

## Gal-Gal PYELONEPHRITIS *ESCHERICHIA COLI* PILI LINEAR IMMUNOGENIC AND ANTIGENIC EPITOPES

BY M. ALEXANDER SCHMIDT, PETER O'HANLEY, AND  
GARY K. SCHOOLNIK

*From the Medical Service, Palo Alto Veterans Administration Medical Center, and the  
Departments of Medicine and Medical Microbiology, Stanford University School of Medicine,  
Stanford, California 94305-2499*

The ability of pathogenic bacteria to adhere to epithelial cells is a prerequisite for the colonization of mucosal surfaces and subsequent infection of the host. Adherence is thought to be mediated by pili, proteinaceous filaments that protrude from the bacterial outer membrane, or afimbrial adhesins. These bind specific "receptor" molecules on host epithelial cells. According to their respective receptor structure, uropathogenic *Escherichia coli* pili have been functionally classified as mannose-sensitive (MS),<sup>1</sup> Gal-Gal-binding (also termed p-fimbriae), and X-binding. Gal-Gal pili function as virulence determinants by binding renal epithelial glycosphingolipids containing the D-Gal-*p*- $\alpha$ -(1-4)-D-Gal-*p*- $\beta$ -(1- moiety (1, 2). Vaccines composed of pure Gal-Gal pili confer homologous protection in the BALB/c mouse and primate pyelonephritis models (3, 4). However, although Gal-Gal pili from heterologous uropathogenic *E. coli* are functionally and structurally similar, they exhibit antigenic heterogeneity. Their serologic diversity could impede the development of a broadly crossreactive pyelonephritis vaccine.

The complete primary structure of Gal-Gal pili from a recombinant strain (HU 849) was recently determined in our laboratory by automated Edman degradation of overlapping peptide fragments (5) and deduced from the DNA sequence of the structural gene by Normark et al. (6). To elucidate the serological properties of Gal-Gal pili, synthetic peptides corresponding to linear regions of the HU 849 pilin amino acid sequence were used to identify the linear immunogenic and antigenic epitopes<sup>2</sup> of the molecule.

This work was supported by a grant from the Cetus Corporation. M. A. Schmidt is supported by grant II-B6-Schm 590/1-1 from the Deutsche Forschungsgemeinschaft. G. Schoolnik is a Fellow of the John A. Hartford Foundation. Address correspondence to G. Schoolnik, Department of Medicine, Division of Infectious Diseases, Stanford University Medical School, Stanford, CA 94305.

<sup>1</sup> *Abbreviations used in this paper:* BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; MBS, m-maleinimidobenzoyl-N-hydroxysuccinimide ester; MS, mannose-sensitive; PBS, phosphate-buffered saline; SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SMCC, succinimidyl 4-(N-maleinimido-methyl)cyclohexane-1-carboxylate; *t*-Boc, tertiary butyl oxy carbonyl.

<sup>2</sup> Throughout this report, the term "immunogenic epitope" refers to a particular domain in the native protein that is recognized by the immune system and gives rise to antibodies able to bind synthetic peptides corresponding to this domain. "Antigenic epitope" is used to describe domains that are recognized in the native protein by antibodies engendered by synthetic peptides corresponding to that region of the protein.

### Materials and Methods

**Bacteria.** *E. coli* strain 3669 (02:K nontypable) was isolated from the urine of a girl with pyelonephritis. *E. coli* J 96 (04:K5:H51), also a human pyelonephritis isolate, is hemolytic, colicin V positive, motile, resistant to the bactericidal action of normal serum, and simultaneously expresses MS and Gal-Gal pili. HU 849 is a recombinant strain prepared from J 96 chromosomal DNA that expresses J 96 Gal-Gal pili and digalactoside-binding activity. The construction of this strain has been described elsewhere by Hull et al. (7). Briefly, a cosmid gene library was prepared from J 96 DNA. Bacteriophage lambda transducing particles carrying recombinant cosmid molecules with portions of the J 96 genome were used to transduce the nonpilated *E. coli* K-12 strain HB 101. A Gal-Gal hemagglutinating clone was identified and its genome subcloned into the vector pACYC184. The hybrid plasmid was transformed into the minicell producing *E. coli* K-12 strain P678-54. The resulting recombinant strain HU 849 and the parent strain J 96 expressed Gal-Gal pili that were functionally, chemically, and serologically identical (8). Clinical isolates were also used in this study. They include 8 *E. coli* strains isolated from the feces of healthy, nonpregnant women, and 12 *E. coli* strains isolated from the urine of patients with acute pyelonephritis. All of the clinical isolates were shown to express Gal-Gal-binding adhesins.

**Pili Purification.** Gal-Gal pili from strains HU 849 and 3669 were purified from organisms grown on tryptic soy agar for 24 h at 37°C by a modification of the method of Brinton (9). The organisms were harvested into ice-cold 0.005 M Tris-HCl buffer, pH 8.3 (T buffer), homogenized (4,000 rpm) for 30 min at 4°C in a Sorvall Omnimixer (Dupont Instruments-Sorvall, Dupont Co., Newton, CN), and the sheared bacteria removed by centrifugation at 12,000 g for 30 min. Pilus filaments were precipitated in 0.05 M Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl, by the addition of MgCl<sub>2</sub> to 0.1 M (TSM buffer), collected by centrifugation at 12,000 g for 45 min, and the pellet dissolved in T buffer. Insoluble contaminants were removed by centrifugation at 12,000 g for 60 min and the pili precipitated from the supernatant in TSM buffer. After six successive cycles of solubilization and crystallization by exposure to T buffer and TSM buffer, respectively, the pili were dialyzed against water and their purity assessed by electron microscopy, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), amino-terminal amino acid sequence analysis, and isopycnic ultracentrifugation. The 163 amino acid, HU 849 pilin sequence was determined by Edman degradation of overlapping pilus peptides (5) and also deduced from the DNA sequence of pap A, the pilus structural gene (6).

