied in genetic complementation tests reported in a companion paper (26) to elucidate the genes determining the presence of *E. coli* type 1 pili.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain AW405 was received from Julius Adler and has the following genotype (4): F⁻ *thr leu his thi lac gal ara xyl str ton tsx*.

Media. The basic minimal medium consisted of 1.0% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.06 M KPO_4 buffer at pH 7.0, 0.025% sodium citrate, 0.0005% thiamine, and 0.2% glucose. Required amino acids were added in a concentration of 40 μg/ml, with the exception of L-threonine which was added at 80 μg/ml.

Minimal agars were prepared by adding 2.0% agar (Difco Laboratories) or 1.5% Difco Noble agar. When necessary, naladixic acid was added in final concentrations of 10 or 20 μg/ml.

Z medium contained 1.0% tryptone (Difco), 0.8% sodium chloride, 0.1% glucose, and 0.1% yeast extract (Difco). To each liter, 0.32 ml of 10 N NaOH was added before autoclaving.

Soft tryptone agar used in chemotactic swarming tests contained 1.0% tryptone (Difco), 0.5% sodium chloride, and 0.35% agar (Difco) (4). Petri dishes each contained 25 ml of medium which was prepared 2 or 3 h before inoculation.

Mutagenic methods. Modifications of the nitrosoguanidine procedure of Adelberg et al. (1) were employed, and two different procedures were used. In the first, logarithmic phase cells in 5 ml of Z medium were centrifuged, and the residue was washed once in 10 ml of saline. The washed cells were suspended in 6 ml of saline and shaken for 5 min at 37°C. A freshly prepared solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added to a final concentration of 200 μg/ml. Cells were shaken in the presence of mutagen for 30 min at 37°C and centrifuged. The pellet was then washed once with 10 ml of saline to remove the mutagen. The final sediment was suspended in 5 ml of Z medium. Cells were either diluted and spread on minimal agar or diluted 1:25 into 5 ml of Z medium, grown overnight with shaking, and then streaked on minimal agar. Colonies were then examined for differences in morphology from the majority (Pil^+) type. An alternative mutagenesis procedure, in which 0.5 ml of Z broth containing 4 mg of the mutagen per ml was added to 4.5 ml of an overnight Z medium culture and incubated without shaking in a closed tube for 30 min at 37°C, was also employed. The culture was then diluted and spread on minimal agar and examined for colony types 16 to 18 h later.

5-Bromouracil was also employed as a mutagen. An overnight culture in Z or minimal liquid medium containing 1% Casamino Acids (Difco) was diluted 1:25 or 1:50 into the minimal medium and grown to the logarithmic growth phase. The cultures were then diluted 1:25 or 1:50 into the same medium containing 250 μg of deoxyadenosine per ml and 100 μg of 5-bromouracil per ml. The test tubes were covered with foil and incubated with shaking for 3 h at 37°C. Dilutions were prepared in saline in a darkened room, and portions were spread on minimal agar. The dishes were wrapped in foil and incubated for 16 to 18 h at 37°C before examination of colony types.

Nitrous acid mutagenesis was accomplished by concentrating stationary-growth-phase cells 20 times and washing and suspending them in 0.1 M acetate buffer of pH 4.5 to 4.6. Freshly dissolved $NaNO_2$ in acetate buffer was added to a final concentration of 0.05 M. After 10 min of incubation at 37°C, the cells were diluted, spread on minimal agar, and incubated for 16 to 18 h for colony formation.

Ethyl methane sulfonate was used as follows (24).

Cells were grown to the mid-logarithmic growth phase in Z medium, centrifuged, and washed once in M-9 buffer. Ethyl methane sulfonate at a final concentration of 0.28 M was added to 2×10^9 cells per ml. The mixture was incubated for 12 min at 37°C and then diluted 1:10 into phosphate buffer, centrifuged, and washed in Z medium. The final suspension was diluted in saline and spread on minimal agar. Colonies were examined 16 to 18 h later.

Mutations were induced with 2-aminopurine as follows. Logarithmic-growth-phase cells (0.1 ml) grown in Z medium were added to 20 ml of Z medium containing 600 μg of 2-aminopurine per ml and incubated with shaking to the stationary growth phase. The cells were diluted in saline and spread on minimal agar. Colonies were examined 16 to 18 h later.

Determination of colony morphology. The isolation of *pil* mutants depended on a difference in colonial morphology between colonies with piliated cells and colonies with nonpiliated cells. The use of 2% agar in minimal medium (or 1.5% Difco Noble agar) facilitated the identification of mutants. Colonies were examined 16 to 18 h after inoculation with slight magnification (5 to ×30) by using a Bausch and Lomb stereomicroscope illuminated with oblique lighting. The lighting was attained by placing the shade of a goose-necked desk lamp equipped with a 150-W bulb approximately 8 inches (ca. 20.32 cm) in front of the microscope. The mirror of the microscope was detached and placed on the table directly in front of the microscope. Light was directed at the concave side of the mirror at an angle greater than 45° and was reflected through the stage and through a petri dish containing colonies growing on the agar surface. When the light and mirror were properly aligned, the colonies did not appear shiny and showed different tints of color.

