ANALYSIS OF PILIN POLYMERIZATION IN A UROPATHOGENIC \textit{ESCHERICHIA COLI} STRAIN CONTAINING TWO PYELONEPHRITIS-ASSOCIATED (PAP) GENE CLUSTERS

by

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This thesis has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

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To the Graduate Council of The University of Utah:

I have read the thesis of Diana Gaudin Wilkins in its final form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the Supervisory Committee and is ready for submission to the Graduate School.

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ABSTRACT

_E. coli_ strain C1212 expresses two distinct pili on its cell surface that are encoded by separate pyelonephritis-associated pili (_pap_) gene clusters. These gene clusters have been demonstrated to produce expression of pili composed of either 17 kilodalton (kd) or 21 kd pilin monomers. The potential for copolymerization of the two pilin monomers, resulting in production of hybrid pili, was examined here.

Pilin structural genes (A-B) and assembly genes (C-G) from each cluster were isolated and ligated to pBR325 and pACYC184 plasmid vectors, respectively. These vectors, harboring specific _pap_ genes, were utilized to produce organisms carrying a set of pilin structural genes from one _pap_ gene cluster and a set of assembly genes from the other (heterologous) _pap_ gene cluster. Pilin monomers expressed by these organisms were examined by immunoelectron microscopy with 6nm and 18nm-diameter gold particles conjugated to a _pap_ monoclonal antibody. Individual pilin monomers were also harvested and purified from overnight cultures of uropathogenic _E. coli_ expressing either pilin-17 or pilin-21. Copolymerization of the two monomers was examined (in vitro) through specific iodination of pilin monomers, incubation and polymerization of monomer mixtures with 0.1M MgCl₂, column chromatography of mixtures on Sepharose CL-6B and Sephadex G-100, and immunoblots of pilin-17 and pilin-21 immunoprecipitates. Data gathered from complementation analysis of heterologous gene clusters and immunoprecipitates suggest that in vitro copolymerization of pilin monomers and hybrid pilin production can occur.
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INTRODUCTION

Specific Aims

The major goal of the proposed study was to further the understanding of the molecular nature of pilin production in uropathogenic *E. coli*. This study had the following specific aims:

a) to complete a complementation analysis of the pilin and assembly regions of the *pap*17 and *pap*21 gene clusters in *E. coli* strain DH5α and

b) to determine whether *pap*-encoded heterologous pili monomers can copolymerize to form hybrid pili.

Significance

Although urinary tract infections are caused by many species of microorganisms, most are due to *E. coli*. However, only a few serogroups (01, O2, O4, O7, O75) cause a high proportion of the infections. Uropathogenic *E. coli* can be differentiated from normal *E. coli* by the presence of virulence factors. Virulence factors enhance both colonization and invasion of the urinary tract and the capacity to produce disease. In particular, uropathogenic *E. coli* express specific adhesins that enable them to reach and colonize the urinary tract. Attachment of microorganisms to uroepithelial cells is an important initial event in establishing infection.

*E. coli* that cause urinary tract infection and acute pyelonephritis often express adhesins that give rise to mannose resistant hemagglutination (MRHA) and cause attachment to uroepithelial cells. In at least three studies, 75%-85% of strains isolated from females with pyelonephritis were capable of agglutinating human red blood cells in the presence of mannose. The majority of these strains produced adhesins that are specific for P-blood group substance and are termed P-adhesins. The receptor structure for this adhesin has been identified as $\alpha$-D- $\beta$Galp-$\alpha$-D- $\beta$Galp present in the globoseries of glycolipids.

MR hemagglutination is believed to result from the interaction of this glycolipid and the bacterial adhesin encoded by the pyelonephritis-associated-pili (*pap*) gene cluster. Analysis of polypeptides expressed by this gene cluster led to the detection of a number of distinct genes. These are shown in Figure 1.
Most adhesins in gram negative bacteria have been associated with the production of pili. It was at first considered that the pilin protein subunit carried the information for receptor-binding specificity. However, it was later shown that the major pilin subunit of pap pili (papA) was not required for the expression of the adhesin. The ability of pili to bind to the surface of uroepithelial cells was associated with three other pap proteins—papE, papF, papG—that are encoded within the same gene cluster as papA. This conclusion was reached through the observation that a nonpolar mutation in the papA gene prevented formation of pap pili but did not affect the amount of glycolipid specific hemagglutination. The papF and papG gene products are required for agglutination by both whole bacterial cells and purified pilin preparations. PapE has been postulated to play a role in anchoring the adhesin, since papE mutants have lost their ability to bind purified pili while whole cells retain it. More recent evidence indicates that the papH gene has a role in anchoring the pilus to the cell and in modulating pilus length. Little conclusive information is available concerning the role of papI while papD is believed to function to localize and stabilize pilin subunits on the cell surface during assembly. The role of papB has not clearly been established; however, it appears to be involved with regulation of the gene cluster since Tn5 insertions within the gene cause reduced expression of pap pili. Additionally, a cyclic AMP (cAMP) binding site has been found within the cluster, thus indicating that pap is under cAMP-CRP control.