**Selection of Synthetic Peptides.** The choice of peptides corresponding to segments of the HU 849 pilus subunit sequence for synthesis was biased with regard to secondary structure and hydrophilicity predictions according to the algorithms of Chou and Fasman (10) and Hopp and Woods (11), respectively. Regions were selected that were predicted to incorporate hydrophilic beta-turns at either end of the peptide sequence. A natural or additional cysteine residue was placed at the N- or C- terminal, distal to the predicted reverse turn, so that the peptide could be coupled in a unique orientation to the carrier protein with a heterobifunctional crosslinker (see below).

**Synthesis and Characterization of Peptides.** Peptides were synthesized by solid phase techniques (12) using tertiary butyl oxy carbonyl (*t*-Boc)-protected amino acids and amino acid polystyrene resins (Peninsula Laboratories, Inc., Belmont, CA). As side chain protecting groups, *O*-benzyl esters were used for Asp, Glu, Thr, and Ser, and tosyl groups were used for Arg and His. Cys was protected by *p*-methoxybenzyl, Lys by *o*-chlorobenzyl, and Tyr by 2,6-dichlorobenzyl. Couplings were performed with a 2.5-3-fold molar excess of *t*-Boc amino acid and dicyclohexylcarbodiimide (DCC). If Asn or Gln was to be coupled, a 2.5-fold molar excess of *N*-hydroxytriazole was also included. The coupling of each amino acid was monitored with ninhydrin (13) and, if necessary, the amino acid was recoupled until >99% efficiency was achieved. Anhydrous hydrogen fluoride in the presence of dimethylsulfide and anisole was used to cleave the protecting groups and the resin simultaneously. The cleaved peptide was extracted with ether to

remove side products and residual organic solvents, isolated from the resin by extraction with 5% acetic acid, and subsequently lyophilized several times. The purity of the final product was determined by reverse phase high performance liquid chromatography on Lichrosorb RP-18 (E. Merck AG, Darmstadt, Federal Republic of Germany) and amino acid analysis. The peptides used in our experiments were found to contain >90% of the expected product and were thus not further purified.

*Conjugation of Peptides to Carrier Proteins.* Each peptide was conjugated to both thyroglobulin and bovine serum albumin (BSA) using *m*-maleinimidobenzoyl *N*-hydroxysuccinimide ester (MBS) and succinimidyl 4-(*N*-maleinimido-methyl) cyclohexane-1-carboxylate (SMCC), respectively, as heterobifunctional crosslinkers. Briefly, 10 mg of the carrier protein were dissolved in 3 ml phosphate-buffered saline (PBS) (pH 7.4) and mixed with 1 ml distilled *N,N*-dimethylformamide containing 5 mg of the respective crosslinker. After 2 h with occasional stirring at ambient temperature, the conjugate was separated from unreacted crosslinker by gel filtration on Sephadex G-25 in 0.1 M phosphate buffer (pH 6.0). To ensure a free sulfhydryl group on the peptide, reduction with sodium borohydride was carried out for 15 min on ice and the excess borohydride subsequently destroyed with HCl. The neutralized and reduced peptide was combined with the carrier-crosslinker conjugate and stirred overnight at room temperature. The resulting peptide carrier conjugate was subsequently isolated by gel filtration on Sephadex G-25 in 0.1 M ammonium bicarbonate buffer (pH 7.4). The molar ratio of conjugated peptides to carrier protein was determined by comparing the amino acid composition of the carrier before and after conjugation.

*Specific Pili and Peptide Antisera.* All hyperimmune sera were prepared in female New Zealand White rabbits. 50–150  $\mu$ g of the peptide-carrier conjugate or purified pili in PBS were emulsified with complete Freund's adjuvant and injected subcutaneously and intramuscularly at multiple sites. Booster injections with the same dose were prepared with incomplete Freund's adjuvant and administered 5–6 wk later. 8 d later the animals were bled by cardiac puncture. For the preparation of specific antipeptide sera, rabbits were solely immunized with the peptide-MBS-thyroglobulin conjugate. The resulting sera were subsequently evaluated in a solid phase binding assay (see below) using the homologous peptide-SMCC-BSA conjugate as antigen.

*Solid Phase Antigen-binding Assay.* Peptide-protein conjugates or pili were coated on polystyrene or polyvinyl microtiter plates. The plates were washed with PBS containing 0.1% Brij 35, treated with dilutions of antisera in 0.1% BSA, and washed again, and the bound antibody was detected enzymatically with alkaline phosphatase-conjugated second antibody, followed by *p*-nitrophenylphosphate as substrate, or radioactively with  $^{125}$ I-protein A. The enzyme reaction was evaluated by determining the optical density at 405 nm and the radio-binding assay by determining the counts per minute (cpm) of microtiter wells that had been cut from the plate and subjected to gamma counting. All assays were repeated several times in duplicate or triplicate.

*Western Blotting.* The electrophoretic transfer of proteins from SDS-polyacrylamide gels to nitrocellulose paper was performed in 25 mM Tris buffer (pH 7.2) essentially as described by Towbin et al. (14) and Burnette (15). After electrophoretic transfer, the nitrocellulose was blocked with 2.5% gelatin in PBS at 37°C overnight. Subsequently, the antiserum was applied in a 1:200 dilution in PBS containing 0.1% nonfat dry milk. After 2 h at room temperature the paper was washed several times with 25 mM Tris buffer (pH 7.2) containing 0.1% nonfat dry milk, and incubated with  $^{125}$ I-protein A at 50,000 cpm/ml in PBS/0.1% nonfat dry milk for 2 h. After washing with 0.1% Brij 35/25 mM Tris/0.1% nonfat dry milk, the blot was dried and developed on Kodak XAR-5 film overnight.

