The EMBO Journal vol.3 no.5 pp.1167 – 1173, 1984

Genes of pyelonephritogenic *E. coli* required for digalactoside-specific agglutination of human cells

Frederik P.Lindberg*, Björn Lund and Staffan Normark

Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden

*To whom reprint requests should be sent
Communicated by B.Daneholt

Most pyelonephritic *Escherichia coli* strains bind to digalactoside-containing glycolipids on uroepithelial cells. Purified Pap pili (pili associated with pyelonephritis) show the same binding specificity. A non-polar mutation early in the *papA* pilin gene abolishes formation of Pap pili but does not affect the degree of digalactoside-specific hemagglutination. Three novel *pap* genes, *papE, papF* and *papG* are defined in this report. The *papF* and *papG* gene products are both required for digalactoside-specific agglutination by whole bacteria cells as well as for agglutination by pilus preparations. Pili prepared from a *papE* mutant have lost their binding ability although whole cells from this mutant retain it, implying an adhesin anchoring role for the *papE* gene product. A mutant with lesions both in the *papA* and the *papE* genes does not mediate digalactoside-specific agglutination. The implications of this finding for pilus biogenesis are discussed.
Key words: adhesin/hemagglutinin/lectin/microbial pathogenesis/pili genetics

Introduction

It has become increasingly clear that carbohydrates attached to the cell surface may act as receptors for lectins or adhesins present on infectious agents like viruses, bacteria and parasites. Such receptor-adhesin interactions are now considered of prime importance for many infectious diseases (Beachey, 1981). For pathogenic Gram-negative bacteria there is evidence that filamentous structures termed pili mediate adherence. However, it has not been determined what portions of these structures specify receptor-specific binding to host cells.

Recently a receptor for uropathogenic *Escherichia coli* was identified (Källenius *et al.*, 1980; Leffler and Svanborg-Edén, 1981). The minimal receptor structure was shown to be the α-D-Gal*p*-(1-4)-β-D-Gal*p* moiety, the digalactoside, present in the globoseries of glycolipids (Svenson *et al.*, 1983). This receptor, to which bacteria may attach in the uroepithelium, is also present on human erythrocytes as a part of the P bloodgroup antigens. p̄ erythrocytes lack this antigen and are not agglutinated by these bacteria (Källenius *et al.*, 1980).

To investigate the mechanism of interaction between the bacterial adhesin and this host receptor we have studied the genetic determinants specifying digalactoside-specific agglutination. A chromosomal fragment isolated from the uropathogenic *E. coli* isolate J96 was cloned (Hull *et al.*, 1981) and shown to carry these determinants and to express Pap pili, pili associated with pyelonephritis (Normark *et al.*, 1983a). The 8.5-kb region required for Pap pilus formation and receptor-specific agglutination has been shown to code for at least eight different polypeptides (Normark *et al.*, 1983a). The pilin, the subunit of the purified pilus, has an apparent mol. wt. of 19.5 kd and is the product of the *papA* gene. This gene has recently been sequenced (Båga *et al.*, 1984), and the amino acid sequence deduced from the 5′ end of the gene agrees exactly with the amino-terminal sequence that has been established for the Pap pilus (O'Hanley *et al.*, 1983). The gene for a 13-kd polypeptide is located immediately before the pilin gene and is named *papB*, while the genes following *papA* are called *papC* and *papD* and express 81-kd and 28.5-kd proteins, respectively (Norgren *et al.*, accompanying paper). Further downstream lie genes for 16.5-kd, 15-kd and 35-kd proteins which are defined in this paper.

Tn*5* insertions abolishing digalactoside-specific hemagglutination, but not Pap pilus formation, were located in the distal part of the *pap* region (Normark *et al.*, 1983a). In addition, a Tn*5* insertion late in the structural gene for the pilin, *papA*, was still weakly positive for hemagglutination. These unexpected results led us to undertake a study, aimed at a genetic definition of the digalactoside-specific adhesin. In this paper we show that the *papA* pilin is not required for receptor-specific hemagglutination. Also, a mutant containing a lesion in the *papE* gene retains its agglutinating ability. A *papA,papE* double mutant, however, does not mediate this phenotype. We also define two additional *pap* genes, *papF* and *papG*, both required for digalactoside-specific binding.

