MANNOSE-SENSITIVE AND GAL-GAL BINDING *ESCHERICHIA COLI* PILI FROM RECOMBINANT STRAINS

Chemical, Functional, and Serological Properties*

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Two major classes of chromosomally encoded *Escherichia coli* pili have been defined functionally by their receptor specificities. Common pili are termed "mannose-sensitive" (MS). They bind Tamm-Horsfall uromucoid and their agglutination of guinea pig erythrocytes is inhibited by D-mannose. In contrast, mannose-resistant (MR) pili agglutinate human erythrocytes in the presence of D-mannose. Most human pyelonephritis *E. coli* isolates express MR pili that bind neutral glycosphingolipid constituents of uroepithelial cells (1). They contain D-Gal p $\alpha 1 \rightarrow 4$ D-Gal p $\beta 1$ and a synthetic analogue of this disaccharide (Syn Gal-Gal) inhibits hemagglutination (2).

Mannose, Gal-Gal, and X pili may co-exist on the same bacterial strain (3, 4). Consequently, the pathogenic significance of functionally distinct pili may be difficult to assess with clinical isolates. Therefore, Hull et al. (4) cloned two distinct *E. coli* chromosomal fragments that encode mannose or Gal-Gal pili, into a nonpiliated K-12 derivative. The functional, serologic, and chemical properties of pili prepared from these recombinants is the subject of this report.

Materials and Methods

Bacterial Strains

E. coli J96 (O4, K6), a pyelonephritis isolate, is hemolytic, colicin V positive, motile, and can simultaneously express MS and Gal-Gal pili (4). The construction of the two recombinant strains, SH48 and HU849, which express MS or Gal-Gal pili, respectively, is described in detail elsewhere (4). P678-54 was employed as a hemagglutination-negative control.

Pili Purification

Pili from strains J96, SH48, and HU849 were purified from organisms grown on trypticase soy agar for 18 h at 37°C according to the method of Brinton (5). After three cycles of precipitation and solubilization in the appropriate buffers (5), the HU849 pilus preparation formed a single protein band in SDS-PAGE according to the method of

* This work was supported by a grant from the Cetus Corporation and Public Health Grant (NIH) AI-18719.

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Laemmli (6). SH48 pili did not enter the stacking gel under these conditions. Instead, the pilus filaments were depolymerized before electrophoresis by the addition of HCl to pH 1.8 according to the method of McMichael and Ou (7), after which two protein bands characteristic of MS pili were detected. Negatively stained pilus preparations were shown by electron microscopy to be composed of homologous filaments.

Amino Acid Analysis

The amino acid compositions of HU849 and SH48 pili were determined after 24-h hydrolysis in 5.7 N HCl at 110°C in vacuo on a Durrum model D500 amino acid analyzer. Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively after performic acid oxidation (8).

Sequence Analysis

The amino terminal amino acid sequences of HU849 and SH48 pili were determined by automated Edman degradation on a Beckman Model 890C liquid phase sequencer. Each phenylthiohydantoin (Pth) derivative was identified and quantitated by reverse-phase high pressure liquid chromatography (HPLC) and confirmed by gas-liquid chromatography (GLC) and/or thin-layer chromatography (TLC).

Enzyme-Linked Immunosorbent Assay (ELISA)

Antibody to each pilus preparation was elicited in rabbits by systemic immunization. A two-step competitive ELISA (9) was employed to deduce, from the slopes of the inhibition curves, the shared antigenicity between the two pilus proteins.

Receptor Binding Assays

A. Hemagglutination (*HA*) was performed with blood group P_1 human and guinea pig erythrocytes by slide agglutination: Whole bacteria (2×10^3 to 2×10^8 CFU/ml) or purified pili (0.1–1.0 mg/ml) were mixed with an equal volume of a 3% (vol/vol) erythrocyte suspension in phosphate-buffered saline, pH 7.2 (PBS).

B. Hapten inhibition of hemagglutination (HAI) by carbohydrate receptor analogues was performed in PBS by mixing 2% (wt/vol) α methyl mannoside (α MM) (Sigma Chemical Co., St. Louis, MO) or 4% (wt/vol) synthetic D-Gal *p* α1 → 4 D-Gal *p*β OR (Syn Gal-Gal) with whole bacteria or isolated pili. The chemical synthesis of the disaccharides D-Gal *p* α1 → 4D-Gal *p* βOR and D-Man *p* α1 → 2D-Man *p* αOR (Syn Man-Man), *vide infra*, as their 8-methoxycarbonyloctyl-glycosides (R = —$(CH_2)_8$ $COOCH_3$) was performed according to the method of Lemieux (10) by Chembiomed Ltd., (Edmonton, Alberta T6G 2G2, Canada). After preincubation for 1 h at 25°C, an equal volume of a 3% (vol/vol) erythrocyte suspension was added and the reactants were gently mixed.

