JOURNAL OF BACTERIOLOGY, Aug. 1984, p. 736–744
0021-9193/84/080736-09$02.00/0

Vol. 159, No. 2

Organization and Expression of Genes Responsible for Type 1 Piliation in *Escherichia coli*

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Received 27 February 1984/Accepted 15 May 1984

The genetic organization of a segment of recombinant DNA conferring the capacity of synthesize *E. coli* type 1 pili was examined. This 11.2-kilobase (kb) segment of DNA, derived from a clinical isolate, conferred a piliated phenotype (Pil$^+$) on a nonpiliated (Pil$^-$) strain of *E. coli* K-12 that lacked DNA homologous to the 11.2-kb region. Insertional mutagenesis, deletion mutagenesis, and subcloning of various regions of the 11.2-kb fragment allowed the localization of five genes, each encoding a polypeptide, that were associated with pilus expression. Three gene products, 17, 86, and 30 kilodaltons (kd) in size, were involved in pilus assembly; assembly of the 17-kd structural (pilin) protein into pili was not seen in mutants lacking either the 86- or 30-kd proteins, but pilin synthesis and proteolytic processing were not affected. The fourth polypeptide, 23 kd in size, appeared to be involved in the regulation of pilus expression because mutants lacking this protein exhibited a 40-fold increase in the amount of pilin antigen per cell. The last protein, 14 kd in size, was not associated with piliation by genetic criteria; however, the 14-kd protein was immunoprecipitated with pili, suggesting an association with pili or immunological cross-reactivity with pilin. Immunoprecipitates of minicell transcription translation products revealed that pilus polymerization was taking place in minicells. This may facilitate the study of the molecular steps in pilus biosynthesis and, as a consequence, provide clues to the assembly of supramolecular structures in general.

Type 1 pili (fimbriae) of *Escherichia coli* and other gram-negative enteric microorganisms are filamentous, proteinaceous appendages, ca. 7 nm wide and 2 μm long (8), and are composed of a single repeating polypeptide subunit, pilin (8). Type 1 pili confer adhesive properties on bacterial cells, mediating binding to a variety of erythrocytes (14, 15, 18, 23, 48, 53) and other eucaryotic cells (21, 34, 42, 44, 46, 54, 57) via an unidentified receptor on the eucaryotic cell surface. The interaction between type 1 pili and the eucaryotic cell is inhibited by mannose, alpha-linked mannose oligosaccharides, or certain mannose analogs (47), indicating that the receptor contains mannose or a compound of similar structure.

The specificity of pili binding to eucaryotic cells of the genitourinary tract has led to speculation that type 1 pili of *E. coli* (20, 44, 57) and similar appendages of other enteric bacteria (1, 22, 48) are involved in establishing infectious foci in extraintestinal infectious disease (5, 34, 42, 44, 45, 55). In support of this notion, two reports have shown a correlation between the ability of *E. coli* to colonize the lower urinary tract of experimentally infected mice and the presence of type 1 pili (26, 29). However, since most (>70%) *E. coli* strains have type 1 pili (13, 16), these structures evidently do not, by themselves, confer virulence, and their role as an adherence factor in the pathogenesis of infectious disease is uncertain. Nonetheless, since the adhesive properties of the majority of gram-negative enteric bacteria are conferred by a class of structures analogous or related to type 1 pili (43), these organelles provide an ideal model for the study of microbial adherence. Also, these structures present a useful model for examining the genetic control, synthesis, processing, and export of proteins (7, 9, 14, 17–19) and the assembly of supramolecular structures.

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In this report we describe the genetic organization of a fragment of recombinant DNA from a clinical isolate of *E. coli* encoding polypeptides required for type 1 pilus expression (Pil). Also, we discuss possible functions of the gene products of the Pil region.

MATERIALS AND METHODS

Bacterial strains and media. An *E. coli* K-12 derivative, P678-54 (2), was employed as the host strain for plasmids encoding the production of type 1 pili. This strain also served as the source of minicells used to assess the protein products produced by the Pil region. Additional properties of strain P678-54 and descriptions of other strains used for plasmid isolation and in vivo mutagenesis are discussed below.

Unless otherwise noted, bacteria were grown in L broth or L agar (37). Antibiotic concentrations for selection and routine maintenance of plasmid-containing strains were as follows (per ml): chloramphenicol, 20 μg; tetracycline, 20 μg; ampicillin, 100 μg; kanamycin, 40 μg; and spectinomycin, 50 μg.

Bacterial plasmids and cloning vectors. The plasmid pSH2 contains an 11.2-kilobase (kb) chromosomal DNA fragment of the *E. coli* clinical isolate J96 inserted into the vector pACYC184 (10). The isolation and initial characterization of the plasmid has been described previously (26, 27). Subsequent manipulations of pSH2 are described below. In addition to pACYC184, the vectors pBR322 (6) and pRN2010 (received from Walter Dallas, Burroughs Wellcome Foundation) were used in this study. The vector pRN2010 is a derivative of pBR322 that contains a gene encoding spectinomycin resistance in place of the gene encoding beta-lactamase.

Plasmid isolation and transformation. Plasmid DNA was prepared by the method of Birnboim and Doly (4). In some cases plasmid DNA was also purified by CsCl density gradient centrifugation by the method of Guerry et al. (25).

Transformation, using plasmid DNA, was carried out according to the method of Lederberg and Cohen (33).