Results

Properties of a frameshift mutant in the structural gene for the major subunit in Pap pili

To assess the actual role of the structural gene encoding Pap pili, *papA*, for hemagglutination, we wanted to construct a derivative with a lesion within the *papA* gene but otherwise isogenic with the wild-type. For this purpose the large *Eco*RI-*Bam*HI fragment of pRHU30, which codes for Pap pili and digalactoside-specific binding to globoside receptors (Normark *et al.*, 1983a), was cloned into pBR322 giving plasmids pPAP5 (Figure 1) and pPAP22, as described in Materials and methods. Both pPAP5 and pPAP22 carry the whole *Eco*RI-*Bam*HI insert, although pPAP22, due to a deletion in vector DNA, carries a unique *Pvu*II site in the *papA* structural gene. To investigate the importance of the *papA* gene product, we constructed plasmid pPAP23 with the frameshift mutation, *papA1*, by introducing an 8 bp long *Xho*I linker at the unique *Pvu*II site in pPAP22 (Figure 2). From the DNA sequence of the *papA* gene (Båga *et al.*, 1984), the mutated gene is predicted to give rise to a translational product carrying the signal peptide and 29 amino acids of the N-terminal portion of the normally 163 amino acid long *papA* pilin followed by 28 erroneous amino acids encoded by the linker and the +1 frame. In *E. coli* HB101, this frameshift mutant, unlike the wild-type, was not agglutinated by antiserum raised against purified Pap pili, nor was any *papA* gene product detected in an *E. coli* minicell expression system (Figure 3). Also, the *papA* frameshift mutation in pPAP23 shows no or very little

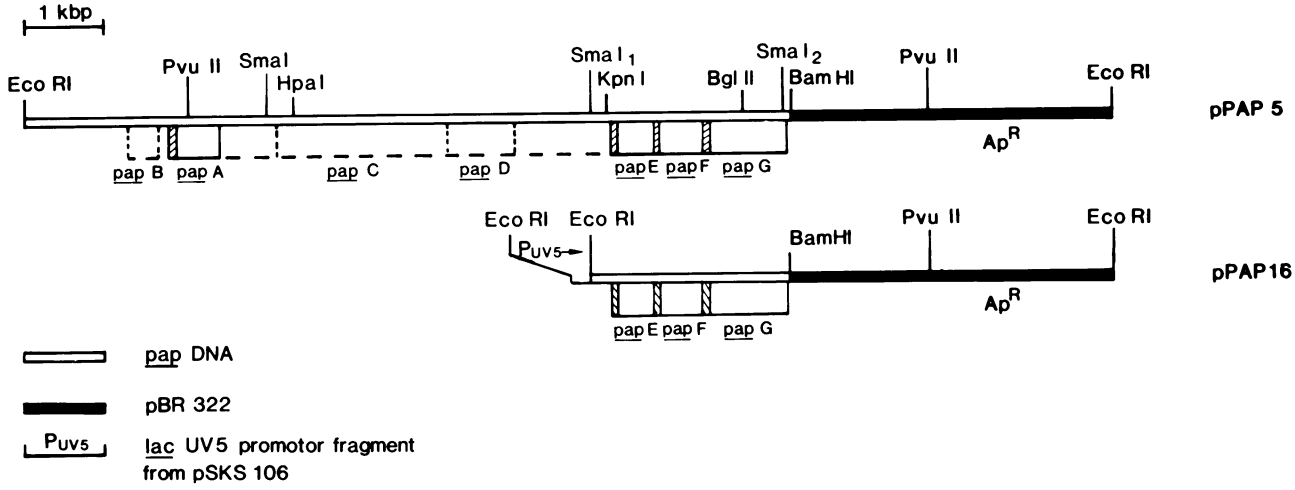

Fig. 1. Restriction map and gene organization of *pap* hybrid plasmids used for *in vitro* mutagenesis. Plasmid pPAP5 carries the entire *Eco*RI-*Bam*HI fragment necessary for expression of Pap pili and digalactoside-specific agglutination of human erythrocytes. Plasmid pPAP16 carries only the *SmaI*$_1$-*Bam*HI fragment under transcriptional control from the *lacUV5* promoter.