Colonies that differed in opacity or morphology from the majority of colonies (containing piliated cells) were streaked on minimal agar and examined again to assure that a single morphological type was present.

Type 1 pilus antiserum test. Type 1 pilus antiserum was prepared in rabbits against a purified preparation of the type 1 pili of *E. coli* strain B american (B_{am}) (9). The antiserum was absorbed with concentrated cells of a nonpiliated derivative of the same strain before use. It had an agglutination titer of 2,000 to 8,000 for piliated cells of *E. coli* B_{am} or *E. coli* K-12 strain AW405 but did not agglutinate nonpiliated cells of either strain. Volumes of 0.04 ml of antiserum diluted 1:500 in saline and 0.04 ml of cell suspension were gently mixed on slide agglutination plates for 15 min at room temperature. The plates were covered with glass lids to prevent evaporation. The reactions were read under slight magnification. Controls consisting of 0.04 ml of cells and 0.04 ml of saline were treated similarly.

Chemotatic swarming. One to three plates of soft tryptone agar were inoculated by stabbing the center of the plate with a loopful of an overnight Z medium culture (4). Inoculated plates were placed in single layers of 37°C. The distance that the culture spread through the soft medium was measured from the center of the inoculum to the leading edge of growth. A measurement was first obtained 4 to 5 h after inoculation and again at several intervals after additional incubation. The difference between the first and final readings was recorded.

Stationary culture. Piliated cells of strain AW405 formed pellicles in stationary Z cultures. Therefore, qualitative tests were performed to determine if cells selected by colonial morphology were *pil* mutants or phase variants in a nonpiliated phase. Single colonies of suspected *pil* mutants, or a loop of liquid medium culture of cells that did not react with type 1 pilus antiserum, were inoculated into 10 ml of Z medium contained in test tubes (18 by 150 mm) covered with plastic caps. The tubes were placed on the shelf at 37°C and examined for 5 to 7 days for the presence of a pellicle, which was presumptive evidence of the presence of piliated cells. In some instances, growth from the tops of the tubes was spread on minimal agar, and the resulting colonies were examined for types resembling those containing piliated cells. If such colonies were present, cells from them were grown in minimal liquid medium and tested with type 1 pilus antiserum. Nonpiliated mutants were not expected to form pellicles.

Nonpiliated isolates were examined further for their ability to revert to a Pil^+ form by a more extensive test. Cells were grown overnight in 5 ml of Z medium with shaking at 37°C and then diluted in Z medium to 1 to 6 cells per ml. A 10-ml portion was added to each of five test tubes, and the cultures were placed without shaking at 37°C. At the end of 5 or 7 days, cells from the tops of tubes that had pellicles were spread on minimal agar and incubated for 16 to 18 h at 37°C. Colonies were then examined for differences in morphology, and several were inoculated into minimal liquid medium, shaken overnight at 37°C, and tested with type 1 pilus antiserum to confirm the presence of Pil^+ cells.

Electron microscopy. Formaldehyde was added to logarithmic-phase liquid cultures in a final concentration of 1.2%. The cultures were diluted in growth medium (also containing 1.2% formaldehyde) to 5×10^7 to 10×10^7 cells per ml. Specimens were prepared by agar filtration (23) and shadowed with platinum-carbon, or stained with uranyl acetate. Observations were made with a Philips EM 300 electron microscope operating at 60 kV.

To facilitate the identification of F pili, cells were labeled before formaldehyde treatment with the male-specific phage R-17 at a ratio of phage to bacterium of approximately 200.

Numbers of type 1 piliated and nonpiliated cells were tabulated during observations on the fluorescent screen at magnifications between 5,000 and 12,000. Photographs of cells were taken at random and enlarged to determine the distribution of pili on cells. The number of pili per cell was counted with the aid of a hand magnifying lens.

Isolation of Nal^R derivatives. Modifications of the method of Hane and Wood (21) were used to isolate Nal^R derivatives. A total of 20 or 30 μg of naladixic acid per ml was added to cells of Pil^- mutants grown to the logarithmic growth phase in Z medium. The

cultures were shaken for 4 h or overnight at 37°C. They were spread without dilution after 4 h of incubation or were diluted 1:50 in saline (overnight cultures) and spread on minimal agar containing 20 μg of naladixic acid per ml. Colonies that arose were either purified by restreaking on naladixic acid-minimal agar or added directly to minimal liquid or Z medium and grown overnight with shaking. They were then tested for reaction with type 1 pilus antiserum. Antiserum-negative isolates were retained.