Restriction endonuclease cleavage of plasmid DNA and agarose gel electrophoresis. Restriction endonucleases were purchased from commercial sources and stored at −20°C. The enzyme reaction mixtures for cleaving DNA were those suggested by Davis et al. (12). Cleaved DNA fragments were analyzed by agarose gel electrophoresis (36). Restriction endonuclease-generated DNA fragments were subcloned, where necessary, after excision from agarose gels, electroelution, and ligation to the appropriate vector following previously published procedures (12).

Insertional mutagenesis of pSH2 by using the transposon Tn*5*. λ::Tn*5* (λ *c*I857 *b*221 *rex*::Tn*5* *O*am3 *P*am80) (52) was used to deliver Tn*5* into *E. coli* K-12 HB101 (*hsdM hsdR*) (12) containing pSH2 by using the methods described by Kehoe et al. (31). Insertion of Tn*5* into pSH2 was detected by combining ca. 10,000 kanamycin-resistant colonies, each colony representing an independent Tn*5* transposition event, and extracting the plasmid DNA. This DNA was then employed to transform *E. coli* LE392 ($hsdM^+$ $hsdR^-$) (56) with selection for kanamycin resistance (Tn*5*) and chloramphenicol (pSH2). Plasmid DNA from these strains was extracted and used to transform strain P678-54 ($hsdM^+$ $hsdR^+$). Approximately 300 kanamycin- and chloramphenicol-resistant colonies isolated in this manner were screened for the Pil^+ or Pil^- phenotype (the screening procedure is described below).

Isolation and radiolabeling of minicells. Minicells from strain P678-54 were isolated after 12 h of growth at 37°C in L broth containing the appropriate antibiotics. Parental cells were separated from minicells by successive differential and sucrose density centrifugation according to the methods described by Gill et al. (24). Minicells were labeled with [^{35}S]cysteine, [^{35}S]methionine, or [^{3}H]leucine for various phases of this study. The labeling medium contained minimal salts (37) supplemented with 0.004% thymine and 0.02% threonine and leucine and contained 1.0% cysteine assay medium, methionine assay medium, or leucine assay medium (Difco Laboratories), depending on the isotope employed in a given experiment. The radiolabeled amino acids were purchased from Amersham Corp., and ca. 60 μCi per reaction mixture was used to label the minicells. Minicell labeling conditions were those described previously (24).

Immunoprecipitation of radiolabeled plasmid-encoded proteins from minicells. Radiolabeled minicells from 100 to 400 ml of original culture were suspended in 10 μl of 2% sodium dodecyl sulfate (SDS)–0.02 M Tris-hydrochloride (pH 7.5) and placed for 3 to 5 min in a boiling water bath. The lysed minicell solution was then diluted to a final volume of 100 μl with Rose buffer (50) (Rose buffer is 1% Nonidet P-40 [Sigma Chemical Co.], 1% sodium deoxycholate, 0.15 M NaCl, 0.01 M Tris-hydrochloride [pH 7.5], and 1% Trasylol [Sigma]). One microliter of anti-type 1 pilus antiserum (from Gary Schoolnik, Stanford University) was added, and the mixture was held at room temperature for 30 min. Approximately 30 μl of protein A-Sepharose beads in Rose buffer plus 0.1% SDS was added. The Sepharose was kept suspended by gentle agitation on a rotary shaker for 1 to 3 h at room temperature. The beads were then collected by brief centrifugation, followed by several washes with Rose buffer plus 0.1% SDS. The final wash was in distilled water. Pili bound to the beads were released by boiling for 5 min in a solution of 2% SDS adjusted to pH 1.8 with HCl. This treatment not only released any bound pili from the beads but also dissociated the pili into pilin subunits. Alternatively, pili were released by boiling the beads in the final sample buffer (32) used for polyacrylamide gel electrophoresis (see below). This latter treatment did not dissociate the pili into pilin subunits.

Determination of precursor forms of Pil-specific polypeptides. Precursor forms of proteins encoded by the Pil region were monitored after radiolabeling of minicells in the presence of 8% ethanol by the methods described by Palva et al. (49).

Polyacrylamide gel electrophoresis and autoradiography. Proteins labeled in minicells were separated by discontinuous polyacrylamide gel electrophoresis with the buffer system described by Laemmli (32). Samples of the lysed, radiolabeled minicells were precipitated in acetone (24) before resuspension and boiling for 3 min in final sample buffer (32).

Immunoprecipitates were not acetone precipitated but were added directly to the wells of a slab gel containing 15% acrylamide (an acrylamide-to-bisacrylamide ratio of 60:1). Electrophoresis was accomplished by applying a constant current of 15 mA across the 1.5-mm thick gel for 8 to 10 h or until the bromophenol blue tracking dye reached the bottom of the gel.

The radiolabeled proteins on the gels were examined by autoradiography. Autoradiography was accomplished by impregnating the gel with the scintillant En^{3}Hance (New England Nuclear Corp.) as directed by the manufacturer, followed by drying and exposure against a sheet of X-Omat R film (Eastman Kodak Co.) at −70°C. Typically, 10^5 cpm of radioactivity was applied to each lane, and an exposure of from 1 to 2 weeks was sufficient to see the labeled bands. Before impregnation with the scintillant, gels were stained with Coomassie brilliant blue to determine the location of molecular-weight-marker proteins (Bio-Rad Laboratories).

Assays for piliation. Cells expressing type 1 pili were detected by using a combination of methods, including bacterial agglutination, hemagglutination of guinea pig erythrocytes, and an enzyme-linked immunoadsorbent assay.