*Gal-Gal-specific Agglutination by Clinical E. coli Isolates.* *E. coli* clinical isolates (see above) were assessed for Gal-Gal-binding activity after overnight growth on L-agar by determining their capacity to agglutinate latex beads adsorbed with Syn Gal-Gal (Chembiomed Ltd., Edmonton, Canada) and by hapten inhibition of hemagglutination by Syn Gal-Gal (4% wt/vol) as described by O'Hanley et al. (8).

## Results

**Choice and Synthesis of Peptides.** To identify linear immunogenic and antigenic determinants within the 163 amino acid, HU 849 pilus subunit sequence, nine peptides were prepared by solid phase Merrifield synthesis, corresponding to residues R 5-12, R 25-38, R 38-50, R 48-61, R 65-75, R 93-104, R 103-113, R 119-131, and R 131-143. Each peptide contained a predicted beta-turn (Fig. 1). Three of these peptides correspond to segments of the Gal-Gal pilus disulfide loop subtended by cysteine residues at positions 21 and 61. They were synthesized because the single gonococcal pilin disulfide loop has been shown to encode immunodominant, strain-specific epitopes (16). The Gal-Gal loop peptides contained small areas of overlapping sequence and correspond to R 25-38, R 38-50, and R 48-61. All peptides were synthesized with either a natural or an additional cysteine residue as the C- or N-terminus, distal to the predicted reverse turn, thus providing a unique orientation by which the peptide was conjugated to its carrier protein. The complete amino acid sequences of the nine synthetic peptides, and the four amino acids predicted to constitute the reverse turn in each, are depicted in Table I.

**Peptides as Antigens.** The synthetic peptides recognized by polyclonal HU 849 pilus antiserum are defined in this report as "immunogenic HU 849 pilus

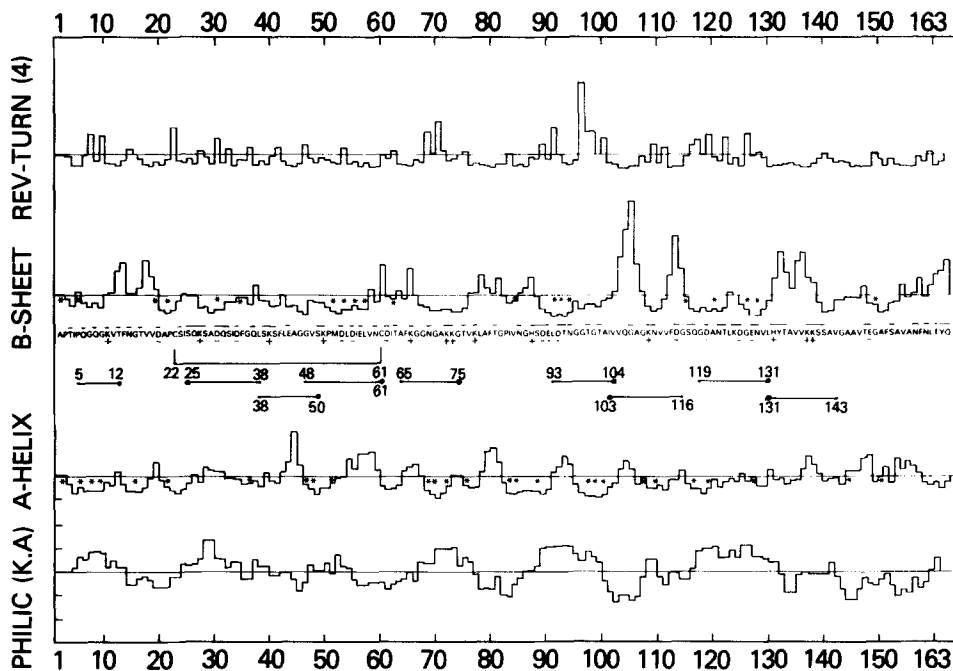


FIGURE 1. Predictive analysis of the secondary structure and local average hydrophilicity of the HU 849 pilin amino acid sequence, based on the Chou and Fasman (10) and Hopp and Woods (11) algorithms, respectively. The hydrophilicity values are derived from a hexapeptide moving average and are plotted at the midpoint of the particular segment. Hydrophilic regions appear as positive peaks above 0. The synthesized amino acid sequences are indicated, with the cysteine conjugation site depicted as (—●). The intramolecular disulfide bridge is denoted by a bracket between cysteine residues 22 and 61.

TABLE I  
Amino Acid Sequences of the Synthetic Peptides and Their Location in  
the HU 849 Pilin Sequence

Residues	Sequence	Reverse turn probability
5-12	<u>P</u> <u>Q</u> <u>G</u> <u>Q</u> GKVT-C*	$1.86 \times 10^{-4\dagger}$
25-38	C-S <u>Q</u> <u>K</u> SAD <u>Q</u> SIDF <u>Q</u> GL	$1.00 \times 10^{-4}$
38-50	L <u>S</u> <u>K</u> S <u>F</u> LEAGGVSK-C	$1.12 \times 10^{-4}$
48-61	V <u>S</u> <u>K</u> P <u>M</u> D <u>L</u> DIELVNC	$1.04 \times 10^{-4}$
65-75	A <u>F</u> <u>K</u> <u>G</u> <u>G</u> <u>N</u> GAKKG-C	$2.52 \times 10^{-4}$
93-104	L <u>D</u> <u>T</u> <u>N</u> <u>G</u> <u>G</u> <u>T</u> GTAIV-C	$2.05 \times 10^{-4}$
103-116	C-I <u>V</u> <u>V</u> <u>Q</u> <u>G</u> A <u>G</u> <u>K</u> <u>N</u> V <u>V</u> FDG	$0.33 \times 10^{-4}$
119-131	G <u>D</u> <u>A</u> <u>N</u> <u>T</u> L <u>K</u> D <u>G</u> <u>E</u> <u>N</u> <u>V</u> L-C	$1.68 \times 10^{-4}$
131-143	C-L <u>H</u> <u>Y</u> <u>T</u> A <u>V</u> <u>V</u> <u>K</u> <u>K</u> <u>S</u> <u>S</u> A <u>V</u>	$0.55 \times 10^{-4}$

\* -C, non-natural cysteine residues added for conjugation purposes. The underlined four amino acids constitute beta-turns as predicted by the Chou and Fasman algorithm (10).