Hemagglutination. The ability of piliated bacteria to agglutinate chicken red blood cells was described previously (7).

RESULTS

Type 1 piliation of the parental strain AW405. Since our goal was the eventual genetic analysis of the processes required for expression of type 1 piliation, we chose an *E. coli* K-12 F^- parental strain carrying multiple chromosomal markers. Strain AW405 was also known to be wild type with respect to chemotaxis and motility (3). Since the type 1 piliation of this strain had not been studied previously, it was first necessary to examine its piliation properties.

Cells of AW405 reacted readily with antiserum that had been prepared against purified type 1 pili from *E. coli* B_{am}. Standing broth cultures of strain AW405 exhibited typical surface pellicle formation after 1 or 2 days of growth.

The presence of type 1 pili on AW405 was confirmed by electron microscope examination (Fig. 1), which additionally revealed that some cells were not piliated. The effects of the type of growth medium and the age of the cells on the number of cells that formed pili in shaken, aerated cultures was studied. Cells previously grown in minimal liquid medium were inoculated into minimal or Z (enriched) medium, grown for 12 h, and then subcultured again as shown in Table 1. The number of piliated cells in the various cultures was determined by electron microscopy. Table 1 shows that more cells formed pili in minimal medium than did those in Z medium regardless of the age of the culture. Twice as many cells formed pili in the early logarithmic-phase Z cultures as did those in older cultures. Conversely, more piliated cells formed in the older minimal medium cul-

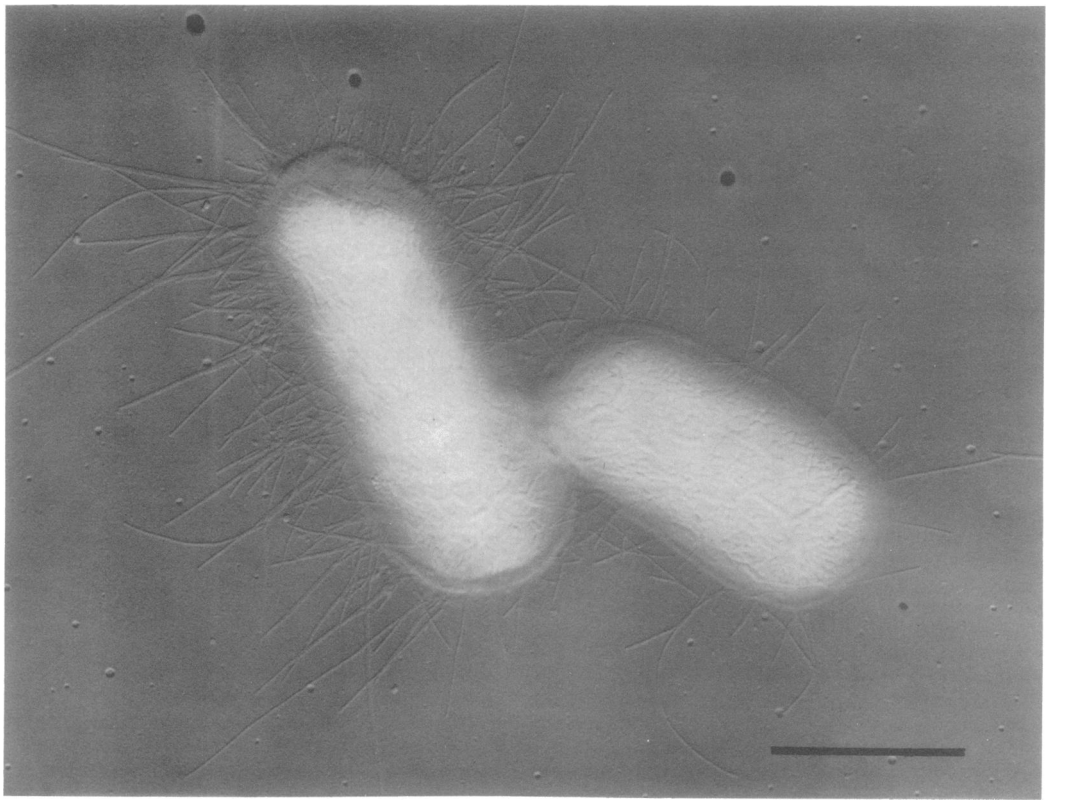

FIG. 1. *Piliated cells of E. coli K-12 AW405 grown in aerated Z broth. Specimen prepared by agar filtration followed by shadowing with platinum and carbon. Bar represents 1 μm.*

ture than those in early to mid-logarithmic-phase cultures. Thus, the expression of piliation and the effect of the age of the cells differed in each medium.