Recent evidence indicates that a single uropathogenic E. coli can contain two complete pap gene clusters. Each of these clusters will produce antigenically distinct pili and P-adhesin activity. In particular, E. coli strain C1212 expresses two distinct pili, composed of two distinct pilin protein monomers. (These are referred to as pilin-17 and pilin-21 with respect to their molecular weights.) When incubated with an appropriate FITC-labeled antibody, 5% of a single colony of C1212 produced pili composed of pilin-17 while 84% produced pili composed of pilin-21.
Double-labeling experiments with antibody showed that coexpression of pili17 and pili21 does occur at low levels (0.3%) and may be regulated by an unknown mechanism.

The differential expression of antigenically distinct pili by uropathogenic *E. coli* may contribute to their virulence potential. Since *E. coli* strain C1212 may at times contain two *pap* gene clusters, it is important from an regulatory standpoint to determine if *pap*-encoded pilin monomers can copolymerize to form hybrid pilin structures in those cells. If hybrid pili are produced, the assembly machinery of one *pap* gene must be able to recognize and assemble heterologous pilin monomers into a complete pilus. This may, perhaps, give rise to the expression of a new and unique antigenic determinant on the cell surface of the invading microorganism. Therefore, an understanding of the complex mechanisms by which pili are assembled and polymerized is necessary to our progress in both prevention and treatment of urinary tract infection.
MATERIALS AND METHODS

**Bacterial Strains and Plasmid Constructions**

*E. coli* strain HB101 (F-, hsdR, hsdM*, recA13, supE44, lacZ4, leuB6, proA2, thi-1, pap*) and DH5α (F-, endA1, hsdR, supE44, recA1, thi-1, pap*) were obtained for construction of appropriate clones. Strains of HB101 harboring the 21 pap gene cluster and the 17 pap gene cluster were also obtained. These are referred to as pDAL201B and pDAL210B, respectively, as shown in Figure 2. pDAL210B and pDAL201B were cut with appropriate endonuclease restriction enzymes to result in fragments containing pilin structural genes or assembly genes. Briefly, the 11.7 kb

![Restriction map of pilin-21 and pilin-17.](image)
plasmid pDAL201B was partially digested with restriction endonuclease \textit{Cla}. Resulting fragments were then digested to completion with \textit{Sal} and \textit{EcoR1}, creating fragments (A-B) and (C-G), respectively. After isolation on 0.7% Low Melting Temperature (LMT) agarose, pilin subunit genes (A-B) were ligated to \textit{EcoR1/Cla1} digested plasmid vector pBR325. Assembly regions (C-G) were ligated to \textit{Cla/Sal}-digested plasmid vector pACYC184. The 17.2 kb plasmid pDAL210B was completely digested with restriction endonuclease \textit{BamH1/Cla1} creating pilin structural subunit genes (A-B) and assembly genes (C-G). Fragments were isolated on 0.7% LMT agarose and ligated to \textit{BamH1/Cla1}-digested plasmid vectors pACYC184 and pBR325. Mini gels were run to verify that all ligations were successful.

\textit{E. coli} strain DH5\textalpha was transformed by a calcium chloride\textsuperscript{7} procedure with each vector/insert and plated to appropriate antibiotic media. Plasmid constructs are characterized in Table 1. Strain HB101 was not chosen as a host strain, as expression of pilin subunits appears to be greatly reduced in recombinant strains. In addition, DH5\textalpha transforms more efficiently than HB101 to result in an average of > 1 x 10\textsuperscript{8} transformants per \mu g DNA.

Four overnight cultures of \textit{E. coli} strain DH5 were then prepared and each transformed with two cleavage fragments: 1) pilin 17/assembly 17, 2) pilin 17/ assembly 21, 3) pilin 21/ assembly 21, 4) pilin 21/ assembly 17.

\textbf{Agglutination Methods}

Presence of adhesin and globoside-binding specificity was determined by positive slide agglutination of P-erythrocytes containing the globoside receptor. Bacteria from agar plates were diluted to 0.5 McFarland Standard with PBS and a single drop placed on an etched-agglutination slide. A drop of a 5% suspension of human red blood cells (P\textsuperscript{+}) in PBS was then added to the slide and rocked gently, on ice, for 15 sec. Presence of adhesin was confirmed by agglutination of the human red blood cells.

\textbf{Immunoelectron Microscopy}

Polyclonal antisera generated to the 17kd and 21 kd pilin monomers were used to examine production of pilin subunits. Anti-\textit{pap17} and anti-\textit{pap21} were labeled with different sizes of
colloidal gold particles, 18nm and 6nm, respectively. The two sizes of gold particles are easily differentiated under the electron microscope. Briefly, gold particles are made by boiling chloroaauric acid with sodium citrate. The size of the resulting gold particles is determined by the ratio of acid to citrate. Gold particles are then coated with Staphylococcal Protein A to form a complex and incubated with appropriate antisera. After incubation of the Gold-Staph A- pap Antibody complex on formvar coated copper grids, the grids are negatively stained with 1% phosphotungstic acid and examined under the electron microscope. All specimens were examined with a Siemens Elmiskop 1A transmission electron microscope (Siemens Corp., Berlin, West Germany). Photographs were processed on Polycontrast Rapid II RC Paper (Kodak).

**Immunofluorescent Microscopy**

Glass slides were precoated with poly-L-lysine by incubation at room temperature for 5 min followed by thorough rinsing with distilled water. Single colonies from "double" transformants in strain DH5α were suspended in PBS (pH 7.4), placed on glass slides and air dried. Dried slides were formalin fixed (4% in PBS) and rinsed again with PBS. Antiseras diluted 1:200-1:500 in BLOTTO buffer was added for 2h at 37°C in a moist petri dish. The slides were washed with PBS, air dried, and analyzed using a Zeiss fluorescent microscope.