Antibody to purified type 1 pili was obtained from rabbits immunized with purified pili from *E. coli* K-12 strain HB101 (12) harboring pSH2 by G. Schoolnik. The antiserum had a bacterial agglutination titer of >1:30,000.

Bacterial agglutinations were usually performed in microtiter trays. Each well contained 60 μl of an overnight broth culture of the cells to be tested together with 60 μl of a 1:160 dilution of antiserum in phosphate-buffered saline. In cases where significant differences in growth rate between strains to be tested were observed, cultures were diluted to a constant optical density of 1.5 (600 nm) with fresh L broth. For rapid tests on isolated colonies, bacterial slide agglutinations were performed by using a small amount of growth from a culture plate and a drop of diluted antiserum. The suspension was mixed with a sterile toothpick, and agglutination was judged relative to control mixtures containing the parental (Pil^+) strain and an identical strain containing just the cloning vector.

Guinea pig erythrocytes were obtained by cardiac puncture. The erythrocytes were washed and stored in Alsevier solution (2.1% glucose, 0.42% NaCl, 0.8% trisodium citrate, 0.06% citric acid) at 4°C. Packed erythrocytes were suspended in a volume of phosphate-buffered saline sufficient to give a 2% final erythrocyte concentration in phosphate-buffered saline. Hemagglutination was tested with microtiter wells by using equal volumes of an overnight broth culture of test bacteria and the erythrocytes.

The inhibitory effects of mannose on the hemagglutination of erythrocytes was monitored by performing the hemagglu-

tination reactions in the presence of alpha-methyl-mannoside (final concentration, 50 mM; Sigma).

Competitive enzyme-linked immunoadsorbent assay measurements were carried out as described by Normark et al. (41), except that overnight broth cultures were tested rather than plate-grown cells. Overnight broth cultures contained 50 mM alpha-methyl-mannoside.

RESULTS

Hull et al. (27) previously reported the isolation of a chromosomal DNA fragment associated with type 1 piliation from the *E. coli* clinical isolate J96. This large DNA fragment (40 kb), when inserted into the cosmid vector pHC79, was found to contain sufficient genetic information to permit the synthesis of type 1 pili by a nonpiliated host bacterium. Subsequent reisolation of restriction endonuclease fragments into the vector pACYC184 (10) led to the isolation of pSH2, which contained an 11.2-kb DNA fragment of the original 40-kb cosmid and conferred piliation (Pil^+) upon the Pil^- *E. coli* K-12 strain P678-54 (2). Using the 11.2-kb region as a probe, we showed that there was no DNA homologus to the 11.2-kb region in strain P678-54 as measured by colony-blot hybridization tests (40), even at reduced hybridization stringency. In contrast, Pil^+ strains of *E. coli* K-12 and *E. coli* Bam (8) showed homology to the 11.2-kb fragment at moderate hybridization stringency (data not shown). Since no homologous DNA was present in P678-54, we used this strain as a host for pSH2 and performed a genetic and biochemical analysis of the piliation conferred by this plasmid.

Pili synthesis in *E. coli* minicells. Immunoprecipitation with antipili antiserum of the radiolabeled polypeptides encoded by the 11.2-kb region in minicells revealed a prominent 17-kilodalton (17-kd) polypeptide (Fig. 1). This polypeptide comigrated with pili purified from strain P678-54 containing the pSH2 plasmid during SDS-polyacrylamide gel electrophoresis. In addition, purified pili competitively inhibited immunoprecipitation of the 17-kd polypeptide (data not shown). Also, the 17-kd polypeptide was not radiolabeled with [^{35}S]methionine; this indicated its similarity to type 1 pili isolated from *E. coli*, which are known to be devoid of methionine (8, 53).

Immunoprecipitates from radiolabeled minicells were also found to contain high-molecular-weight material that was resolved as a "ladder" on SDS-polyacrylamide gels (Fig. 1), provided that samples of the minicell translation products were processed under standard conditions for electrophoresis (32). As was the case with the 17-kd polypeptide, the ladder of bands was not radiolabeled with [^{35}S]methionine, and the ladder was not seen if purified, unlabeled, pili were added before immunoprecipitation (data not shown). However, if the immunoprecipitates were boiled at pH 1.8 before electrophoresis (a condition that dissociates pili into pilin subunits) (35), only a single band, migrating at 17 kd, was seen; the high-molecular-weight ladder was not seen (Fig. 1). The only other band present after this treatment was just above the 17-kd polypeptide band (Fig. 1, lane C) and probably represents a partially reduced form of the 17-kd polypeptide since this band was not always seen.

These experiments indicate that in minicells a pilin monomer was synthesized and was assembled into a higher-molecular-weight form (the ladder) which displayed the dissociation characteristics of purified pili. We tentatively conclude that the ladder represents polymerized pilin and that each "rung" of the ladder represents a pilus fragment plus or minus one pilin subunit.