C. Latex agglutination (*LCA*) was employed to define pilus receptor specificity. Syn Gal-Gal or Syn Man-Man was adsorbed to latex beads (Chembiomed Ltd.). Lactose-adsorbed latex was used as an agglutination-negative control. Equal volumes of whole bacteria or isolated pili were mixed with a 1% (vol/vol) latex suspension in PBS. A11 HA, HAI, and LCA assays were read after 5 min.

Results

Pilus Protein Structure. Pili purified from strain J96 were depolymerized according to the method of McMichael and Ou (6) and three proteins of M_r 19 kdaltons, 17.5 kdaltons, and 17 kdaltons were noted by SDS-PAGE. When the pili were prepared according to the method of Laemmli (5) without acid depolymerization, only the 17.5-kdalton protein was detected. HU849 Gal-Gal pili migrated with the 17.5-kdalton protein; SH48 MS pili only entered the gel after acid depolymerization and migrated with the 19- and 17-kdalton proteins.

The amino acid compositions of the pili from the recombinant strains were similar (Table I). Two cysteine residues per subunit were detected for both pilus

TABLE I

Amino Acid Composition of Mannose-Sensitive (SH48) and Gal-Gal Binding (HU849) Pili of the Pyelonephritic E. coli Strain J96

Amino acid	Integral number: residues per subunit*	
	SH48 (MS)	HU849 (Gal-Gal)
Ala	35	17
Val	14	17
Leu	14	9
Ile	5	6
Pro	2	5
Phe	8	7
Trp	ND‡	ND
Met§	ND	1
Asx	18	19
Glx	16	13
Lys	4	10
Arg	2	ND
His	2	2
Gly	21	21
Ser	9	11
Thr	20	12
½ Cys§	2	2
Tyr	2	2

* Based on a mol wt of 18 kdaltons (SH48 pili) or 17.5 kdaltons (HU849 pili) estimated by SDS-PAGE.

‡ *ND*, No amino acid was detected.

§ Cysteine was analyzed as cysteic acid and methionine as methionine sulfone.

preparations and these form an intra-chain disulfide bond since no sulfhydryl group was detected by amino acid analysis after alkylation of unreduced pili (11). The presence of a disulfide loop has been detected in other *E. coli* pili (9) and may be a conserved feature of the tertiary structure of these proteins. The amino acid compositions reported here and elsewhere (9) indicate the presence of one methionine residue per Gal-Gal pilus subunit. In contrast, MS pili lack this residue (7, 12) and cannot be intrinsically labeled with [^{35}S]methionine (Paul Orndorff, personal communication).

The amino-terminal amino acid sequences of the pili from the recombinant strains were determined through residue 46 (Table II). When the cysteine residues are aligned, 13 of the first 46 positions (27%) were homologous. The amino acid sequences of SH48 MS pili (Table II) and *E. coli* BAM (9) are identical through residue 26. In contrast, only 32% of the first 26 residues of HU849 pili and Gal-Gal pili from the uropathic *E. coli* strains 3669 and 3048 (9), are homologous. Proline exists within the first 21 residues (Table II) of the three Gal-Gal pili for which N-terminal amino acid sequences are available (9) and may have significance for secondary structure by helix termination or reverse-turn nucleation.

Serologic Properties of Pili. <5% shared antigenicity was detected between the two pilus proteins (Fig. 1); therefore, the homologous regions that exist within the N-terminal 46 residues do not specify an immunodominant antigenic determinant.