**Construction of Clones for In Vivo Copolymerization Analysis**

pDAL 210B containing the complete 17 pap gene cluster was digested with Sma 1, Kpn 1, and Cla 1 to obtain a 1.6kb fragment containing the pilin-17 genes (A-B). Plasmid vector pSKS106 was digested to completion with EcoR1/BamH1. Fragments were isolated on 0.7% LMT Agarose gel, removed from the gel, and ligated with T4 DNA ligase according to the following protocol: Gel slices containing the relevant DNA fragments are melted at 70°C for 15 min and then combined in appropriate proportions to give a final volume of 10μl. After equilibration of the molten gel slices to 37°C, 10μl of ice-cold, 2X concentrated buffer containing T4 DNA ligase is added, mixed quickly, and incubated at 15°C for 24 h. To introduce ligated products into E. coli MC1061 cells, the gel
containing the reaction mixture was remelted at 70°C and diluted by a factor of 10 into ice-cold TCM (10mM Tris, pH 7.5, 10mM MgCl₂, 10mM CaCl₂) prior to carrying out the standard calcium chloride transformation procedure.

*E. coli* strain MC1061 (araD139, ΔlacX74, galU*, galK*, hsr-, hsm+, strA) was transformed by the calcium chloride transformation procedure with the plasmid vector pPY1025, which carried lacI (a lac super-repressor). Spectinomycin resistant colonies were picked, grown overnight at 37°C, and transformed with the previously prepared ligation mixture. Amp<sup>r</sup>/Spec<sup>r</sup> colonies were transferred to fresh Mac/IPTG/Amp/Spec media with nitrocellulose paper and grown overnight at 37°C. Plates were examined for the presence of white (lac-) colonies indicating potential *pap* pilin-17 gene inserts. White colonies were picked and plasmids harvested by the Birney Plasmid Protocol. Plasmid preparations were run on 0.7% agarose gels to determine presence and size of DNA inserts.

**Pili Purification**

21kd and 17kd pili from HB101 clones were purified from organisms grown on Luria Bertani (LB) agar for 24h. at 37°C by a modification of the method of Brinton. The organisms were harvested into ice-cold 0.05M Tris-HCl buffer, pH 8.3 (T buffer) and homogenized at 8,000 rpm for 15 min at 4°C in a Sorvall Omnimixer. The sheared bacteria were removed by centrifugation at 12,000 x g for 30 min. Pili were precipitated overnight at 4°C in 0.05M Tris-HCl buffer, pH 7.0, containing 0.15M NaCl, by the addition of MgCl₂ to 0.1M (TSM buffer). Pili were collected by centrifugation at 12,000 x g for 45 min and the pellet resuspended in T buffer. Insoluble contaminants were precipitated for 12 h at 4°C and removed by centrifugation at 12,000 x g for 60 min. Pili were then reprecipitated from the supernatant in TSM buffer. After three successive cycles of solubilization and precipitation by exposure to T buffer and TSM buffer, respectively, pili were analyzed by SDS-PAGE to determine purity of preparation. Purified pili preparations were quantitated by the BCA Protein Assay™ (Pierce Chemicals, IL). As determined by this assay, pilin-17 preparations contain 15 mg/ml of protein and pilin-21 preparations contain 40 mg/ml.

**Iodination of Pilin Subunits**

Pilin-17 and pilin-21 monomers were iodinated by the *Iodobeads*™ (Pierce Chemicals, IL) protocol. An aliquot (10 μl) of 125I-labeled (5.0 x 10⁷ cpm/μl) was added to 30 μl of Tris-HCl (1M
stock, pH 8.0). One *iodobead™* was added to the buffered solution and allowed to stand for 5 min at room temperature. A total of 10 µl of purified 17 or 21 monomers (150µg and 440µg, respectively) was added to the reaction mixture, and iodination was allowed to proceed for 15 min. Termination of iodination was accomplished by separation of the reaction volume from the *iodobeads™* with sterile plastic pipettes into a sterile Eppendorf™ tube.

To estimate the efficiency of ¹²⁵I-incorporation, TCA precipitation of protein was performed. A total of 1 µl of labeled preparation, 100 µl of 0.1% BSA, and 1 ml of 10% TCA was added to a 1.5 ml Eppendorf™ tube and incubated on ice for 20 min. The mixture was then filtered over a glass filter, rinsed twice with 10% TCA and once with 95% ethanol. The filter was removed and counted on a Beckman Gamma Counter to obtain cpm/preparation. As a control, iodinated preparations without exposure to TCA were filtered and counted. The percent iodination yield was calculated to be approximately 26%, with a ratio of molecules of iodine/molecules of pilin of 0.3.

Verification of specific iodination of 17 and 21 monomers was accomplished by the following method. Labeled preparations (0.5 µl) were loaded to a 12% SDS-PAGE gel and run overnight at 5mAmps. Gels were then stained with Coomassie Blue, dried, and exposed to film overnight (Kodak XAR-5).