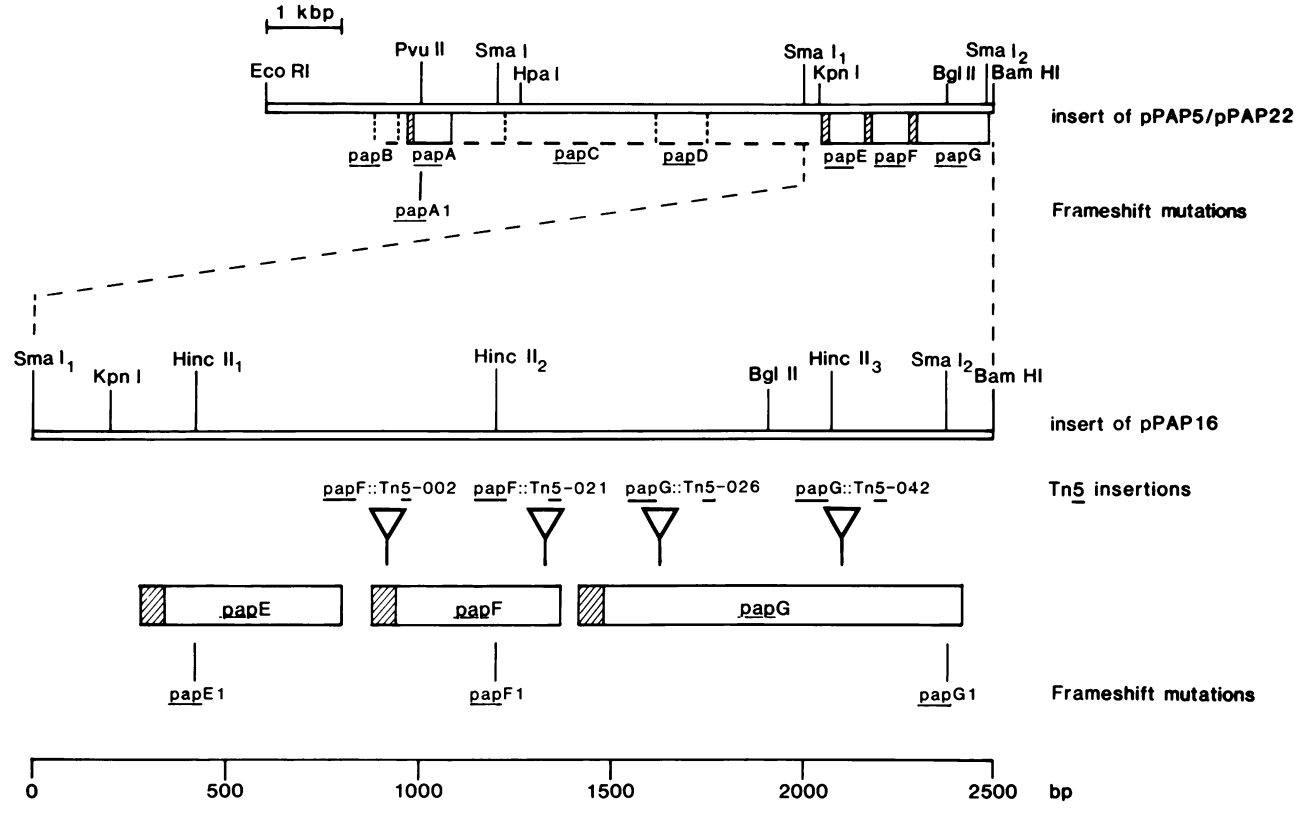

Fig. 2. The top half of the figure shows the whole *pap* region as found in pPAP5 or pPAP22. pPAP22 is identical to pPAP5 except that it lacks the *Bam*HI-*Pvu*II part of the vector DNA. This plasmid is the parent of the *papA1* derivative pPAP23. Also the position of the *papA1* mutation is shown. The lower part of the figure shows the physical map of the *SmaI*$_1$-*Bam*HI region. The position of the Tn*5* insertions in this region are shown. All destroy the capacity to mediate hemagglutination. Below this, the position of the *papE, papF* and *papG* genes are shown. The hatched area represents the putative signal peptides which are believed to be encoded by those genes (data not shown). Also the position of the *papE1*, *papF1* and *papG1* mutations are shown. They have been introduced separately into both pPAP5 and pPAP16.

polarity effects on more distal genes, as judged by protein expression in minicells.