Binding Properties of Whole Bacteria and Isolated Pili. *E. coli* strain J96 agglutinated human and guinea pig erythrocytes and latex particles adsorbed with Syn Man-Man and Syn Gal-Gal. Hapten inhibition of J96 hemagglutination by αMM and Syn Gal-Gal was demonstrated (Table III). SH48 agglutinated only guinea

TABLE II

Amino Terminal Amino Acid Sequence

Recombinant strain	Receptor specificity	Residue no. 1	2	3	4	5	6
HU849	Gal-Gal*	Ala	Pro	Thr	Ile	Pro	Gln
SH48	Mannose	—	Ala	Ala	Thr	Thr	Val

	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
HU849	Pro	Gln	Gly	Gln	Gly‡	Lys	Val	Thr	Phe	Asn	Gly	Thr	Val	Val	Asp
SH48	Thr	Val	Asn	Gly	Gly	Thr	Val	His	Phe	Lys	Gly	Glu	Val	Val	Asn
	20	21	22	23	24	25	26	27	28	29	30	31			
HU849	Ala	Pro	Cys	Ser	Ile	Ser	Gln	Lys	Ser	Ala	Asp	Gln			
SH48	Ala	Ala	Cys	Ala	Val	Asp	Ala	Gly	(Thr)§	Val	Asp	Gln			
	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
HU849	Ser	Ile	Asp	Phe	Gly	Gln	Leu	Ser	Lys	Ser	Phe	Leu	Glu	Ala	Gly
SH48	Thr	Val	Gln	Leu	Gly	Gln	Val	Arg	Thr	Ala	Thr	Leu	Ala	Gln	Glu

* Gal-Gal: D-Gal *p* α 1 → 4D-Gal *p* β1.

‡ Underlined residues are conserved.

§ Residue identified by reverse-phase high-pressure liquid chromatography of Pth derivative, only.

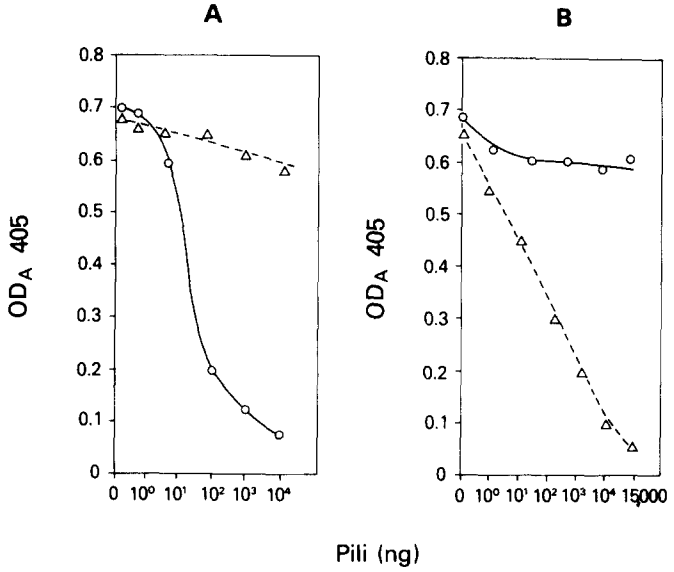

FIGURE 1. The antigenic relatedness of Gal-Gal pili from recombinant strain HU849 and MS pili from recombinant strain SH48 by a two-step competitive ELISA (15). (*A*) Inhibition of the binding of Gal-Gal pili antibody to microtiter wells coated with Gal-Gal pili by increasing concentrations of soluble Gal-Gal pili (○) or MS pili (△). (*B*) Inhibition of the binding of MS pili antibody to microtiter wells coated with MS pili by increasing concentrations of soluble MS pili (△) or Gal-Gal pili (○).

pig erythrocytes and latex particles adsorbed with Syn Man-Man. HU849 agglutinated only human erythrocytes and latex particles adsorbed with Syn Gal-Gal. The purified pili exhibited the binding specificities of the recombinant strain from which they were prepared (Table III). Thus, the binding properties of the parent strain and both recombinants are conferred by two pilus proteins that are genetically, chemically, antigenically, and functionally distinct.

TABLE III

Agglutinating Properties of E. coli Strains and Isolated Pili

Agglutinin / Bacteria	Erythrocyte species: Human HA*/HAI‡	Erythrocyte species: Guinea pig HA/HAI	Disaccharide adsorbed latex beads: Carbohydrate§ Syn Gal-Gal	Syn Man-Man	Lactose
J96	+/Syn Gal-Gal	+/αMM	+	+	−
HU849	+/Syn Gal-Gal	−/−	+	−	−
SH48	−/−	+/αMM	−	+	−
P678-54	−/−	−/−	−	−	−
Pilus protein					
HU849	+/Syn Gal-Gal	−/−	+	−	−
SH48	−/−	+/αMM	−	+	−

* Hemagglutination of human or guinea pig erythrocytes: + positive; − negative. Minimal hemagglutinating concentrations of bacteria for human and guinea pig erythrocytes were 5×10^4 CFU/ml and 2.5×10^3 CFU/ml, respectively. The minimal hemagglutinating concentration of pilus protein for human and guinea pig erythrocytes was 250 μg/ml.