**Column Chromatography with Pilin-17 and Pilin-21**

**Column Assay #1**

¹²⁵I-labeled pilin-17 monomers (3µg/µl) and 0.1% BSA were loaded onto a Sephadex G-10 (1cm x 50cm) in the presence of 0.05mM Tris-HCl, pH 8.0. Fractions (0.5ml) were collected at a rate of 0.25 ml/min. The radioactivity eluted from the column (cpm/fraction) was monitored with a Technical Associates Model TBM-3S Geiger counter.

**Column Assay #2**

¹²⁵I-labeled pilin-17 monomers (3µg/µl), 1mg Blue Dextran, and 0.1% BSA were loaded onto a Sepharose CL-6B (Pharmacia) column, 1cm x 64cm, in the presence of 6M Guanidine-HCl (ultrapure). Fractions (180, 0.5ml each) were collected at a rate of 0.5ml/min. The radioactivity eluted from the column (cpm/fraction) was measured on a Beckman Model - Gamma Counter and results tabulated. Each fraction was analyzed by SDS-PAGE for the presence of pilin monomers. Each
fraction (50 μl) was added to 50 μl of 2X Laemmli Sample Buffer, mixed, and boiled for ~5 min. A 25 μl fraction was then loaded onto the gel, and allowed to run overnight at 5 mAmps. Gels were stained the next day with Coomassie Blue, dried, and exposed to film (Kodak XAR-5) for 48 h at -70°C.

Column Assay #3

125I-labeled pilin-17 monomers (3μg/μl), 1mg transferrin, and 0.1% BSA were loaded onto a Sephadex G-100 column, 1cm x 100cm, in the presence of 4M Urea. Fractions (196, 0.5ml each) were collected at a rate of 0.5ml/min. The radioactivity eluted from the column (cpm/fraction) was measured on a Beckman Model- Gamma Counter and results tabulated. (A 1mg lysozyme standard was also run to determine the approximate elution time of pilin monomers). Each fraction obtained was analyzed by 12% SDS-PAGE for the presence of pilin monomers. Each fraction (50 μl) was added to 50 μl of 2X Laemmli Sample Buffer, mixed, and boiled for ~5min. A 25 μl fraction was then loaded onto the gel, and allowed to run overnight at 5mAmps. Gel were stained the next day with Coomassie Blue, dried, and exposed to film (Kodak XAR-5) for 48 h at -70°C. The experiment was then repeated with pilin-21 monomers.

Column Assay #4

125I-labeled pilin-17 monomers (3μg/μl) and 0.1% BSA were loaded onto a Sephadex G-100 column, 1cm x 100cm, in the presence of 0.05M Tris-HCl, pH 8.0. Fractions (145, 0.5ml each) were collected at a rate of 0.5ml/min. The radioactivity eluted from the column (cpm/fraction) was measured with a Beckman Gamma Counter and results tabulated. Radioactivity retained on the column was noted with a Technical Associates Model TBM-3S Geiger Counter.

NONiodinated pilin-17 monomers (3μg/μl) and 0.1% BSA were then loaded to the same column, as described immediately above. Fractions (122, 0.5 ml each) were collected at a rate of 0.5 ml/min. The presence of pilin monomers eluting during chromatography/filtration was determined with a Beckman U.V. Spectrophotometer, absorbance at 280nm.

Preparation of Pilin Antibody-Protein A Matrix

Protein A Sepharose CL-6B™ (Pharmacia) was mixed with monoclonal pilin-21 antibody in 0.1M Borate Buffer, pH 8.2, for 30 min at room temperature with gentle shaking, after
which the Sepharose beads were washed with excess Borate Buffer. The Sepharose was washed with 0.2M triethanolamine, pH 8.2, and then resuspended in 20 volumes of dimethylpimelimidate dihydrochloride (Pierce) freshly prepared in 0.2M triethanolamine, with pH adjusted to 8.2. The mixture was agitated gently at room temperature for 45 min and the reaction stopped by centrifugation of the beads (500 x g for 1 min) and resuspension in an equal volume of ethanolamine, pH 8.2. After 5 min, the cross-linked beads were washed three times with 0.1M Borate Buffer, pH 8.2, supplemented with 0.02% sodium azide.

**Immunoprecipitation of Pilin-17 and Pilin-21**

Purified preparations of 17 and 21 pilin monomers were mixed in various concentrations and allowed to polymerize overnight in the presence of 0.1M MgCl₂ and 0.15M NaCl at 4°C. The mixes included: 15µg/15µg; 30µg/15µg; 75µg/15µg; 150µg/15 µg of pilin-17 and pilin-21, respectively. This corresponded to an equimolar, 2x, 5x, and 10x concentration of pilin-17 monomers with respect to pilin-21. Control samples were also prepared with: pilin-21 monomers only, pilin-17 monomers only, polymerized 21 pilin, and polymerized 17 pilin. Polymerized pili were removed by rapid centrifugation for 5 sec in an Eppendorf™ centrifuge. A total of 1 µl of the polymerized preparations was added to 10 µl of prewashed monoclonal pilin-21 Antibody-Protein A Sepharose beads, 10 µl of plain Sepharose, and 50 µl of fresh BLOTTO buffer. Tubes were rocked overnight, at 4°C, and then washed once with 1X Kaplan Buffer wash, three times with PBS, and resuspended in 50 µl of 2X Laemmli Sample Buffer. Preparations were then loaded to a 12% SDS-PAGE gel and run for 7 h at 25 mAmps. When complete, gels were electroblotted to nitrocellulose (Schleicher and Schuell) in 2 L Blotting Transfer Buffer containing 28.8g glycine to 1.6L, 6.05g Trizma Base, methanol to 2L, and 0.1% SDS for 1 h at 5 Amps. Blots were then rinsed briefly, soaked in BLOTTO buffer for 1 hr and reacted with 1:400 polyclonal antiserum overnight with gentle rocking. The next day, nitrocellulose blots were rinsed in BLOTTO for 0.5hr with three changes, reacted with ¹²⁵I-Protein A (3.75 x 10⁷ cpm) in a total volume of 50 ml and rocked gently for 2 h. After rinsing for 45 min with three changes of BLOTTO buffer, blots were dried briefly on Schleicher and Schuell blot paper, wrapped in Saran Wrap on a fresh sheet of blot paper, and placed into a film cassette with XAR-5 (Kodak) film and developed for 48 h at -70°C.
RESULTS