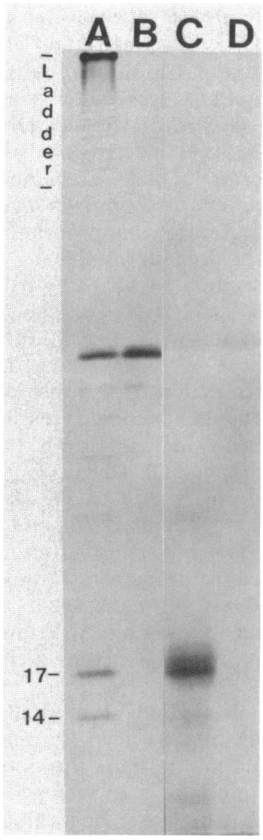

FIG. 1. Immunoprecipitates of the protein products of pORN104 and the vector pRN2010. The plasmid pORN104 contains the 11.2-kb region from pSH2 cloned into the vector pRN2010 (see the legend to Fig. 2C for a further description of the plasmids). Minicells containing pORN104 and minicells containing pRN2010 were pulse-labeled with [^{35}S]cysteine, and the products were immunoprecipitated with antibody raised against purified type 1 pili. The immunoprecipitates were then either directly separated by SDS-gel electrophoresis or boiled for 5 min at pH 1.8 to dissociate pili into pilin subunits and then separated. An autoradiogram of the gel is shown. Lane A, immunoprecipitated products of pORN104; lane B, immunoprecipitated products of pRN2010; lane C, immunoprecipitated products of pORN104 after boiling at pH 1.8; lane D, immunoprecipitated products of pRN2010 after boiling at pH 1.8. The ladder of bands, denoted on the left, is described in the text. Also denoted on the left are the molecular sizes in kd.

In addition to the 17-kd polypeptide, a 14-kd polypeptide was also immunoprecipitated (Fig. 1). This 14-kd polypeptide did not appear to be a major constituent of pili because dissociation of the higher-molecular-weight polymers did not yield a significant amount of the 14-kd polypeptide (Fig. 1). Also, the 14-kd polypeptide was radiolabeled with [^{35}S]methionine, and the ladder of bands showed no labeling with this compound (data not shown). A vector-encoded polypeptide, ca. 50 kd in size, was also seen on immunoprecipitation (Fig. 1). This polypeptide represents a prominent product of the cloning vector and was probably precipitated nonspecifically by antipilus antiserum.

Identification and localization of additional gene products of the 11.2-kb region involved in pili expression. To better define the region required for piliation, Tn*5* insertion mutations were introduced at random into pSH2 (52). In addition, we isolated a series of deletion derivatives and also prepared subclones of several regions of the 11.2-kb insert of pSH2.

Deletion mutations were obtained by taking advantage of Tn*5* insertion mutations that inactivated the gene encoding a 23-kd protein and conferred a hyperpiliated phenotype (Pil^{++}) upon the host strain (see below). Hyperpiliated mutants displayed a strikingly different colonial morphology from that produced by strains harboring the parental plasmid. Hyperpiliation was unstable, and hyperpiliated mutants frequently gave rise to progeny having the parental colonial morphology. In all cases examined, these revertants had accrued additional mutations in the Pil region that effectively lowered piliation. Thus, we were able to use the hyperpiliated phenotype as a genetic and physical tool to isolate other mutations affecting piliation in pSH2. In this instance, we took advantage of Pil^{++} revertants that had deletions of various segments of the Pil region. (A more detailed account of the isolation and analysis of hyperpiliated mutants is provided elsewhere [P. E. Orndorff and S. Falkow, submitted for publication].)

Subcloning of segments of the Pil region was accomplished by restriction endonuclease digestion and religation to an appropriate vector. To obtain specific regions of Pil, we often took advantage of restriction endonuclease sites within a nearby Tn*5* insertion (30).

The various Tn*5* insertion mutants, deletion derivatives, and subclones of pSH2 were introduced into strain P678-54, and their translation products were examined in minicells. These data are summarized in Fig. 2.

The 11.2-kb DNA fragment directed the synthesis of seven identifiable polypeptides when pORN103 was analyzed in minicells (Fig. 3A). Two of the polypeptides, denoted by arrows (Fig. 3A), were not required for piliation and could be eliminated either by Tn*5* insertion mutagenesis or by sub-