† Probability of each turn.

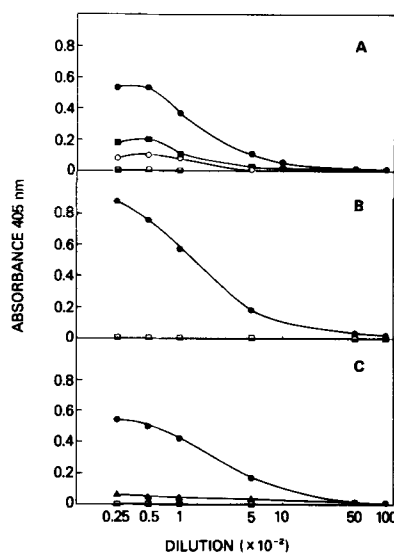


FIGURE 2. Reaction of anti-HU 849 pilus antiserum with peptide-BSA conjugates in an ELISA as described in Materials and Methods. The pilus antiserum was assayed for its ability to bind peptides R 25-38 (O), R 38-50 (●), and R 48-61 (■), corresponding to the cysteine loop (A); peptide R 65-75 (B); and peptide R 103-116 (C) coupled via SMCC (●) and via water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (▲) to BSA. The absorbance at 405 nm was corrected for nonspecific binding due to BSA and preimmune sera as indicated (◻). Peptides R 5-12, R 93-104, R 119-131, and R 131-143 were not bound by anti-HU 849 antiserum.

epitopes." Each of the nine BSA-peptide conjugates was assessed by enzyme-linked immunosorbent assay (ELISA) with dilutions of HU 849 antiserum. As depicted in Fig. 2, A-C, five out of the nine peptides R 25-38, R 38-50, R 48-61, R 65-75, and R 103-116, were bound by polyclonal HU 849 pilus antiserum, indicating that the corresponding sequences in the pilus subunit encode immu-

nogenic epitopes. The antigenicity of R 103–116 (Fig. 2C) was compared as the peptide-BSA conjugate coupled through the N-terminal cysteine by SMCC or through free amino and/or carboxyl groups by water-soluble carbodiimide (EDAC). The EDAC-coupled conjugate was not bound by HU 849 antiserum, indicating that the lysine (R 110) and/or the aspartate (R 115) residues might be required for antigenicity. This result also indicates that recognition of the biological properties of some peptide-carrier conjugates can be significantly influenced by the coupling strategy used.

To compare the relative antigenicity of these peptide conjugates for polyclonal anti-HU 849 serum, a solid phase binding assay was conducted using  $^{125}\text{I}$ -protein A to detect bound antibody. The relative number of counts bound are indicated in Table II. Peptides R 25–38, R 38–50, R 48–61, and R 103–116 bound between 11.5 and 21.7% of the total bound counts and thus constitute only weakly immunogenic HU 849 pilus epitopes. With respect to the three peptides that jointly compose the disulfide loop, the strongest response was directed against the middle segment (peptide R 38–50), which bound 21.7% of the counts. The disulfide loop peptides flanking R 38–50 (R 25–38 and R 48–61) bound 11.6 and 11.5%, respectively. A more prominent immunogenic epitope was found to reside in a peptide just distal to the disulfide loop (residues R 65–75) that accounted for more than 37% of the bound radioactivity.

*Peptides as Immunogens.* Polyclonal peptide antisera were engendered by immunizing rabbits with peptide-MBS-thyroglobulin conjugates and were then tested for their capacity to bind the immunizing peptide and intact pili, thus defining the peptide as an antigenic epitope. As shown in Fig. 3, all peptides elicited a strong specific immune response as judged by ELISA, where the corresponding peptide-SMCC-BSA conjugate was used as the solid phase antigen. However, only four antipeptide sera crossreacted with solid phase, intact HU 849 pili (Fig. 4). The most strongly crossreacting antisera were elicited by peptides R 5–12 and R 93–104 (Fig. 4, A and C). Antisera against peptides R 65–75 and R 119–131 bound HU 849 pili to a lesser extent (Fig. 4, B and D). Therefore, peptides R 5–12 and R 93–104 appear to encompass two prominent antigenic epitopes, and peptides R 65–75 and R 119–131, two minor antigenic epitopes. R 65–75 was the only peptide encompassing an immunogenic as well as an antigenic HU 849 pilus epitope.

*Recognition of Antigenic Epitopes by Western Blotting.* To determine if the peptide antisera bind pilus subunits under denaturing conditions, HU 849 pili were boiled in 2-mercaptoethanol and SDS according to the method of Laemmli

TABLE II  
*Binding of Anti-HU 849 Polyclonal Serum to Synthetic Peptides Corresponding to the Amino Acid Sequence of the HU 849 Pilus Subunit*

	BSA	5-12	25-38	38-50	48-61	65-75	93-104	103-116	119-131	131-143
cpm*	519	881	1,073	2,003	1,062	3,461	713	1,612	863	700
Binding	—	—	+/-	+	+/-	+	—	+	—	—
Relative percent	—	—	11.6	21.7	11.5	37.5	—	17.5	—	—

\* Wells were sensitized with a solution of the various peptide-BSA conjugates (1 mg/ml), washed, treated with a 1:50 dilution of anti-HU 849 polyclonal antiserum, exposed to 50,000 cpm of  $^{125}\text{I}$ -protein A, and washed; the wells were then cut from the plate and counted. Data are averages of several experiments done in duplicate.