E. coli HB101 harboring pPAP23 agglutinates human P_1-erythrocytes, as well as digalactoside-coated latex beads. No agglutination occurred using latex beads coated with α-D-Man*p*-(1-2)-D-Man*p*, lactose, or $\bar{p}$-erythrocytes, which lack the receptor carbohydrate (Källenius *et al.*, 1980). Hence, pPAP23/HB101 appears to express the same receptor binding specificity as HB101 carrying the wild-type *pap* operon on pPAP22 or pPAP5. To roughly quantify the minimum number of bacteria required for agglutination, we added washed P_1-erythrocytes to serial, 2-fold dilutions of bacteria. The maximal concentration tested (3 x 10^9 bacteria/ml) did not give agglutination when tested with the HB101 host

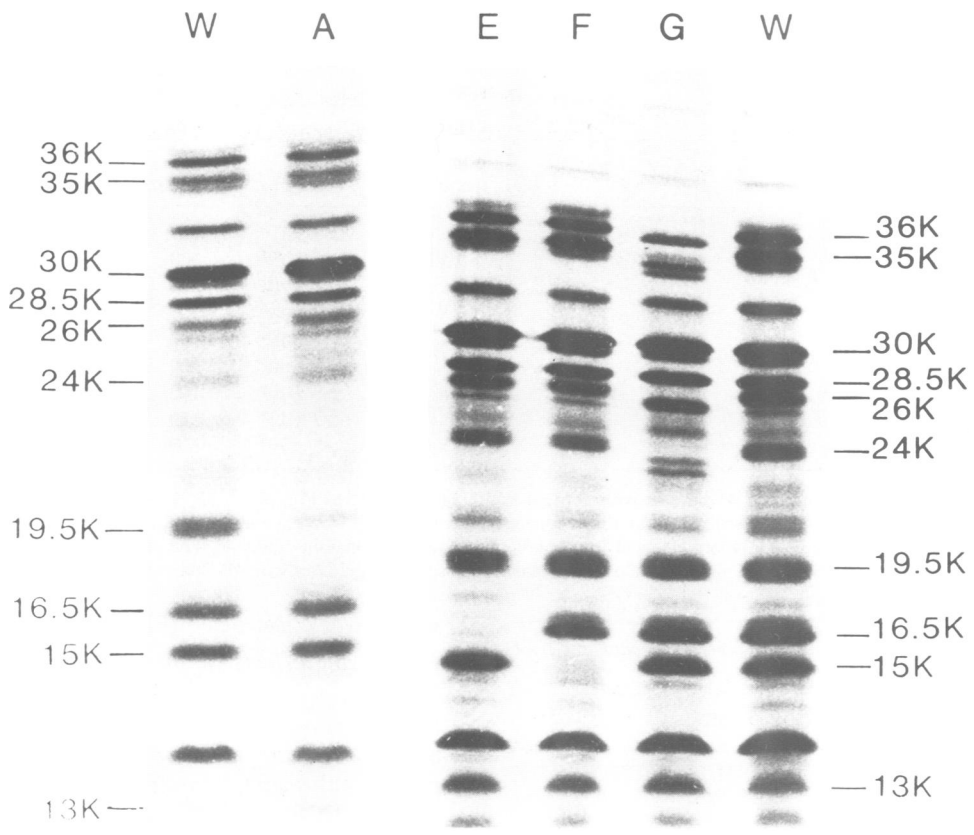

Fig. 3. Autoradiogram of SDS-polyacrylamide gels of [^{35}S]methionine-labeled proteins expressed from the different plasmid derivatives in an *E. coli* minicell expression system. Apparent mol. wts. in daltons of the relevant proteins are indicated in the figure. Proteins below 10 kd or above 40 kd are not shown. **Lanes W** and **A** in the left part of the figure contain proteins expressed from the wild-type (pPAP5) and the *papA1* mutant (pPAP23), respectively. As can be seen, the pilin (19.5 kd) is not expressed from the mutant. In the right part of the figure the wild-type **(lane W)** is shown parallel to the *papE1* (pPAP15), *papF1* (pPAP14) and *papG1* (pPAP7) mutants in **lanes E, F** and **G**, respectively.

alone. For HB101, carrying either the wild-type operon on pPAP5 or the *papA1* mutant (pPAP23), the corresponding levels were 1 x 10^7 bacteria/ml, in both cases (Table I). Thus inactivation of the pilin gene, *papA*, did not diminish the degree of digalactoside-specific agglutination in the assay used.