A quantitative difference in the number of pili produced by cells was sought. Cells in the mid-logarithmic phase of their second subculture in Z medium were examined for the number of pili on individual cells. The data of Table 2 indicate that single cells had from 0 to 140 pili per cell, and joined (dividing) cells had from 0 to 180. Free pili were also present in the cell preparations.

Typical colonies of strain AW405 on minimal agar were uniform in size and slightly raised, had even edges, and appeared opaque under the oblique lighting used. We refer to this morphology as S. Occasionally, translucent, flat, striated colonies (R) could be noted among the usual S colonies of AW405. Since we hoped to be able to distinguish Pil$^-$ mutants of strain AW405 on the basis of colonial morphology, these were examined further.

TABLE 1. *Expression of type 1 piliation by AW405 Pil$^+$ in aerated minimal and Z liquid media*

Medium	Inoculum for subcultures[a]	Optical density of subculture examined	Electron microscope observations	
			No. of cells	% Pil$^+$
Minimal	12-h culture		567	90
Minimal		0.09	830	68
Minimal		0.32	783	61
Z	12-h culture		519	30
Z		0.09	296	50
Z		0.64	402	22

[a] Inoculum for 12-h minimal and Z liquid media consisted of cells grown to the mid-logarithmic growth phase in minimal liquid medium.

TABLE 2. *Distribution of type 1 pili on AW405*

Distribution of pili (No. of pili/cell)	No. of isolated single cells	No. of isolated dividing (joined) cells
0	36	22
1 to 10	17	2
11 to 20	1	1
21 to 30	6	1
31 to 40	1	2
41 to 50	2	1
51 to 60	8	0
61 to 70	4	0
71 to 80	14	0
81 to 90	6	8
91 to 100	13	0
101 to 110	6	6
111 to 120	3	2
121 to 130	2	5
131 to 140	2	2
141 to 150	0	1
151 to 160	0	4
161 to 170	0	1
171 to 180	0	3

Characterization of strain AW405 R colonies. Colonies obtained by streaking cells from R-type colonies of AW405 on minimal agar medium were primarily R type, but a few colonies with a different morphology often appeared. These were more compact and even edged and somewhat similar to the S colony of strain AW405. Because they exhibited a more glossy appearance at early stages of colonial development, however, they are termed G colonies.

Repurification of G-type colonies by restreaking on minimal agar resulted in a preponderance of G-type colonies and a few R colonies. Thus, even after several purifications on agar, R colonies could give rise to a few G colonies, and G colonies could always give rise to R colonies.

Under the assumption that these colonial morphologies might be associated with type 1 piliation, cells from both colonial forms were tested for their ability to react with type 1 pilus antiserum. Progeny of R colonies that were grown in broth reacted only faintly, or not at all, with the antiserum; however, progeny from G colonies reacted strongly. Pellicle formation of stationary broth cultures inoculated with cells from either R or G colonies was identical. The surface pellicle in each case formed within 1 to 2 days after inoculation. Thus, R-colony isolates can be considered to consist primarily of cells in a nonpiliated growth phase which can vary to a piliated phase associated with G-colony morphology. Therefore, we term R-colony isolates "phase variants" (for definitions see below).

Chemotaxis of AW405 and its phase variants. Strain AW405 is motile and chemotactic and, consequently, during incubation its cells migrate through soft tryptone agar in an expanding circle from the point of inoculation (2, 3, 4). Phase-variant cells from R and G colonies also were chemotactic, and their swarming was compared to that of AW405 (Table 3). There was no difference in movement between the S and G types of piliated cells. When either an R- or G-phase variant was used in the initial inoculum, a majority of colonies formed by leading edge cells were the Pil$^-$ R type. Cells from the site of inoculation formed mainly the G colonies typical of the Pil$^+$ phase of the variant. If cells were picked from the leading edge of the expanding circle of strain AW405 growth and purified on minimal agar, colonies of the nonpiliated R type were readily observed.

TABLE 3. *Swarming of AW405 Pil+ and its colonial phase variants in soft tryptone agar*

Isolate	Major phase of inoculum	Movement in 2 h (mm)
1. Pil+ wild type	Pil+	3.5
2. R-phase variant of no. 1	Pil-	5.5
3. Pil+ wild type	Pil+	4.5
4. R-phase variant of no. 3	Pil-	6.0
5. Phase variant A; G form	Pil+	3.5
6. Phase variant A; R form	Pil-	6.5
7. Phase variant B; G form	Pil+	4.5
8. Phase variant B; R form	Pil-	6.5

Isolation of Pil- mutants of AW405. In an effort to find Pil- mutants of strain AW405, colonies formed on minimal agar plates after mutagenesis were examined for aberrant morphology. To distinguish mutants from the phenotypically Pil- phase variants, the following protocol was used. Strain AW405 was treated with one of a variety of mutagenesis procedures and spread on minimal agar. Colonies were then chosen on the basis of morphology and repurified on minimal agar by streaking. An isolated colony was picked and grown overnight, with shaking, in Z broth and tested with type 1 pilus antiserum. If the antiserum test was negative, cells were inoculated into Z medium and grown in still culture at 37°C to encourage the development of a pellicle. Isolates that formed pellicles within 3 days were discarded under the assumption that they were either phase variants or mutants with high reversion rates. Cultures that did not form pellicles or that formed pellicles only after extended incubation were retained, assigned PL1000 series strain numbers, and tested further. Many of the mutants were examined in the electron microscope for confirmation of the absence of both free and attached pili.