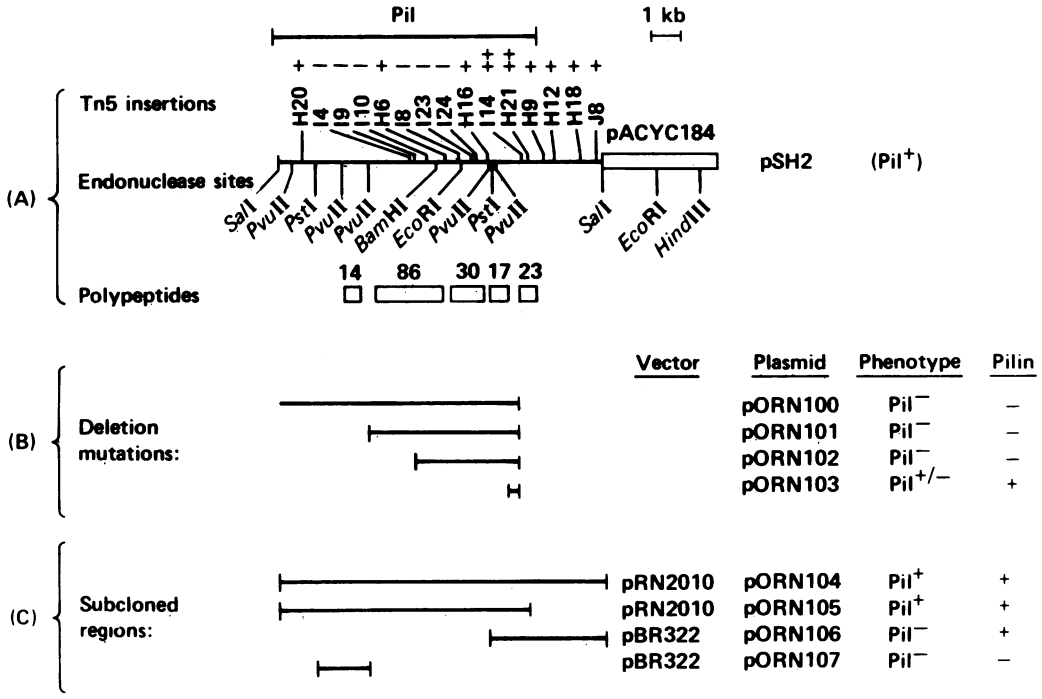

FIG. 2. (A) Denoted are (i) the positions of Tn*5* insertions into pSH2, (ii) the positions of endonuclease sites in pSH2 (adjacent sites of less than 200 base pairs are not listed), and (iii) the positions of the stuctural genes for the 86-, 30-, 23-, 17-, and 14-kd polypeptides. The positions of Tn*5* insertions were determined by *SalI-EcoRI* double digestions of pSH2::Tn*5*. Each insertion is denoted by a letter and a number. The designations above each insertion denote a Pil^+ (+), Pil^- (−), or a hyperpiliated (++) phenotype. The positions of the structural genes for the proteins were determined by the positions of Tn*5* insertions which eliminated expression of those proteins in minicells. Additional methods of localizing the genes for the proteins are described in the text. (B) Deletion mutations in pSH2 (the bars indicate the regions deleted). Spontaneous deletion mutations were discovered in plasmid pSH2 that contained Tn*5* insertions which inactivated the gene for the 23-kd polypeptide (e.g., H21 or I14). These insertions conferred a hyperpiliated phenotype (Pil^{++}). Pil^- revertants frequently had deletions of the Pil region of varying lengths starting a few hundred base pairs to the right of the *PvuII* site (to the right of the gene for the 17-kd polypeptide) and extending leftward. The length of the deletions was determined by using restriction endonuclease sites in pSH2 and in Tn*5* insertion H21. The designations of plasmids containing these deletion mutations, the phenotype conferred by the deletion mutations in strain P678-54, and the presence or absence of pilin in immunoprecipitates of the protein products in minicells are designated on the right. Pil^- denotes a completely nonpiliated phenotype, showing neither bacterial agglutination in the presence of antibody raised against purified pili nor hemagglutination of guinea pig erythrocytes. $Pil^{+/-}$ denotes a very low level of piliation (very weak agglutination of guinea pig erythrocytes). A plus (+) in the column denoting the presence of pilin indicates the presence of the processed form of pilin, as determined by the presence of a radiolabeled band migrating at 17 kd on polyacrylamide gels after labeling of minicells with [^{35}S]cysteine and immunoprecipitation. (C) Subclones of pSH2. Subcloned regions are designated by bars. To the right are listed the vectors to which the regions were joined, the phenotypes of the subclones in strain P678-54, and the presence or absence of pilin in immunoprecipitates. Plasmid pORN104 contained the entire 11.2-kb insert of pSH2 and was joined to pRN2010 at the *SalI* endonuclease site (inactivating the tetracycline transacetylase gene). Plasmid pORN105 contained the region starting in Tn*5* insertion H21 (the *HindIII* site in IS*50*) and extending to the *SalI* site in pSH2. Plasmid pORN106 contained the region starting from the *HindIII* site in IS*50* of insertion H16 and extended to the *SalI* site in pSH2. Plasmid pORN107 contained the region starting at the *PstI* site in IS*50* of the Tn*5* insertion H21 in pORN101 and the *PstI* site in pORN101 (i.e., this region represents the region just to the left of the deletion in pORN101).

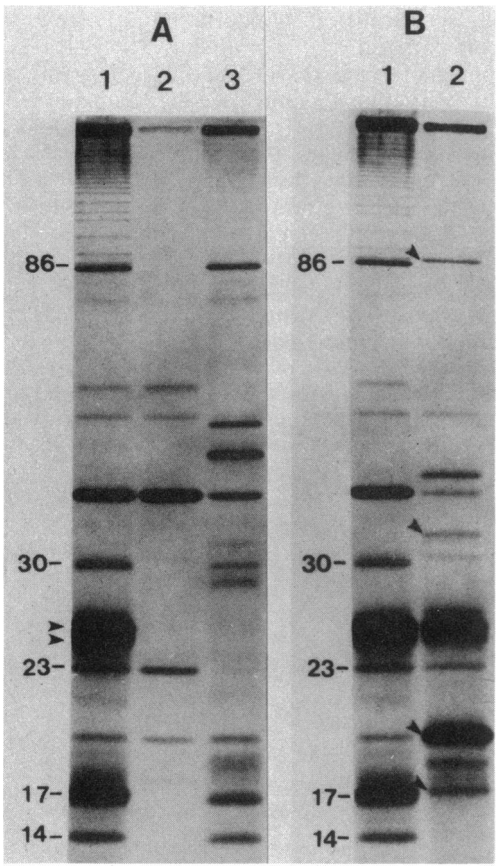

FIG. 3. (A) Protein products of pORN104, pORN105, and the vector pRN2010 (see the legend to Fig. 2C for a description of the plasmids). Minicells containing the plasmids were pulse-labeled with [^{35}S]cysteine, and the radiolabeled products were separated by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the gel is shown. Lane 1, products of pORN104; lane 2, products of pRN2010; lane 3, products of pORN105. The arrows indicate two proteins not involved in piliation. The column on the left denotes the molecular sizes in kd. (B) Precursor forms of proteins encoded by pORN104. Minicells containing pORN104 were pulse-labeled with [^{35}S]cysteine in the presence or absence of 8% ethanol, and the products were separated by SDS-polyacrylamide gel electrophoresis. An autoradiogram is shown. Lane 1, radiolabeled products of pORN104; lane 2, radiolabeled products of pORN104 labeled in the presence of 8% ethanol. The arrows denote bands in lane 2 that are the presumed precursor forms of the protein bands which migrated at 86, 30, 17 and 14 kd in lane 1. The column on the left indicates the molecular sizes in kd.