Complementation Analysis

The first portion of the experiment was designed to determine if complementation of heterologous pilin and assembly regions of the *pap* 21 and *pap* 17 gene cluster could occur. Double transformations were performed, as discussed previously, with both homologous and heterologous pilin regions and assembly regions. Briefly, pilin structural genes (A-B) and assembly genes (C-G) from each cluster were isolated and ligated to pBR325 and pACYC184 plasmid vectors, respectively. These plasmid constructs, harboring specific *pap* genes, were transformed into *E. coli*. The resulting *E. coli* transformants contained a set of pilin structural genes (A-B) from one *pap* cluster and a set of assembly genes (C-G) from the other *pap* gene cluster.

To determine if pili had been produced in these organisms, slide preparations of fresh cultures (18-24 h) were incubated with an appropriate FITC-labeled polyclonal antibody and examined by fluorescence microscopy. Control strains that produced 17 or 21 pilin, and had not been subjected to digestion by restriction endonucleases, exhibited 4+ fluorescence (> 90% of organisms fluorescing). Unfortunately, the degree of fluorescence of double transformants was poor, 1+, even when homologous fragments were incubated with complementary antibody. It is assumed that homologous pilin and assembly regions should be capable of producing a functional pilin structure. The problem encountered may perhaps be due to a combination of factors. First, polyclonal antisera must be diluted since antibody generated to the *pap* 17 and *pap* 21 gene clusters exhibits some weak cross-reactivity, even after absorption with heterologous pilin. The necessary antibody dilution might then result in a poor fluorescence signal.

Secondly, construction of *pap* subclones involved restriction endonuclease cleavage at a *cla1* site, which lies within the *pap*H region. According to Baga et al., a decrease in the ratio of *pap* A to *pap* H gene products results in a large fraction of cells producing shortened pili. Those observations lead one to suppose that the poor sensitivity of the fluorescence assay may be due, in part, to this shortened pilus length. Further data (unpublished) gathered in Dr. David Low's
laboratory, University of Utah, indicate that this shortened pilus length may be caused by a decreased quantity of pilin secreted by the cell.

Alternatively, an immunoelectron microscopy technique was utilized to achieve greater sensitivity. Clones were grown overnight at 37°C on LB agar and examined as described. Data are presented in Table 2 and Figure 3. These preliminary results indicate that complementation between pilin monomers and heterologous assembly gene products probably does occur. (E. coli strain DH5 (pap)) was also examined to ensure that gold particles did not label the native type 1 pili produced by this strain.) It was clearly shown by gold labeling that 21 assembly regions are capable of producing a 17 pilus from 17 monomers. The reverse complementation experiment, however, is not as clear. The clones do produce large quantities of pilin; however, they are not individually labeled with gold particles. The 6nm gold particles remain trapped within a "mat" of pilin. The problem may partially be explained by the fact that a) 17 assembly genes are on a low copy pACYC184 plasmid vector, while 21 pilin genes are on a high copy pBR325 vector, b) an excess of antigen is present so that antibody is unable to individually label pili, and c) the pap 21 polyclonal antibody is not solely directed against 21 monomers.

This problem was later addressed in our laboratory (Low et al., Molecular Microbiology, in press). The complete pap 17 DNA sequence (pDAL211B) was introduced, in single copy vector pMF3, to E. coli strain HB101. This organism was also transformed with pDAL238B, harboring the pilin-21 structural genes and regulatory regions (no assembly regions). When examined by immunoelectron microscopy, this organism expresses both pilin-17 and pilin-21 on its cell surface. pDAL211B by itself can only express pilin-17, while pDAL238B is incapable of producing pilin at all. It is therefore apparent that complementation of assembly 17 regions with pilin-21 structural regions can occur.

**Copolymerization of Pilin Monomers**

The second goal of the study was to determine if the two pilin monomers (pilin-17 and pilin-21) could copolymerize to form a hybrid polymer. Three approaches were attempted to address this question.

First, an in vivo method was attempted whereby E. coli strain MC1061 was transformed with three compatible plasmids: a) pilin-21 structural genes under lacP5 control, b) the complete pap 17 gene cluster, and c) lacI, a super-repressor. If copolymerization occurs, the organism will express
### TABLE 1. Characterization of plasmid constructs.