Initially, colonies that resembled the R type associated with the Pil- phase variants were sought and tested. Usually, such isolates resulted in cultures that formed pellicles readily and were, therefore, phase variants of the R type described previously. Four isolates (PL1001, PL1003, PL1014, and PL1016), which produced translucent colonies with striations similar to the R colonies, did not form pellicles. At the same time, colonies exhibiting other morphological characteristics distinguishable from wild type were also purified and tested. This led to the discovery of a new colony type, ST (slightly transparent), associated with lack of type 1 piliation. ST colonies differed from the typical strain AW405 S type by a very slight change in opacity. The distinguishing transparency difference was evident only when young colonies (16 to 18 h of incubation) were examined. As the eyes of the investigators became experienced at detecting this subtle morphological property, Pil- isolates were selected with greater ease. Seventy-five mutants were obtained for study. In addition, one such colony, chosen on the basis of this morphology, proved to be a phase variant. This isolate, PL1008, produced pellicles in stationary culture and gave rise to occasional colonies of the G type containing piliated cells. Approximately one G colony per 500 ST colonies was observed. Since this appeared to be a new type of phase variant, in terms of colony morphology, it was also retained for further study. The properties and relationships among piliated and nonpiliated derivatives of strain AW405 are summarized in Table 4.

Pil- mutants were obtained from strain AW405 after all of the mutagenesis procedures were employed. The isolation of Pil- derivatives from nonmutagenized AW405 was also attempted in three different experiments. Four additional mutants were isolated among several thousands of colonies on minimal agar by choosing colonies that differed from the parental S type by the variation in opacity described above.

Characterization of Nal^R derivatives. In preparation for the genetic analysis of our mutants described in the accompanying paper, a nalidixic acid-resistant derivative of each of the Pil- isolates was selected. These were assigned corresponding strain numbers in a series starting with PL2000. Thus, the Nal^R derivative of isolate PL1001 is called PL2001 and so on. Subsequent characterization of the Pil- strains was performed on these derivatives to insure that the Pil- phenotype was unaffected by the addition of a drug resistance marker.

All Nal^R derivatives were examined in the electron microscope and determined to be free from either loose or attached type 1 pili. Mutant PL2003 produced minicells, as did its parent, PL1003.

To obtain some measure of the stability of the mutants, five cultures of each strain were inoculated with 1 to 6 cells per ml and allowed to grow without aeration for 5 to 7 days. This was considered to be sufficient time to select for even a low frequency of revertants in the population. All five cultures of the phase variant PL2008, as expected, formed pellicles within 2 days. In contrast, as can be seen in Table 5, Pil- mutants ordinarily did not form surface pellicles. Twenty-four of the mutants gave rise to

pellicles in a minority of the trials. Clearly then, although many of the mutants appear to be revertable, all of them are considerably more stable than the phase variants.

Chemotactic swarming of Pil⁻ mutants. The chemotactic swarming ability of Nal^S Pil⁻ mutants and their Nal^R derivatives was similar to that of strain AW405 and to each other (Table 6). Twenty other Nal^R Pil⁻ isolates were also tested; eighteen were chemotactic and two (PL2003 and PL2015) appeared to have lost motility since they did not move through the agar. Thus, in contrast with the results obtained with phase variants, it appears that there is no correlation between piliation and chemotactic swarming. It would seem then that presence or absence of piliation, by itself, has no affect on swarming.

Hemagglutination of AW405. Well-piliated cultures of strain AW405 did not agglutinate chicken red blood cells. Under the same conditions, *E. coli* $B_{am}P^+$ showed strong hemagglutination (7). When cultures of AW405 were concentrated approximately 10-fold by centrifugation and resuspended, weak hemagglutination was observed.