cloning without noticeable effect on the Pil$^+$ phenotype (Fig. 3A, lane 3). The remaining five polypeptides (14, 86, 30, 17, and 23 kd) were associated with piliation. One of the polypeptides (23 kd) migrated at the same position as a vector-encoded protein. However, the vector-encoded protein was not synthesized in the recombinant plasmid. As evidence of this, the polypeptide products of pORN104 (Fig. 3A) are shown. Plasmid pORN104 is a subclone made from insertion mutant H21 (Fig. 2A) and lacks the Pil-specific 23-kd polypeptide. (Transcription and translation of pSH2 containing insertion mutation H21 in minicells had shown the 23-kd protein to be absent [data not shown].) The pORN104 plasmid was constructed by taking advantage of the *Hin*dIII endonuclease site in IS*50* of Tn*5* insertion H21 and the *Sal*I site in pSH2. The transcription-translation products of this plasmid show no band at 23 kd (Fig. 3A). However, this subclone does show two additional polypeptides migrating at ca. 60 kd. These are probably polypeptide products produced from the transcription start signals in IS*50* (28, 51).

The tentative intracellular location of the Pil-specific proteins was determined by examining the various proteins for precursor (higher-molecular-weight) forms of the various proteins. A precursor form suggests the presence of a signal sequence involved in protein translocation (49). The 86-, 30-, 14-, and 17-kd polypeptides were synthesized in precursor form in the presence of 8% ethanol (Fig. 3B), indicating that these polypeptides were probably envelope proteins (49). Each precursor denoted in Fig. 3B appeared to have an increase in molecular weight corresponding to the addition of 20 to 25 amino acids. Identification of the precursor bands of the 14- and 17-kd proteins comes from differential labeling experiments with [^{35}S]methionine and [^{35}S]cysteine (data not shown). Precursors for the 30- and 86-kd proteins were inferred on the basis of proximity and the corresponding band intensity of the denoted precursors to the mature forms. As discussed below, at least two of these proteins (86 and 30 kd) appear to be involved in pilus assembly.

Representative evidence supporting the location of each of the genes is shown in Fig. 4. Fig. 4A shows Tn*5* insertions I10 and I24, which eliminated the 86- and 30-kd polypeptides, respectively. Fig. 4B shows immunoprecipitates of the transcription-translation products of subclones pORN105, pORN106, and pORN107, which determine the location of the genes encoding the 14- and 17-kd proteins. (To avoid possible confusion as to whether proteins migrating at 17 and 14 kd were indeed the products of the genes of interest, we took advantage of the fact that both proteins were precipitable by antipilus antiserum; see Fig. 1). The location of the 23-kd protein was, as stated earlier, inferred from Tn*5* insertion mutation H21 (see discussion of Fig. 3A).

Possible functions of the 86-, 30-, and 23-kd accessory proteins. Tn*5* insertions that eliminated the 30- and 86-kd proteins resulted in a Pil$^-$ phenotype (i.e., they showed no hemagglutinating activity, nor did they agglutinate in antipilus antiserum) and showed no polymerization of pilin into higher-molecular-weight forms in minicells (Fig. 4A). However, pilin was still synthesized and was present in its mature (processed) form, as evidenced by the presence of labeled pilin antigen migrating at 17 kd when minicells containing Tn*5* insertion mutants I10 or I24 were examined (Fig. 5). We conclude from this that the 86- and 30-kd proteins are involved in pilus assembly because their absence did not appear to interfere with pilin synthesis or processing, yet no pilin polymerization was observed.

Insertions eliminating the 23-kd polypeptide were not associated with a Pil$^-$ phenotype. Rather, such mutants exhibited a unique small, compact colony morphology, and the cells from such colonies clumped spontaneously when grown in static broth culture. These cells possessed ca. 40-fold more pilin antigen per cell than the parental strains harboring pSH2 (Table 1). Mutants lacking the 23-kd protein due to Tn*5* insertion showed a high reversion rate (as indicated by a change back to normal colony morphology). Most of the revertants were Pil$^-$, and all revertants had accrued additional mutations in the Pil region. As noted earlier, some of these revertants contained extensive deletions of Pil DNA.