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<tr>
<td>PDAL210B</td>
<td>PAP 17 ASSEMBLY (C-G) IN PACYC184</td>
<td>PILIN17-/ADHESIN+</td>
<td>PDG101</td>
</tr>
<tr>
<td>PDAL210B</td>
<td>PAP 17 PILIN (A-B) IN PBR325</td>
<td>PILIN17+/ADHESIN-</td>
<td>PDG201</td>
</tr>
</tbody>
</table>

### TABLE 2. Characterization of complementation products.

<table>
<thead>
<tr>
<th>TRANSFORMANTS</th>
<th>ANTI-PAP21 LABELED</th>
<th>ANTI-PAP17 LABELED</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDG100/200 (DH5)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PDG 100/201 (DH5)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PDG200/101 (DH5)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PDG201/101 (DH5)</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
FIGURE 3. Pilin. 1) Homologous pilin-21 and assembly genes in *E. coli* strain DH5α.
2) Homologous pilin-17 and assembly 17 genes in *E. coli* strain DH5α.
3) Heterologous pilin-21 and assembly 17 genes in *E. coli* strain DH5α.
4) Heterologous pilin-17 genes and assembly 21 genes in *E. coli* strain DH5α.
5) Pilin-21 genes in *E. coli* strain DH5α.
6) Pilin-17 genes in *E. coli* strain DH5α.
7) DH5α (*pap-*) native type 1 pili only.
Homologous Pilin-21 and Assembly 21

Homologous Pilin-17 and Assembly 17

Heterologous Pilin-21 and Assembly 17

Heterologous Pilin-17 and Assembly 21
Pilin-21 only

Pilin-17 only

DH5α (pap−)
pilin-17 only, until induction with isophenylthiogalactoside. Expression of pilin-21 would result from
the IPTG induction of the lacPuv5. Prior to induction, intact pili could be sheared from the bacteria
and immunoprecipitated with monoclonal pilin-21 antibody-Sepharose beads. The preparations
would be examined by immunoblotting (see Materials and Methods) for presence of pilin-17 and
pilin-21 (controls). There should be no bands visible on the final film since monoclonal pilin-21
Antibody would be incapable of precipitating pilin-17 monomers alone. Next, organisms can be
induced with IPTG, and the experiment repeated. If copolymerization occurs, a pilin-21 and pilin-17
band should be visible on the final film.

Numerous attempts were made to introduce the three compatible plasmids into the MC1061
host strain. This hybrid, however, was unable to survive to produce mature colonies on
MacConkey solid agar. An effort was made to transfer the immature colonies to fresh IPTG/XGal
media with nitrocellulose filter papers as soon as they were visible on the agar surface. White
colonies (lac−) were picked and grown overnight in the presence of IPTG/Amp/Spec, after which
Birney plasmid preparations9 were performed. Plasmid preparations were examined on 0.7%
Agarose gels and found to contain no DNA inserts. It is believed that the clones obtained probably
represent deletions that have resulted from IPTG induction (data not shown).

Alternatively, an in vitro method was used, in which 125I-labeled pilin monomers were
examined by gel filtration column chromatography. Iodinated preparations of pilin-17 and pilin-21
were prepared as described above and assayed for purity of preparation. The pilin-17 preparation
contained polymerized pilin-17 (mw >100 kilodaltons (kd), pilin monomers (mw of ~17 kd), and a
small quantity of an additional protein, probably papE or F protein (mw of ~ 16 kd). Genetic
evidence indicates that mutations in the papE and papF genes do not affect pilin production, only
anchoring of the adhesin and agglutination of whole bacterial cells. Recent data generated in our
laboratory suggest that papE and papF gene products are tightly associated with the tip of the
pilus structure (unpublished).

Initially, 125I-labeled pilin-17 and 0.1% BSA were loaded onto a Sephadex G-10 column in
0.05mM Tris-HCl, pH 8.0. It was observed that although a small fraction of free I2 was eluted from
the column, most of the radioactive counts were retained at, or near, the top of the column.
Addition of strong denaturing agents, such as SDS, only slightly increased the number of
radioactive counts eluted from the column. Two possibilities might explain this phenomenon: a) the
pilin itself has a high affinity for the column material, or b) the pilin is in a polymerized form and is
unable to filter through the column.
Eshdat et al.\textsuperscript{12} described a protocol for the dissociation and reassembly of type1 pili in the presence of Guanidine-HCl. Since P-pili and type1 pili are approximately 27% homologous,\textsuperscript{13} it was decided to modify the protocol and use it to ensure dissociation of pilin-17. Additionally, a new column material was chosen, Sepharose CL-6B, which would facilitate elution of the pilin from the column, allowing for separation of larger proteins or protein complexes.

A total of 11 µl of $^{125}$I-labeled pilin-17 ($\sim 2.5 \times 10^6$ cpm), Blue Dextran (1mg), and BSA (0.1%) was loaded onto a Sepharose CL-6B (Pharmacia) column, 1 cm x 64 cm. Fractions were counted on a Beckman Gamma Counter and results tabulated (Figure 4.) When column fractions were examined by SDS-PAGE, pilin monomers were shown to be present in fractions # 51-55. An additional band of radioactivity (mw ~ 65 kd) appeared in fractions # 47-59. This band was of much less intensity than the pilin monomer band and was believed to be polymerized pilin. From the data obtained, it appeared that although pilin was dissociated by the Guanidine-HCl, a longer column and different column material were needed to achieve adequate separation of polymerized pilin from pilin monomers. Furthermore, to ensure normal functional pilin activity for future experiments, it was necessary to remove the radiolabeled pilin from the presence of Guanidine-HCl. However, a greater than 75% loss of radioactivity was observed in the pilin fractions when dialyzed against T buffer. When dialyzed against Laemmli Sample Buffer using a microdialyzer, the Guanidine-HCl precipitated directly on the dialysis membrane. It is apparent that the separation and chromatography of pilin monomers must be performed in the presence of a dissociating agent other than Guanidine-HCl.