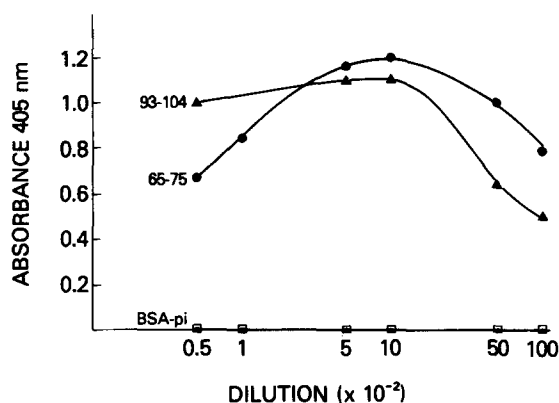


FIGURE 3. Reaction of antiserum against peptide-MBS-thyroglobulin conjugates with homologous peptide-SMCC-BSA conjugates. The ELISA data for R 93-104 ( $\blacktriangle$ ) and R 65-75 ( $\bullet$ ) are exemplary of the other antipeptide antibody/peptide reactions. The reactions were corrected for nonspecific binding to BSA ( $\square$ ). The reactivity of the preimmune sera (*pi*) is also indicated ( $\blacktriangle$ ).

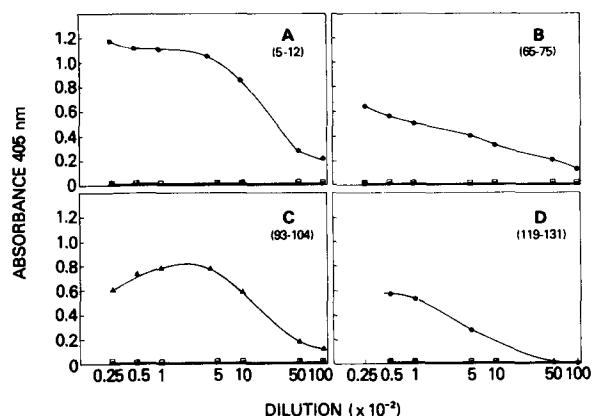


FIGURE 4. Reaction of anti-peptide sera with intact HU 849 pili by ELISA. Anti-peptide sera to R 5-12 (A), R 65-75 (B), R 93-104 (C), and R 119-131 (D) showed significant binding. Nonspecific binding to BSA and by each preimmune serum is indicated ( $\blacksquare$ ).

(17), electrophoresed in 15% SDS-polyacrylamide gels, and transferred to nitrocellulose (Fig. 5). Antisera elicited by peptides R 5-12 and R 93-104 bound the HU 849 pilus subunit under these conditions. However, anti-R 65-75 and anti-R 119-131 did not bind the denatured pilus subunit, indicating that their epitopes were destroyed by this treatment and thus that the antigenicity of these regions may depend on their conformation in native HU 849 pili. Antisera elicited by two of the loop peptides (R 25-38 and R 38-50) crossreacted weakly with the denatured pilus subunit.

*Shared Antigenicity between Heterologous Gal-Gal Pili.* The *E. coli* pyelonephritis isolate 3669 (18) expresses Gal-Gal pili that exhibit about 80% amino acid sequence homology with the HU 849 pilus subunit (D. A. Low, personal communication). Further, a 3669 pilus peptide corresponding in part to the region R 65-75 of the HU 849 pilus sequence also constitutes a prominent immunogenic

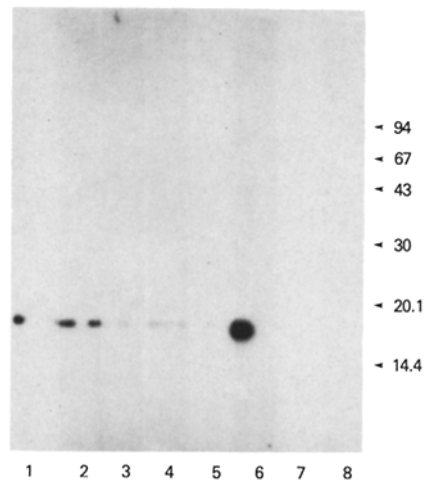


FIGURE 5. Western blot of HU 849 pili with anti-peptide sera. The peptide antisera were diluted 1:200 in 0.1% nonfat milk/PBS.  $^{125}\text{I}$ -protein A was used to detect bound antibody. (1) Anti-HU 849 serum (dilution, 1:5000); (2) anti-R 5-12; (3) anti-R 25-38; (4) anti-38-50; (5) anti-R 65-75; (6) anti-R 93-104; (7) anti-R 119-131; (8) preimmune serum.

epitope of the 3669 pilus subunit, analogous to this region in the HU 849 sequence (Table II). However, antibody to purified HU 849 and 3669 Gal-Gal pili does not bind the heterologous peptide from this region (data not shown), indicating that these peptides may encode strain-specific immunogenic epitopes. The shared antigenicity between the two pilus proteins was examined in greater detail with each of the HU 849 synthetic peptide antiserum. Despite the sequence homology between the HU 849 and 3669 pilus subunits, none of the nine HU 849 synthetic peptides engendered antibodies that recognized isolated 3669 pili in a solid phase binding assay, indicating that the two pilus proteins share few or no linear epitopes that are antigenic in intact pili. When single Gal-Gal-binding *E. coli* colonies from clinical isolates were subjected to Western blotting, none was recognized by polyclonal anti-HU 849 serum. However, a protein with a molecular weight similar to that of a pilus subunit was detected by anti-R 93-104 in 4 of the 8 Gal-Gal-specific fecal strains and in 7 of the 12 Gal-Gal-specific pyelonephritis isolates subjected to Western blotting. In 8 of the 10 Gal-Gal-binding pyelonephritis isolates, anti-R 5-12 serum detected a protein with a molecular weight similar to that of a pilus subunit. This indicates that common linear epitopes may be antigenic in denatured pilus subunits. In all strains, however, R 93-104 antibody also detected several other apparently non-pilus bands of higher molecular weight, whereas anti-R 5-12 serum bound only pilin.