DISCUSSION

The classification of bacterial pili is currently being reinvestigated and revised (C. C. Brinton, Jr., unpublished data) because properties such as morphology, function, or host strain are not good primary criteria for determining evolutionary relationships among pili. Pili of different morphologies, serological specificities, and phage specificities can have similar functions, as, for example, conjugal pili of the F (13) and I (25) types. Pili on different hosts can be identical in structure and serologically related as well, as in the pili of *E. coli* and *E. coli–Salmonella typhimurium* diploids (11). Additional criteria such as serological specificity, amino acid sequence of pilus subunits, and genetic relatedness will have to be used to ade-

TABLE 4. *Summary of the properties and relationships among piliated and nonpiliated derivatives of E. coli K-12 AW405*

Designation	Phenotype[a]	Colony appearance[b] refracted light	Progeny colonies on minimal agar	Source of derivatives	Behavior in still liquid culture	Chemotactic swarming in soft tryptone agar
Parent: stably piliated	Pil⁺	S ("smooth"): uniform in size; slightly raised; even edges; opaque	S colonies: rare R or ST colony	Parent strain from J. Adler; revertants of *pil* mutants to Pil⁺ in still cultures	Forms pellicles after 1 or 2 days in a single tube test	Same rate as parent
pil Mutant	Pil⁻	ST ("slightly transparent"): uniform in size; slightly raised; even edges; slightly less opaque than S colonies	ST colonies	Parent strain before or after mutagenesis	May form pellicle in 5-7 days in 1 to 2 replicates out of 5; may not form a pellicle in 7 days in any of 5 replicates	Same rate as parent
Phase variant, P⁻ phase	Pil⁻	R ("rough"): larger than S, ST, or G; translucent; flat; striated	R colonies mixed with 2% or less G colonies	Spontaneously from parent strain; advancing front of chemotaxis medium; G colonies derived from R	Forms pellicles in 1 or 2 days in a single tube test	Faster rate than parent
Phase variant, P⁺ phase	Pil⁺	G ("glossy"): uniform in size; more compact than S colonies; even edges; smaller than S colonies; glossy surface	G colonies mixed with 5–20% R colonies	R-phase variant; center of inoculation of R colony on chemotaxis medium	Forms pellicles in 1 or 2 days in a single tube test	Same rate as parent

[a] Phenotype is designated as Pil and genotype as *pil* following recommendations of Demerec et al. (16).
[b] Minimal agar.

TABLE 5. *Mutagenic origin and revertibility of Pil⁻ mutants of AW405*

Mutant strain no.	Mutagen used[a]	Revertibility[b]	Mutant strain No.	Mutagen used[a]	Revertibility[b]	Mutant strain no.	Mutagen used[a]	Revertibility[b]
PL2001	NTG	0/5	PL2032	5-BU	2/5	PL2062	HNO_2	0/5
PL2003	NTG	0/5	PL2033	HNO_2	0/5	PL2063	HNO_2	2/5
PL2004	5-BU	2/5	PL2034	HNO_2	0/5	PL2064	EMS	1/5
PL2005	5-BU	1/5	PL2035	HNO_2	1/5	PL2065	EMS	1/5
PL2006	5-BU	1/5	PL2040	HNO_2	2/5	PL2066	EMS	0/5
PL2007	5-BU	0/5	PL2043	2-AP	0/5	PL2068	EMS	0/5
PL2008	5-BU	5/5	PL2044	2-AP	3/5	PL2069	EMS	0/5
PL2012	NTG	2/5	PL2045	2-AP	0/5	PL2070	EMS	0/5
PL2014	NTG	0/5	PL2046	2-AP	0/5	PL2071	EMS	0/5
PL2015	5-BU	0/5	PL2047	2-AP	2/5	PL2072	EMS	0/5
PL2016	NTG	0/5	PL2048	2-AP	2/5	PL2073	EMS	0/5
PL2017	NTG	0/5	PL2049	2-AP	0/5	PL2074	2-AP	0/5
PL2019	5-BU	1/5	PL2052	HNO_2	1/5	PL2075	2-AP	2/5
PL2020	5-BU	1/5	PL2053	2-AP	0/5	PL2076	2-AP	0/5
PL2021	5-BU	0/5	PL2054	2-AP	0/5	PL2077	2-AP	1/5
PL2022	5-BU	0/5	PL2055	2-AP	1/5	PL2078	2-AP	0/5
PL2024	HNO_2	1/5	PL2056	2-AP	1/5	PL2079	2-AP	0/5
PL2025	HNO_2	0/5	PL2057	2-AP	0/5	PL2080	2-AP	1/5
PL2027	5-BU	0/5	PL2058	HNO_2	1/5	PL2081	2-AP	0/5
PL2029	5-BU	2/5	PL2059	HNO_2	0/5	PL2082	2-AP	0/5
PL2030	5-BU	0/5	PL2060	HNO_2	0/5	PL2083	2-AP	0/5
PL2031	5-BU	2/5	PL2061	HNO_2	1/5	PL2084	2-AP	0/5

[a] NTG, nitrosoguanidine; 5-BU, 5-bromouracil; HNO_2, nitrous acid; 2-AP, 2-aminopurine; EMS, ethyl methane sulfonate.