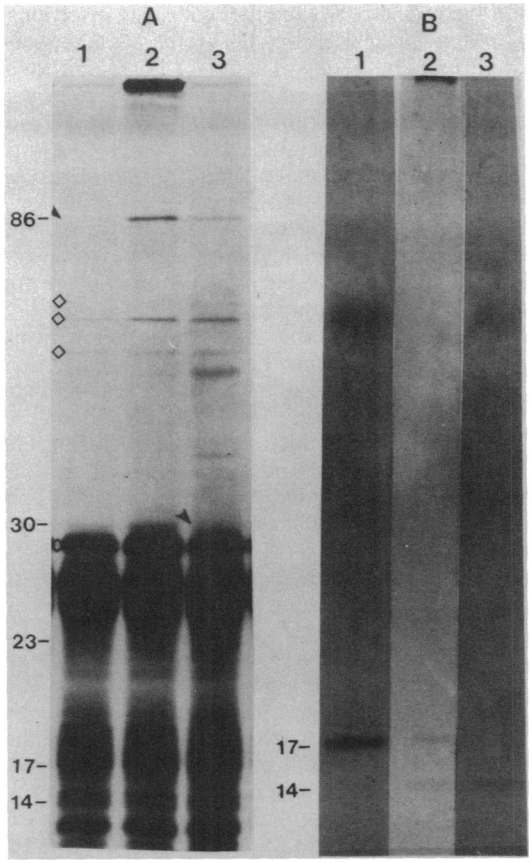

FIG. 4. Location of the 86-, 30-, 17-, and 14-kd proteins. (A) Minicells from pSH2::Tn*5* insertion mutants containing insertions I10 and I24 (see Fig. 2A for the location of the insertion mutations) were isolated and pulse-labeled as described in the text, and the [^{35}S]cysteine-labeled proteins were separated by SDS-gel electrophoresis. An autoradiogram is shown. Lane 1, products of insertion mutant I10; lane 2, the product of insertion mutants H6; lane 3, products of insertion mutant I24. The arrows denote protein bands that are absent in I10 or I24 when compared with H6 (insertion mutant H6 is Pil$^+$ and is shown here as a control). The diamonds denote Tn*5*-encoded proteins. The left-hand column denotes the molecular sizes in kd. (B) Minicells containing plasmids pORN105, pORN106, and pORN107 were radiolabeled with [^{35}S]cysteine as described in the text. The protein products were immunoprecipitated and separated by SDS-gel electrophoresis as described in the text. Lane 1, products of pORN106; lane 2, products of pORN105; lane 3, product of pORN107. The column on the left denotes the molecular sizes in kd.

DISCUSSION

We have presented evidence which indicates that the plasmid pSH2 contains the structural (pilin) gene and additional genetic information for the assembly and expression of type 1 pili. The plasmid confers piliation on a host strain lacking DNA homologus to pSH2, and, when sequestered in minicells, the plasmid has sufficient genetic information to direct the assembly of pilin subunits into higher-molecular-weight forms that have properties identical to polymerized type 1 pili.

We found that there were at least four genes, encoding the 86-, 30-, 17-, and 23-kd polypeptides, involved in pilus expression. The 17-kd protein was found to be the structural gene for pilin (i.e., it encoded the antigenic component of pili); we have recently succeeded in isolating specific mutations in this gene and have obtained the nucleotide sequence, confirming that it indeed encodes the pilin subunit (P. E. Orndorff and S. Falkow, manuscript in preparation). Tn*5* insertion mutations that abrogated expression of piliation were found to be associated with the loss of either the 86- or the 30-kd proteins. Since pilin was still synthesized in these cells, it seems likely that these proteins are not involved in the transcriptional control of pilin biosynthesis. Also, the pilin monomers evidently reached a processing site within the cell envelope of such strains, since no precursor form of pilin was detected. Consequently, we believe that the 86- and 30-kd proteins are involved in some aspect of pilus assembly. In line with this idea was the observation that the ladder of polymerized pili disappeared when either of these proteins was eliminated by mutation. Elimination of the 23-kd protein by Tn*5* mutagenesis produced what we term a hyperpiliated (Pil^{++}) phenotype. These mutants had a distinctive colonial morphology and possessed ca. 40-fold more

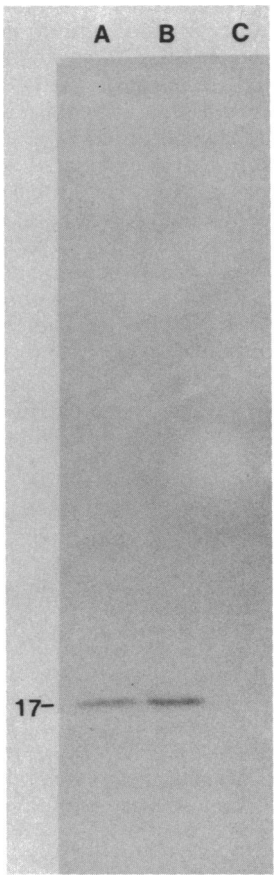

FIG. 5. Immunoprecipitation of pilin from Tn*5* insertion mutants containing insertions I10 and I24 (see Fig. 2A for positions of the insertions). Minicells containing plasmids having Tn*5* insertion mutations I10 and I24 were labeled with [^{35}S]cysteine as described in the text. The products were immunoprecipitated with antibody raised against purified pili, and the immunoprecipitate was separated by SDS-gel electrophoresis. An autoradiogram is shown. Lane A, products of insertion mutant I10; lane B, products of insertion mutant I24; lane C, products of the vector pACYC184. The column on the left denotes the molecular sizes in kd.

TABLE 1. Effect of Tn5 insertion mutation H21 on the amount of pilin antigen per cell[a]

Plasmid[b]	Amt of pilin antigen[c]
pACYC184	0
pSH2	7.5
pSH2::Tn5 (H21)	300.0

[a] Strain P678-54 was used as the host strain for pACYC184, pSH2, and pSH2::Tn5 (H21). Cultures in logarithmic growth were diluted in L broth to the same optical density at 540 nm and six serial 10-fold dilutions were made in L broth. Competitive enzyme-linked immunoadsorbent assays were performed in triplicate on each dilution by using hyperimmune antiserum to purified pili as outlined in the text.