Genetic characterization of the distal region of pap DNA required for agglutination

Tn*5* insertions in the distal part of *pap* DNA abolish hemagglutination but allow Pap pili to be formed (Norgren *et al.*, accompanying paper). We assumed that the genes mediating agglutination would be located in this region. To investigate further the importance of the proteins encoded here we subcloned the *Sma*I$_1$-*Bam*HI fragment, shown in Figure 1, into pBR322 (see Materials and methods). Since the resulting plasmid did not complement Tn*5* insertions in the *Sma*I$_1$-*Bam*HI region, the cloned fragment, in order to ensure adequate transcription of the genes on the fragment, was put under the transcriptional control of a *lac*UV5 promoter which was inserted into the plasmid as an *Eco*RI fragment derived from pSKS106 [Casadaban *et al.*, 1983; (see Materials and methods)]. This construct, pPAP16 (Figure 1), complemented the four non-hemagglutinating Tn*5* mutants with insertion points in the *Sma*I$_1$-*Bam*HI fragment, as shown in Table II. The localization of these mutations is shown in Figure 2.

To define further the genes on the *Sma*I$_2$-*Bam*HI fragment, we constructed a detailed restriction map of the pPAP16 insert with an accurate localization of relevant Tn*5* insertions (Figure 2). We also made three frameshift mutational derivatives of pPAP5 containing lesions in this region (Figure 2 and Materials and methods). Two mutants plasmids, pPAP14 and pPAP15, carry *Xho*I linkers in the *Hinc*II$_2$ site and *Hinc*II$_1$ sites, respectively. In a third mutant, pPAP7, *pap* DNA from *Sma*I$_2$ to *Bam*HI (Figure 2) is deleted.

The proteins expressed from pPAP5 and its three mutant derivatives were [^{35}S]methionine-labeled in *E. coli* minicells and the proteins expressed were analyzed on an SDS-polyacrylamide gel (Figure 3). Plasmid pPAP7, when compared with pPAP5, did not express the 35-kd polypeptide. Instead a new polypeptide of 34 kd was seen. Since the mutation in pPAP7 truncates the *pap* region at the *Sma*I$_2$ site this would map the 3′ end of the gene coding for the 35-kd protein, *papG*, between the *Sma*I$_2$ and the *Bam*HI sites (Figure 2). This is the last gene in the *pap* region. Also a 26-kd and a 24-kd polypeptide, as well as the 36-kd precursor of the PapG protein, are affected by the deletion mutation at the *Sma*I$_2$ sites, *papG1*, and new polypeptides of ~1 kd lower mol. wts. are seen.

The *Hinc*II$_2$ mutation in pPAP14 abolishes expression of the 15-kd protein (Figure 3), as do Tn*5* insertions 002 and 021 (Norgren *et al.*, accompanying paper), which accurately maps the gene, *papF*, for this protein (Figure 2). No other polypeptides were affected by the *Hinc*II$_2$ mutation *(papF1)* in pPAP14. The minicell preparation of the *Hinc*II$_1$ linker insertion mutant, pPAP15, does not produce the 16.5-kd polypep-

Table I. Effect of *papA, papE, papF* and *papG* mutations on receptor-specific hemagglutination

Strain	Mutation	Pilus antigen	Hemagglutination				
			Cells		Pili		
			p̄-Ery (bact/ml)	P_1-Ery (bact/ml)	p̄-Ery (μg/ml)	P_1-Ery (μg/ml)	Ratio (p̄/P_1)
HB101	(host)	−	>3 x 10^9	>3 x 10^9	ND	ND	ND
pPAP5 /HB101	wt	+	>3 x 10^9	1 x 10^7	400	2	200
pPAP23/HB101	*papA1*	−	>3 x 10^9	1 x 10^7	ND	ND	ND
pPAP15/HB101	*papE1*	+	>3 x 10^9	2 x 10^7	200	50	4
pPAP14/HB101	*papF1*	+	>3 x 10^9	>3 x 10^9	32	16	2
pPAP7 /HB101	*papG1*	+	>3 x 10^9	>3 x 10^9	200	200	1
pPAP19/HB101	*papF1, ΔpapG*	+	>3 x 10^9	>3 x 10^9	100	50	2
pPAP20/HB101	*papE1, Δ(papF-papG)*	+	>3 x 10^9	>3 x 10^9	50	50	1
pPAP26/HB101	*papA1, papE1*	−	>3 x 10^9	>3 x 10^9	ND	ND	ND

Pilus antigen was monitored by agglutination of whole cells with antisera raised against purified Pap pili. Cells positive in this assay also express Pap pili as monitored by electron microscopy (data not shown). The conditions for hemagglutination of whole bacteria cells or purified Pap pili are described in Materials and methods. Cells harbouring pPAP14 express significantly less pili than cells containing the wild-type plasmid. Due to this the pilus preparations from pPAP14/HB101 cells are less pure and this might explain the increased non-specific agglutination.