### Discussion

Uropathogenic *E. coli* exhibit a constellation of pathogenic features: they usually belong to a restricted number of O and K antigen serogroups (19, 20); they are resistant to the bactericidal action of normal human serum (21, 22); they secrete hemolysin (23, 24) and produce colicin V (25, 26); and they attach to uroepithelial cells in vitro (27, 28). Epithelial cell adherence is the in vitro



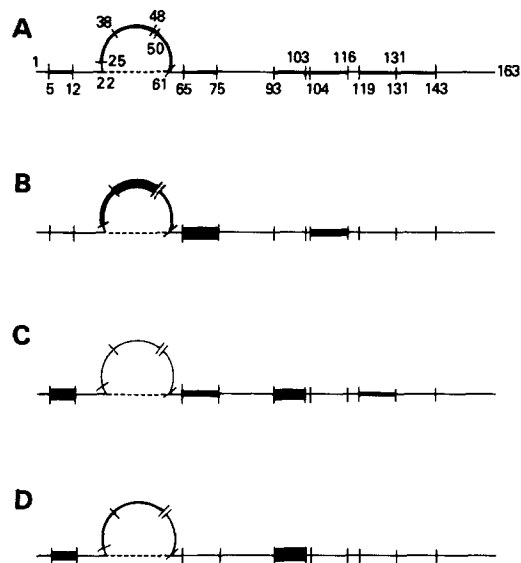


FIGURE 6. Mapping of linear immunogenic and antigenic epitopes in the HU 849 pilus subunit. The linear depiction of the HU 849 pilus subunit shows the location of the synthesized peptides (A), location of immunogenic epitopes (B), location of antigenic epitopes (C), and the recognition of denatured HU 849 pilin by antisera engendered by synthetic peptides, detected by Western blotting (D). The thickness of the bar represents the relative amount of binding.

analogue of mucosal colonization, an event that appears to precede and be a prerequisite for subsequent pathogenic steps. It is mediated by bacterial adhesions and the epithelial cell surface molecules to which they bind.

Two major classes of chromosomally encoded *E. coli* adhesins have been defined functionally by the receptor specificity of their associated pili. MS pili bind Tamm-Horsfall uromucoid, and their agglutination of guinea pig red cells is blocked by D-mannose. Gal-Gal pili agglutinate human red cells in the presence of mannose by binding glycosphingolipids that contain the D-Gal- $p$ - $\alpha$ -(1-4)- $p$ - $\beta$ -(1- . . .) moiety. Of these Gal-Gal pili appear to be significant determinants of uropathogenicity: (a) Gal-Gal pili are expressed by most pyelonephritis isolates (29); (b) renal epithelium contains Gal-Gal pilus receptor compounds (3); (c) Gal-Gal, but not MS, pilated recombinants colonize renal epithelium in the absence of acute ureteric reflux (3); and (d) Gal-Gal, but not MS pilus vaccines prevent pyelonephritis in the unobstructed murine and primate urinary tract (3, 4).

In the present studies, we used synthetic peptides corresponding to nine segments of the HU 849 recombinant *E. coli* Gal-Gal pilus sequence (5, 6). Linear antigenic and immunogenic epitopes were identified with these reagents and are mapped onto the Gal-Gal pilus amino acid sequence as depicted in Fig. 6. Three immunogenic determinants were identified that collectively bound >75% of the HU 849 antibodies directed against the nine peptides: one within the disulfide loop (R 38–50); one adjacent to the disulfide loop between R 65–75, which is the most prominent; and one towards the C-terminal (R 103–116). Only R 65–75 proved also to be an antigenic epitope; i.e., antibodies to peptide R 65–75 bound native HU 849 pili. In addition, two prominent (R 5–12 and R 93–104)

and one minor (R 119–131) antigenic epitopes were detected. These results indicate that an immunogenic Gal-Gal pilus epitope is not necessarily also an antigenic epitope, a result concordant with the analysis of the antigenic structure of gonococcal pili (16).

The use of synthetic peptides to identify the immunogenic epitopes of a protein is applicable only to epitopes encoded by linear segments of the sequence. In most proteins, including pili (16), "linear" epitopes appear to comprise a relatively small fraction of the molecule's entire antigenicity. Most of the antigenicity seems to be determined by residues that are not contiguous in sequence, but are juxtaposed in space as a result of secondary, tertiary, and, in polymeric proteins like pili, quaternary structural features. Antibodies to synthetic peptides can be successfully used to identify antigenic epitopes and as sequence-specific immunological reagents for structure-function analysis, provided they elicit crossreacting antibodies of sufficiently high affinity. Only if a synthetic peptide adopts, or can be induced to adopt, a conformation that is identical or very similar to the conformation of the corresponding sequence in the parent protein, will it engender high affinity antibodies to the parent protein or be recognized by antibodies elicited by the parent protein.

These considerations led to the selection of synthetic Gal-Gal pilus peptides derived from regions of the pilus sequence that were predicted to contain hydrophilic beta-turns. In contrast to alpha-helices or beta pleated sheets, beta-turns are the feature of secondary structure formed principally by forces contained in the linear amino acid sequence, particularly by the four residues actually comprising the beta-turn. Therefore, peptides corresponding to beta-turn regions of the protein should be thermodynamically prone to also adopt a beta-turn conformation. Furthermore, beta-turn structures appear to be present in immunodominant regions of several other proteins (30–32). Consistent with this notion, all synthetic pilus peptides (Table I) that contain a stretch of four amino acids with a calculated probability for a reverse turn  $>1.3 \times 10^{-4}$  (10) engendered antibodies that crossreacted with HU 849 Gal-Gal pili, thus defining the corresponding domain as an antigenic epitope.