[b] Number of standing cultures out of five from which piliated cells were recovered from the surface 5 to 7 days after inoculation (see text).

TABLE 6. *Swarming of Nal^S and Nal^R Pil⁻ mutants of AW405 in soft tryptone agar*[a]

Isolate	Movement in 4 h (mm)
Expt 1	
Nal^S Pil$^+$ control	7.5
Nal^S Pil$^-$ mutant	
PL1001	6.5
PL1004	7.9
PL1006	8.7
PL1007	7.8
PL1014	7.5
PL1017	8.6
PL1019	8.3
PL1025	7.5
Expt 2	
Nal^S Pil$^+$ control	10.3
Nal^R Pil$^+$ control	8.9
Nal^R Pil$^-$ mutant	
PL2001	5.7
PL2004	7.5
PL2006	7.2
PL2007	6.8
PL2014	8.7
PL2017	7.9
PL2019	8.7
PL2025	7.9

[a] Nal^S and Nal^R Pil$^-$ mutants were tested on different days. Control Pil$^+$ strains were included in each experiment.

quately define the precise relationships among different pili.

E. coli K-12 AW405, chosen as the parent strain in these studies, is a well-piliated strain when grown under controlled conditions. A disadvantage of using strain AW405 for pilus studies is that it has a long history of laboratory passage and mutagenization. Thus, AW405 may lack some of the functions associated with the type 1 piliation of strains more recently isolated from clinical sources.

Strain AW405 pili are closely related serologically to the prototype type 1 pili of *E. coli* B strains previously studied, so new information may be integrated with previous knowledge. The pili of *E. coli* $B_{am}P^+$ hemagglutinate (7), but AW405 pili do not hemagglutinate and thus appear to have lost an adhesive property. It is tempting to infer that type 1 pili on the originally isolated ancestor of AW405 were organelles of adhesion and, perhaps, virulence. We consider strain AW405 and $B_{am}P^+$ to belong to the type 1 pilus family, but their exact relationships to each other and to other *E. coli* somatic pili are not yet clear.

One property of piliated cells, which we have confirmed for strain AW405 and used extensively as a criterion for stability of nonpiliated isolates, is their selective survival and formation of pellicles in unshaken liquid media, as first reported by Duguid and his colleagues (17,

18). Gemski and Brinton (Gemski, Ph.D. thesis) studied this phenomenon further and found that a more rapid relative growth rate of piliated-phase cells versus nonpiliated-phase cells at the low oxygen tensions prevailing in unshaken media appeared to be mainly responsible for the rapid outgrowth of piliated cells. Results obtained in the present study indicate that pili may be directly responsible for the increased relative growth rate of piliated cells at low oxygen tension, since reversion of *pil* mutants was also manifested by the outgrowth of piliated cells in stationary cultures. Pellicle formation is probably a secondary effect of outgrowth due to the strong tendency of pili to adhere to each other resulting in autoagglutination of piliated cells at high cell densities.

An important contribution of this paper was the development of methods to distinguish between mutants and phase variants that affect piliation. Phase variation is a regulatory phenomenon controlling the phenotypic expression of piliation and, as elucidated by this study (see below), other cell properties as well. Phase variants can exist in either a piliated (P^+) or a nonpiliated (P^-) phase. Each phenotypic state is metastable and can be maintained by cloning. Each phase can arise from the other spontaneously and at random at a rate per cell generation which can be different in each direction. The rates of phase change are subject to the environmental influences of temperature, medium, and growth conditions as in liquid versus solid medium. The rates of phase change are, in general, much higher than the rates of mutation. A phase variant might lose by mutation the ability to undergo phase variation and thereby become stably piliated or stably nonpiliated. Strain AW405 may be a stably piliated mutant of a phase variant since phase variants can be readily isolated from it. We have found that phase variants are the rule rather than the exception for strains of *E. coli* as well as for other bacteria freshly isolated from clinical or natural sources.

pil mutants, on the other hand, are the result of mutational alterations in the structure of genes controlling pilus production. These genes may be structural or regulatory. In AW405, they are clustered at a locus near 98 min on the *E. coli* K-12 chromosome (5) as defined by complementation and mapping studies (14, 26). *pil* mutants, since they are not subject to environmental influences, can be studied by the usual genetic techniques of recombination and complementation, whereas phase variation must be characterized by cloning and special selective techniques.

Our results suggest that phase variation may be indicative of a general form of cell differentiation in which several properties change. One of these is chemotactic swarming. The strong selection for P^- phase cells over P^+ phase cells in soft tryptone agar and the greater absolute chemotactic swarming rate of P^- phase cells compared to P^+ phase cells could be interpreted in two ways. Either the presence of pili retards swarming, or the P^+ phase is retarded for some other reason. Pili per se cannot be the retarding factors since *pil* mutants have the same chemotactic swarming rate as the strain AW405 parent (Table 6). We therefore conclude that other changes in the cell controlled by the same regulatory systems are responsible for the different chemotactic swarming rates of the two phases.