$^{125}$I-labeled pilin-17 monomers, (3 µg), 0.1% BSA and 1 mg transferrin were then loaded onto a Sephadex G-100 column (1cm x 100 cm) in the presence of 4M Urea. Fractions were collected and counted on a Beckman Gamma Counter and results tabulated. Two main areas of radioactivity were observed (Figure 5). Column fractions were examined by SDS-PAGE and exposure to film for 48 h. Pilin monomers were present only in fractions #44-50 and appear to be associated with BSA in some manner. It would be expected, under normal conditions, for pilin monomers to be eluted from the column much later than the BSA. A lysozyme molecular weight standard (m.w.~14 kd) eluted significantly later than the BSA (>24 fractions). The experiment was repeated with $^{125}$I-labeled pilin-21 (8µg) and similar results obtained. A small peak of radioactivity was observed in fraction # 43-47, a fraction eluting immediately after the 0.1%BSA (fraction # 40-44, Figure 6).
FIGURE 4. Pilin-17 on Sepharose™ CL-6B in the presence of guanidine-HCl.

FIGURE 5. Pilin-17 on Sephadex™ G-100 in the presence of 4M urea.
To investigate this phenomenon and determine if iodination in some way affects the normal affinity of pilin monomers for each other, 1 µl (3 µg) of $^{125}$I-labeled pilin-17 and 0.1% BSA were loaded on a Sephadex G-100 column in the presence of T buffer only. Absorbance of each fraction was monitored and tabulated (Figure 7). Only a small fraction of the total radioactivity loaded onto the column was eluted, in fractions #43-44. Most of the radioactive counts were located at, or near, the top of the column as measured with a Technical Associates Geiger counter. When the same experiment was repeated with noniodinated pilin-17, absorbance of pilin monomers was observed in fractions #61-63 (Figure 8). These observations of pilin behavior suggested that iodination of the pilin monomers in some way altered the normal affinity of pilin monomers for each other. Due to this behavior, it is impossible to investigate the ability of these monomers to copolymerize using this method. In order to make firm conclusions regarding the copolymerization of pilin monomers, one must be sure that the natural affinity of pilin monomers for one another remains unaltered.

Because iodination affected pilin polymerization in some unknown manner, an alternative method for detecting pilin monomers was employed. Immunoprecipitation of monomers with monoclonal antibody to pilin-21 was utilized to investigate this copolymerization question.

By titration of pilin monomers with a constant amount of antibody, it was determined that a minimum of 1.5 µg of pilin must be present in a preparation for adequate detection on a Western Blot. Various concentrations of pilin-17 monomers were added to a constant amount of pilin-21 monomers. These concentrations included: an equimolar, 2x, 5x, and 10x excess of pilin 17 monomers with respect to pilin-21. Pilin mixtures were allowed to polymerize overnight at 4°C in the presence of 0.1M MgCl$_2$ and 0.15M NaCl. Polymerized pilin were then rapidly centrifuged for 5 sec in an Eppendorf™ centrifuge. Appropriate polymerized pilin dilutions (1 µl) were added to 10 µl of prewashed, monoclonal pap21 Antibody-Protein A Sepharose, 10 µl of plain Sepharose, and 50 µl of BLOTTO buffer, and rocked overnight at 4°C. Upon completion of incubation, mixtures were spun rapidly in an Eppendorf™ centrifuge to separate Sepharose beads and polymerized pilin from the free pilin monomers. Mixtures were washed once with Kaplan buffer wash, three times with PBS and then resuspended in 50 µl of 2X Laemmli Sample Buffer prior to SDS-PAGE analysis of mixtures. Mixtures (25 µl) were loaded onto SDS-PAGE gels and subjected to electrophoresis (20 mAmps) for 12 h.

Immunoblotting was then performed, as described above, with rabbit polyclonal pap21 and pap17 antisera, respectively, on each gel. Results are presented in Figure 9. As shown by the
FIGURE 6. Pilin-21 on Sephadex™ G-100 in the presence of 4M urea.