Synthetic peptides have also been used to determine the linear antigenic structure of gonococcal pili (16). Two peptides that jointly compose the gonococcal pilus intramolecular disulfide loop and correspond to regions of sequence heterogeneity were found to encode separate strain-specific, immunodominant epitopes. In contrast, the disulfide loop in Gal-Gal *E. coli* pili seems not to be an area of particular immunological interest, since only a minor immunogenic and no antigenic epitope was detected in this region. Instead, the region between R 65 and R 75 was found to encode a prominent immunogenic and antigenic determinant. A corresponding region in Gal-Gal pili prepared from *E. coli* strain 3669 contains stretches of unconserved sequence and also constitutes a major immunogenic epitope (M. A. Schmidt, P. O'Hanley, D. A. Low, unpublished observation). Therefore, the most prominent linear immunogenic epitopes of *E. coli* Gal-Gal pili from strains HU 849 and 3669 and gonococcal pili reside in areas of hydrophilic beta-turns that contain regions of sequence heterogeneity. For both organelles, the immunodominance of unconserved regions may have arisen under the selective pressure of the host immune system.

Gal-Gal pili used as pyelonephritis vaccines have prevented infection by the homologous strain in the BALB/c mouse and monkey models (3, 4). Heterologous challenge studies have not been reported; however, serological crossreactivity among Gal-Gal pili from different strains was investigated with polyclonal anti-pili sera by Western blotting and ELISA and was found to be rather limited (M. A. Schmidt, unpublished observation). Similarly, peptides corresponding to the HU 849 amino acid sequence elicited few antibodies that bound heterologous pili. Except for the anti-R 93-104 and the anti-R 5-12 sera, none of the peptide antisera recognized other Gal-Gal pili by Western blotting. This suggests that antigenic variation may be even more profound among *E. coli* Gal-Gal pili than gonococcal pili, where several peptides have been found to elicit crossreacting, receptor-blocking antibodies (16, 33).

Mutational analysis of the Gal-Gal pilus operon by Lindberg (34) and Norgren (35) may lead to a more crossreacting Gal-Gal adhesin vaccine. They have determined that cistrons encoding the pilus subunit and the adhesin per se are distinct and, by inference, that the Gal-Gal adhesin may be a heteropolymer composed of the adhesin protein, the pilus subunit, and perhaps the gene product of an additional cistron. Preliminary evidence indicates that the putative adhesin may be highly conserved. Synthetic peptides corresponding to regions of this gene product are currently under study by our laboratories.

### Summary

The linear immunogenic and antigenic structure of *E. coli* Gal-Gal pili from the recombinant strain HU 849 was investigated with nine synthetic peptides corresponding to regions of the pilus sequence predicted to contain hydrophilic beta-turns. Five peptides, as bovine serum albumin conjugates, were found by anti-HU 849 pilus serum and were thus designated "immunogenic epitopes." Peptides corresponding to R 25-38, R 38-50, and R 48-61 (which jointly comprise the single intramolecular disulfide loop), and R 103-116, were bound in low titer. A prominent immunogenic epitope was specified by a peptide corresponding to R 65-75. Four peptides, as thyroglobulin conjugates, elicited antisera in rabbits that bound intact HU 849 pili. These were designated "antigenic epitopes." Two prominent antigenic epitopes were localized to peptides corresponding to R 5-12 and R 93-104, whereas peptides corresponding to R 65-75 and R 119-131 represented two minor antigenic epitopes. None of the peptide antisera bound Gal-Gal pili from heterologous strains except anti-R 93-104 and anti-R 5-12. In 8 of the 10 Gal-Gal-binding pyelonephritis isolates tested, anti-R 5-12 detected a protein with an apparent molecular weight of 18,000 co-migrating with several Gal-Gal pili. Anti-R 93-104 detected a corresponding protein in 4 of 8 fecal and 7 of 12 pyelonephritis Gal-Gal-binding isolates; however, it also bound apparently unrelated proteins of higher molecular weight.

We thank Dr. Stanley Falkow for recombinant *E. coli* strain HU 849, Dr. Catharina Svanborg-Eden for *E. coli* strain 3669, and Dr. Kenneth Vosti for the clinical isolates used in this study.