A second property change associated with variation in phase is an alteration in colony morphology. We confirm here that differences in colonial form between the piliated and nonpiliated phases exist as first observed by Brinton (7, 12). An important distinction, however, was found in the magnitude of the difference in colonial form between P^+ and P^- phase variants of AW405 compared to the difference in colony type between strain AW405 and its *pil* mutants. A very subtle difference in the opacity of *pil* mutant colonies compared with parent colonies was seen compared with a much larger difference between the colonies of the P^+ and P^- phases of phase variants. Therefore, the change from S to R cannot be ascribed entirely to the presence of pili, which suggests that phase variation controls both piliation and other surface properties affecting colonial form. To summarize, phase variation in AW405 appeared to coordinately control piliation, colonial form, and chemotactic swarming rate. On the other hand, mutation of *pil* genes leading to total lack of piliation had a very minor effect on colonial form and no effect on chemotactic swarming.

Whereas phase variation determines the production or nonproduction of pili, additional regulatory mechanisms appear to determine the number of attached pili per cell (Tables 1 and 2). Environmental factors such as temperature, medium, and the stage of the growth cycle can affect this number. It is not understood at present how this kind of regulation is effected, and it may involve several mechanisms. To distinguish this kind of regulation from phase variation we shall call it "quantitative regulation" of piliation. The number of attached pili per cell depends upon a balance between their rate of outgrowth and their rate of release. Any factor that changes this balance can change the num-

ber of pili. Quantitative regulation and phase variation can combine to determine the extent and distribution of piliation of a given clone or culture.

Thus, piliation of bacteria can be lost or gained by at least four known reversible mechanisms: phase variation, plasmid loss or gain, *pil* gene mutation or reversion, and quantitative regulation. It is not always easy to distinguish among them when piliation is the only marker. Our results emphasize that great caution is necessary when ascribing functions to pili. Whereas loss of a function associated with a shift from the P^+ to the P^- phase is acceptable presumptive evidence that the function requires pili, it is not conclusive proof. Loss of the function by *pil* mutants should also be demonstrated, if possible, by using a mutant in the structural gene for the pilus rod subunit itself. In addition, assays to determine extent of piliation should take into account both phase variation and quantitative regulation and be carried out under the same or similar conditions in which the functional test is performed; otherwise, one cannot know whether the tested cells are piliated or not since strains assumed to be stably piliated or nonpiliated may not remain that way under the conditions prevailing when function is measured.

A reversible loss of piliation by phase change can easily be mistaken for loss by mutation or plasmid segregation. Under environmental conditions that favor the nonpiliated phase, such as nonselective growth on solid medium, the nonpiliated state can appear to be genetically stable. For instance, an early report by Brinton et al. (14) suggested that type 1 pilus genes might be plasmid determined because of the high rate of change to the nonpiliated state and its apparent irreversibility. It was subsequently shown that this change was reversible by using techniques such as still culture or counter-current distribution selective for piliated cells.

Evans et al. (19), in a study of the somatic pili of human enteropathogenic strain *E. coli* H10407, compared the piliated parent strain with a nonpiliated "laboratory passed" derivative which they described as a mutant, the "P" strain. They reported that the P strain was less pathogenic for infant rabbits than for the parent and that it had lost a plasmid possessed by the parent strain. They claimed these results in support of the hypothesis that the pili of *E. coli* H10407 are plasmid-determined virulence factors. We have extensively characterized the somatic pili of *E. coli* H10407 and have shown them to be members of the chromosomally determined type 1 pilus family along with the pili of *E. coli* $B_{am}P^+$ and *E. coli* AW405. We have found *E. coli* H10407 to be a reversible phase variant analogous to variants of AW405 described in this report. A direct comparison of our strain of H10407, the Evans strain of H10407, and the Evans P strain of H10407 show all three to be identical with respect to piliation and phase variation. It is therefore most likely that the P strain as characterized by Evans et al. is the nonpiliated phase of the parent strain. The conditions under which Evans et al. tested the piliation of their strains were quite different from the in vivo conditions in which they tested virulence. In vivo conditions strongly favor the piliated phase, and it is likely that the P strain was piliated in vivo. Thus, their results give no information confirming or denying a role of the pili of *E. coli* H10407 in virulence, however likely this may be by analogy with other systems.

The colonial form which we found to be associated with our strain AW405 *pil* mutants was also found to be associated with stable loss of piliation in another *E. coli* K-12 strain and, therefore, could be expected to serve as an effective screening procedure for other *pil* mutants. The mutants we isolated were characterized further and shown to be stable and amenable to complementation and recombinational analysis (26).

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