‡ Hapten inhibition of hemagglutination by Syn Gal-Gal (4% wt/vol) or αMM (α methyl mannoside) (2% wt/vol).

§ Agglutination of latex beads adsorbed with Syn Gal-Gal or Syn Man-Man or Lactose: + positive; − negative.

Discussion

Chromosomal genes that encode MS or Gal-Gal pili have been cloned from an uropathic *E. coli* strain and the recombinants used in a murine urinary tract infection model (13, 14). The Gal-Gal piliated recombinant strain attached to exfoliated uroepithelial cells in vitro (15) and colonized renal tissue in vivo (13, 14). A vaccine composed of pure Gal-Gal pili blocked renal colonization and invasion by the parent strain (14). In contrast, the MS recombinant bound Tamm-Horsfall uromucoid, but did not colonize the renal epithelium (14). Furthermore, a MS pilus vaccine conferred no protection. Pili were purified from these recombinants and their chemical, functional, and serologic properties determined.

The N-terminal amino acid sequences of MS and Gal-Gal pili exhibit considerable homology and are highly homologous with the published N-terminal sequences for five MS and five MR pili from other uropathic *E. coli* (9, 16). Two common structural features characterize these sequences. All exhibit the pattern -Gly-X_1-Val-X_2-Phe-X_3-Gly-X_4-Val-Val-Asx-Ala-X_5-Cys-, where the intervening residues (X) tend to be charged or polar uncharged; and all appear to possess two cysteine residues in disulfide linkage. C-terminal homology between MS and MR pili also exists. The HU849 Gal-Gal pilus C-terminal amino acid sequence was determined by automated Edman degradation of a tryptic fragment (O'Hanley, unpublished observation) and aligned for maximal homology with the published C-terminal sequence of Type IB and IC pili (16, 17). The Gal-Gal and MS sequences are -*Phe-Asn*-Leu-Thr-*Tyr-Gln*-COOH and -Thr-*Phe-Asn*-Val-*Tyr-Gln*-COOH, respectively. These structural features suggest that pili arose from a common ancestral gene and that the conserved regions are functionally or physically critical. In contrast, plasmid-encoded pili from enteropathic *E. coli* exhibit no apparent homology with MS or Gal-Gal pili (17) and may therefore be evolutionarily unrelated.

The functional differences between MS and Gal-Gal pili suggest that relatively little homology exists in portions of these molecules that encompass the receptor

binding domain. Distinct receptor specificities may confer a selective advantage in ecologic niches characterized by different host molecules in mucus or on cell surfaces. The discovery of chromosomally determined MR pili of "X" specificity (3) indicates that the binding repertoire of pili may be extensive.

The antigenic differences between MS and Gal-Gal pili (Fig. 1) indicate that homologous regions of the sequence are immuno-recessive. Unconserved regions must, therefore, determine the serologic diversity of these pili. This may be a concomitant of their separate carbohydrate binding specificities if their antigenic and receptor binding domains are co-extensive. However, the carbohydrate binding domains and the antigenic determinants of lectin like proteins appear to be structurally dissimilar. The carbohydrate combining site usually consists of an hydrophobic cleft in the protein's surface; whereas, antigenic determinants are frequently specified by the region of greatest average hydrophilicity. Serologic diversity may have resulted from selective pressure imposed by the host immune system. Therefore, the immunodominant antigenic determinant and the receptor binding domain of *E. coli* pili may be chemically and evolutionarily distinct.

Summary

Chromosomal genes encoding the MS and Gal-Gal binding properties have been cloned into separate recombinants and their respective pili characterized. Hapten inhibition of hemagglutination with synthetic carbohydrate receptor analogues and carbohydrate-adsorbed latex agglutination studies indicate that Gal-Gal and MS pili collectively exhibit the binding properties of the parent strain. MS pili migrated in SDS-PAGE with an M_r of 19 kdaltons and 17 kdaltons; the M_r of Gal-Gal pili was 17.5 kdaltons. The pili are chemically similar by amino acid composition and when the N-terminal cysteines are aligned, 8 of the 13 residues between positions 9 and 22 are homologous. Further, carboxy-terminal sequence homology was inferred from the carboxypeptidase digestion of a MS pili and the sequence of a carboxy-terminal tryptic peptide from Gal-Gal pili.

Received for publication 11 July 1983 and in revised form 6 September 1983.

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