*Received for publication 28 November 1984.*

## References

1. Leffler, H., and C. Svanborg-Eden. 1980. Chemical identification of a glycosphingolipid receptor for *Escherichia coli* attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 8:127.
2. Kallenius, G., R. Mollby, S. B. Svenson, J. Winberg, A. Lundblad, S. Svenson, and B. Cedergren. 1980. The p<sup>k</sup> antigen as receptor for the hemagglutination of pyelonephritogenic *Escherichia coli*. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 7:297.
3. O'Hanley, P., D. Lark, S. Falkow, and G. K. Schoolnik. 1984. Molecular basis of *Escherichia coli* colonization of the upper urinary tract in BALB/c mice: Gal-Gal pili immunization prevents *E. coli* pyelonephritis in the BALB/c mouse model of human pyelonephritis. *J. Clin. Invest.* 75:347.
4. Roberts, J., K. Hardaway, B. Kaack, E. Fussell, and G. Baskin. 1984. Prevention of pyelonephritis by immunization with P-fimbriae. *J. Urol.* 131:602.
5. O'Hanley, P., K. Watt, I. Romero, D. Lark, and G. K. Schoolnik. 1984. Primary structure of globoside-binding pili: antigenic determinants. *Clin. Res.* 32:377A. (Abstr.)
6. Baga, M., S. Normark, J. Hardy, P. O'Hanley, D. Lark, O. Ollson, G. Schoolnik, and S. Falkow. 1984. Nucleotide sequence of the papA gene encoding the pap pilus subunit of human uropathogenic *Escherichia coli*. *J. Bacteriol.* 157:330.
7. Hull, R., R. Gill, P. Hsu, B. Minshe, and S. Falkow. 1981. Construction and expression of recombinant plasmids encoding type I and D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. *Infect. Immun.* 33:933.
8. O'Hanley, P., D. Lark, S. Normark, S. Falkow, and G. K. Schoolnik. 1983. Mannose-sensitive and Gal-Gal-binding *Escherichia coli* pili from recombinant strains. *J. Exp. Med.* 158:1713.
9. Brinton, C. C. 1965. The structure, function, synthesis, and genetic control of bacterial pili. *Trans. NY Acad. Sci.* 27:1003.
10. Chou, P. Y., and G. D. Fasman. 1978. Empirical predictions of protein conformation. *Annu. Rev. Biochem.* 47:251.
11. Hopp, T. P., and K. R. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA.* 78:3824.
12. Erickson, B. W., and R. B. Merrifield. 1976. Solid-phase peptide synthesis. In *The Proteins*. H. Neurath, R. L. Hill, C.-L. Boeder, editors. Academic Press, Inc., New York. 2:257.
13. Kaiser, E., R. L. Colescott, C. D. Bossinger, and P. I. Cook. 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 34:595.
14. Towbin, H., Th. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350.
15. Burnette, W. N. 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195.
16. Rothbard, J. B., R. Fernandez, and G. K. Schoolnik. 1984. Strain-specific and common epitopes of gonococcal pili. *J. Exp. Med.* 160:208.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. *Nature (Lond.)*. 227:680.
18. Korhonen, T. K., H. Leffler, and C. Svanborg-Eden. 1981. Binding specificity of

- piliated strains of *Escherichia coli* and *Salmonella typhimurium* to epithelial cells, *Sacharomyces cerevisiae* cells and erythrocytes. *Infect. Immun.* 32:796.
19. Vosti, K., L. Goldberg, A. Monto, and L. Rantz. 1964. Host-parasite interaction in patients with infections due to *Escherichia coli*. I. The serogrouping of *E. coli* from intestinal and extraintestinal sources. *J. Clin. Invest.* 43:2377-2385.
  20. Vosti, K., A. Monto, and L. Rantz. 1965. Host-parasite interactions in patients due to *Escherichia coli*. II. Serologic response of the host. *J. Lab. Clin. Med.* 66:613.
  21. Olling, S. 1977. Sensitivity of gram-negative bacilli to the serum bactericidal activity: a marker of host-parasite relationship in acute and persisting infections. *Scand. J. Infect. Dis.* 10(Suppl.)1.
  22. Bjorksten, B., and B. Kaijser. 1978. Interaction of human serum and neutrophils with *Escherichia coli* strains: differences between strains isolated from urine of patients with pyelonephritis or asymptomatic bacteriuria. *Infect. Immun.* 22:308.
  23. Evans, D., D. Evans, C. Hohne, M. Noble, H. Lior, and L. Young. 1981. Hemolysin and K antigens in relation to serotype and hemagglutination type of *E. coli* isolated from extraintestinal infections. *J. Clin. Microbiol.* 13:171.
  24. Hughes, C., J. Hacker, A. Roberts, and W. Goebel. 1983. Hemolysin production as a virulence marker in symptomatic and asymptomatic urinary tract infections caused by *E. coli*. *Infect. Immun.* 39:546.
  25. Brooks, H., F. O'Grady, M. McSherry, W. Cattel. 1980. Uropathogenic properties of *Escherichia coli* in recurrent urinary tract infections. *J. Med. Microbiol.* 13:57.
  26. Davies, D., F. Falkiner, and K. Hardy. 1981. Colicin V production by clinical isolates of *E. coli*. *Infect. Immun.* 31:574.
  27. Duguid, J., S. Clegg, and M. Wilson. 1979. The fimbrial and nonfimbrial hemagglutinins of *E. coli*. *J. Med. Micro.* 12:213.
  28. Kallenius, G., R. Mollby, S. B. Svenson, I. Helin, H. Hultberg, B. Cedergren, and J. Winberg. 1981. Occurrence of P-fimbriated *Escherichia coli* in urinary tract infections. *Lancet.* 2:1369.
  29. Vaisanen, V., J. Elo, L. Tallgren, A. Siitonen, P. Makela, L. Svanborg-Eden, G. Kallenius, S. B. Svenson, H. Hultberg, and T. Korhonen. 1981. Mannose-resistant hemagglutination and P-antigen recognition characteristic of *E. coli* causing primary pyelonephritis. *Lancet.* 2:1366.
  30. Kuntz, I. D. 1972. Tertiary structure in carboxidase. *J. Am. Chem. Soc.* 94:8568.
  31. Levitt, M., and C. Cothia. 1976. Structural patterns in globular proteins. *Nature (Lond.)* 261:552.
  32. Rose, G. D. 1978. Predictions of chain turns in globular proteins on a hydrophobic basis. *Nature (Lond.)* 272:586.
  33. Schoolnik, G. K., J. Y. Tai, and E. C. Gotschlich. 1983. A pilus peptide vaccine for the prevention of gonorrhea. *Prog. Allergy.* 33:314.
  34. Lindberg, F. P., B. Lund, S. Normark. 1984. Genes of pyelonephritogenic *E. coli* required for digalactoside-specific agglutination of human cells. *EMBO (Eur. Mol. Biol. Org.) J.* 3:1167.
  35. Norgren, M., S. Normark, D. Lark, P. O'Hanley, G. K. Schoolnik, S. Falkow, C. Svanborg-Eden, M. Baga, and B. E. Uhlin. 1984. Mutations in *E. coli* cistrons affecting adhesion to human cells do not abolish papili fiber formation. *EMBO (Eur. Mol. Biol. Org.) J.* 3:11590.