[b] Plasmid denotes the plasmid that was resident in strain P678-54. The position of insertion mutation H21 is shown in Fig. 2A.

[c] The values for pilin antigen were taken from the bacterial dilution which produced 50% inhibition of the antibody binding to the solid (antigenic) phase. The reciprocal values are shown. Thus, higher values represent an increased amount of pilin antigen per cell. The values are measured against strain P678-54 containing pACYC184 and consequently represent the relative differences in piliation between strains containing pSH2 and the insertion mutant (H21).

pilin antigen per cell than the same host strain harboring the parental plasmid. This indicates to us that the 23-kd protein (i) was not required for piliation and (ii) may be involved in suppression of pilin synthesis or expression. (We provide a much more detailed examination of the role of the 23-kd protein in piliation in a separate publication [Orndorff and Falkow, submitted for publication].) One other gene, encoding a 14-kd polypeptide, was associated with the Pil region. This polypeptide was an envelope protein and was immunoprecipitated by antipilus antiserum. The most likely cause for the immunoprecipitation of the 14-kd protein is that the 14-kd protein showed some structural similarity with pilin. However, we cannot rule out the possibility that the 14-kd protein was copurified with pili in undetectable, albeit sufficient, amounts to provoke an immune response, thus eliciting antibodies against both the 17- and 14-kd proteins. Unfortunately, we were unable to specifically eliminate the production of the 14-kd polypeptide and, as a consequence, cannot be sure of the involvement of this protein in piliation. However, considering the properties of the 14-kd protein and the proximity of the gene for this protein to other genes involved in piliation, it would seem likely that this protein is somehow involved in piliation.

Tn5 mutagenesis revealed the presumptive location of the structural genes for the 86-, 30-, and 23-kd proteins, and the evidence from deletion mutations supported the location and relative order of the genes. As a technical note, the expression of Pil-encoded polypeptides in minicells was poor when the vector pACYC184 was employed. This problem was never satisfactorily solved, and one consequence was the relatively poor resolution of the polypeptide products shown in Fig. 4A. The problems of poor resolution were only solved by subcloning the Pil region onto pRN2010. Subsequent subcloning of various regions of Pil onto this plasmid confirmed the location of the 14-, 86-, 17-, and 23-kd proteins. However, substantiating evidence for the gene encoding the 30-kd protein was not sought, and its location is based upon Tn5 insertion and spontaneous deletion data generated by using the pACYC184 vector.

We have assumed that there are at least three transcriptional units in the Pil region because Tn5 insertions H6 and H16 retain a piliated (Pil^+) phenotype and are flanked by insertions producing a nonpiliated (Pil^-) or a hyperpiliated (Pil^{++}) phenotype (Fig. 2A). This indicates to us that genes lying in the region between innocuous Tn5 insertions constitute a transcriptional group. Using the same reasoning, we can state that the genes of the 11.2-kb region involved in piliation constitute an autonomous transcriptional group because Tn5 insertions on either end of the Pil region (e.g., H20 or J8) have no detectable effect on pilus biosynthesis. If transcription were dependent upon promoter sequences in the vector DNA, we would expect at least one or both of these insertions to abrogate piliation. However, since Tn5 insertion does not invariably exert a polar effect on downstream transcription (3), our conclusions on the transcriptional units remain tentative.

The organization of the type 1 Pil region bears some resemblance to the region encoding K88 pilus production (31, 39). Similar numbers of accessory genes are required for pili synthesis in both cases. Mooi et al. (38) have described a model for K88 pilus production in which 17-, 27-, and 81-kd proteins were involved in pilus assembly. Interestingly, the genes for those proteins were clustered together in the K88 Pil region, much as the genes for the 14-, 30-, and 86-kd proteins were clustered in the type 1 Pil region (however, not in the same order). From our experiments we surmised that the 30- and 86-kd proteins were involved in pilus assembly, and it is possible that the 14-kd protein is involved as well. Also, both sets of proteins from the K88 region and the Pil region appear to be envelope proteins and have species of similar molecular weights. We have not yet examined the possibility that DNA homology exists between the genes involved in the production of these two pilus types. There does not appear to be a gene product in the K88 region analogous to the 23-kd protein of the type 1 Pil region (i.e., there are no mutations in the K88 region reported to yield hyperpiliated mutants) (31, 39). This may reflect a fundamental difference in the strategies for controlling piliation.

In addition to the genes for type 1 pili, the genetic information for pyelonephritis-associated pili (Pap) has been isolated from *E. coli* J96 (27), and the organization of that region has been described by Normark et al. (41). When compared to the K88 and type 1 regions, the Pap region has little similarity in organization; it has many more accessory proteins. However, there does exist a large (81-kd) protein required for piliation (41). Also, another pilus operon from a uropathogenic *E. coli* strain has a similar large (71-kd) protein associated with piliation (11). These large proteins might serve as "anchors" for the growing pilus, as suggested by Mooi et al. (38). If this is true, it would suggest that there are certain similarities in the cellular strategy for pilus construction.

ACKNOWLEDGMENTS

We thank G. Schoolnik and D. Lark for providing purified type 1 pili, antiserum, and assistance with the competitive enzyme-linked immunoadsorbent assay. Also, we thank W. Dallas for providing the vector pRN2010.

This work was supported by grant PCM-8306654 from the National Science Foundation.

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