FIGURE 7. I-125 labeled pilin-17 on Sephadex™ G-100 in the presence of T buffer only.
FIGURE 8. Noniodinated pilin-17 on Sephadex™ G-100 in the presence of T buffer only.
Figure 9. Immunoblot of immunoprecipitation reactions. The upper band present in all lanes represents a 38kd protein (not pilin), which cross-reacts with pilin-17 polyclonal antibody. It is believed to be papG. Lane A: Homologous pilin-21; Lane B: Homologous pilin-17 (no pilin-21 antibody beads); Lane C: Equimolar concentrations of heterologous pilin-17 and pilin-21; Lane D: Homologous pilin-17 (with pilin-21 antibody beads); Lane E: Heterologous pilin-17 and pilin-21 (2X concentration); Lane F: Heterologous pilin-17 and pilin-21 (5X concentration); Lane H: Heterologous pilin-17 and pilin-21 (10X concentration); Lane I: space; Lane J: Homologous pilin-21; Lane K: Heterologous pilin-17 and pilin-21 (2X concentration); Lane L: Heterologous pilin-17 and pilin-21 (5X concentration); Lane M: Homologous pilin-17 (with pilin-21 antibody beads); Lane N: Heterologous pilin-17 and pilin-21 (10X concentration); Lane O: space; Lane P: Equimolar concentrations of heterologous pilin-17 and pilin-21.
Western blots, 17- and 21-pilin monomers do appear to be able to copolymerize. Control mixtures containing 17- or 21- polymerized pilin, only, incubated with monoclonal 21 Sepharose beads, show that no cross-reactivity of 21 monoclonal antibody Sepharose beads with pilin-17 monomers occurs (lanes D and M). It can also be noted (lanes A and J) that pilin-21 monomers do not cross-react with rabbit polyclonal pilin-17 antibody. A distinct band of radioactivity can be observed in those lanes corresponding to heterologous (pilin-17 and pilin-21) mixes (lanes C, E, F, and H). This band clearly indicates the presence of pilin-17 in the preparations, which could only have been present if the monomers were copolymerized prior to incubation with monoclonal pilin-21 Antibody. An additional band of radioactivity was observed in all mixtures and controls, with a molecular weight of approximately 38 kd. This band is believed to represent another pap protein (not pilin) with which the rabbit polyclonal 17 antibody cross-reacts. PapG protein is believed to be tightly associated with pilin and it is possible that small traces of this protein are present in a purified pilin preparation.
DISCUSSION

The purpose of this study was to examine the process of pili assembly in the uropathogenic *E. coli* strain C1212. In particular, the production and assembly of pilin associated proteins encoded by the two *pap* (pyelonephritis-associated-pili) gene clusters was examined. First, pilin structural genes (*pap* A-B) and assembly genes (*pap* C-G) were isolated from uropathogenic strains that harbored two different *pap* gene clusters. Using electron microscopy, it appeared that the assembly genes of one *pap* gene were capable of producing an intact pilus from the pilin structural genes of the heterologous gene cluster. This finding led me to then examine the possibility that a strain harboring both gene clusters might, perhaps, form copolymers, or hybrid pili, from the pilin structural genes of each *pap* gene cluster. The possibility of such a phenomenon occurring would have important implications for the ability of an invading microorganism to evade the host’s immune system. The ability of the pilin subunits to copolymerize could lead to the expression of a new and unique epitope on the surface of this microorganism, facilitating attachment to, and colonization of, the urinary tract.

The data obtained by immunoprecipitation experiments with $^{125}$I-labeled pilin suggest that such polymerization may indeed occur, at least in vitro. However, to show conclusively that copolymerization does occur, an in vivo model must be examined. Such a model may involve the incorporation of radiolabeled substrates into the growth medium, which will result in production of radiolabeled pilin. The pilin may then be harvested and examined by both column chromatography and electron microscopy.

It will also be necessary to further investigate the mechanism by which $^{125}$I-labeling of pilin monomers alters the normal behavior of pilin polymerization. It has been reported that iodination of proteins can alter their affinities for cell surface receptors, as well as alter their biological activities. The phenomenon I observed by column chromatography, whereby *pap* pilin associates itself with BSA when iodinated, has not been previously described. BSA can bind hydrophilic proteins and it is possible that pilin iodination increases this interaction.

Another problem was encountered with interpretation of amount of incorporation of $^{125}$I into purified pilin preparations. A percent iodine yield was calculated to be ~26% based on column yields, while a TCA precipitate on the reaction mixture indicated a yield of >80%. When examining the data obtained by the column chromatography procedures, it appears that a large fraction of the
purified preparations loaded onto the column consisted of free I₂ (measured by cpm/fraction). The TCA quanitation was repeated and verified, giving the same amount of relative incorporation. At present, no reason for this discrepancy can be determined. As mentioned above, further investigation into the nature of pilin radiolabeling must be undertaken.

Finally, immunoprecipitations using prepolymerized pilin-17 and pilin-21 mixtures must be examined to show that the two types of pilin monomers do not associate with each other. Specifically, these mixtures can be examined with and without the presence of 0.1 M MgCl₂. It has been demonstrated by the procedure for pilin purification of Brinton that pilin monomers polymerize only in the presence of 0.15 M NaCl and 0.1 M MgCl₂. It can be inferred that such a procedure can be used to isolate pilin from immunoprecipitation reactions. A necessary condition for use of this assay is that prepolymerized heterologous pilin mixtures do not interact with each other due to "nonspecific" associations, such as may be caused by close physical proximity of pilin monomers (or pilis) in an incubation mixture. Immunoblots of the preparations will show that the immunoprecipitation reaction is not only sensitive and specific for the monomers of interest, but is also technically accurate.

In conclusion, the data suggest that: a) it is possible for uropathogenic E. coli that harbor two pap gene clusters to assemble intact pili from heterologous assembly regions, and b) copolymerization of pilin monomers can occur, at least in vitro. Further studies, as discussed briefly above, must be performed to show that this copolymerization can occur in vivo. Production of hybrid pili may lead to the expression of an antigenic determinant that allows for increased survival of an invading microorganism. It may also be possible that expression of this antigenic determinant is negatively regulated, as evidenced by the low coexpression frequency (0.3%) demonstrated by Low et al. This negative regulation of hybrid pilin production may, in fact, be a survival mechanism for the invading organism.
LITERATURE CITED