Table II. Complementation analysis of mutants in the *papE, papF* and *papG* genes

Plasmid	Mutation	pSN002 *papF*::Tn*5*-002	pSN021 *papF*::Tn*5*-021	pSN026 *papG*::Tn*5*-026	pSN042 *papG*::Tn*5*-042
pPAP16	wt	+	+	+	+
pPAP18	*papE1*	+	+	+	+
pPAP17	*papF1*	−	−	+	+
pPAP4	*papG1*	+	+	−	−

papE1, papF1 and *papG1* derivatives of pPAP16, which carry the *SmaI*$_1$-*Bam*HI region under *lacUV5* promoter control, were transformed into HB101 harboring the Tn*5* derivatives of pRHU845 (Normark *et al.*, 1983a). Complementation was monitored by agglutination of P_1-erythrocytes, and digalactoside-coated latex beads.

tide (Figure 3). The gene for this protein is called *papE* and the described frameshift mutation will be referred to as *papE1*. The truncation of the *papG* gene products by the *SmaI*$_2$-*Bam*HI deletion shows that this gene is transcribed from left to right in Figure 2. Polarity effects exerted by Tn*5* insertions in *papE* and *papF* on *papG* (Norgren *et al.*, accompanying paper) show that the transcription of all three genes is in this direction.

Complementation analyses

To confirm the position of the Tn*5* mutations relative to the *papF* and *papG* genes, we tried to complement Tn*5* mutations 002, 021, 026 and 042 (Figure 2) with the mutated *SmaI*$_1$-*Bam*HI region. For this purpose, *papE1, papF1* and *papG1* derivatives of pPAP16, which carry the *SmaI*$_1$-*Bam*HI region under *lac*UV5 promoter control, were constructed as described in Materials and methods. These were then transformed into HB101 carrying the Tn*5* derivatives of pRHU845 (Normark *et al.*, 1983a), and assayed for digalactoside-specific agglutination. As shown in Table II the *papE1* derivative complemented all Tn*5* mutations as did the parent pPAP16. The *papF1* plasmid complemented Tn*5* mutations 026 and 042, whereas, the plasmid carrying *papG1* complemented mutations 002 and 021. This defines the 002 and 021 Tn*5* insertions as mutations in *papF* and shows that Tn*5* insertions 026 and 042 reside in the *papG* gene. It also clearly shows that *papF* and *papG* are separate, independently *trans*-complementable genes. From these data the gene map of this region was constructed (Figure 2).

Phenotypic effects of the papE1, papF1 and papG1 mutations

Norgren *et al.* (accompanying paper) have shown that Tn*5* insertions in *papF* and *papG* abolish hemagglutination completely, although pili are formed. Since all these insertions, in contrast to the mutants presented in this paper, are highly polar on more distal genes, the individual importance for hemagglutination of the *papE, papF* and *papG* gene products could not be assessed. To do this, we used the non-polar linker insertion mutant derivatives of pPAP5 in hemagglutination tests. As shown in Table I, neither the *papF1* not the *papG1* derivative showed an agglutinatin of P_1-erythrocytes, demonstrating that both the *papF* and the *papG* gene products are necessary for agglutination. The *papE1* mutation, however, did not affect the hemagglutination titer showing that the *papE* product is dispensable for this phenotype. Having shown that neither *papA* nor *papE* is required for receptor-specific agglutination, we made the *papA1,papE1* double mutant pPAP26. Surprisingly, this construction in HB101 did not agglutinate P_1-erythrocytes, or digalactoside-coated latex beads.

Pap pili purified from HB101 carrying pPAP5 and its mutational derivatives

As expected we did not succeed in preparing pili from the *papA1* derivative (pPAP23), using the same purification procedure as for the wild-type. Pili were, however, obtained from the *papE1, papF1* and *papG1* mutants. Pap pili were also produced in HB101 carrying plasmids pPAP19 *(papF1, ΔpapG)* or pPAP20 *(papE1, Δ(papF-papG))*. On an SDS-polyacrylamide gel the apparent size of the pilin was the same in all preparations (data not shown). When the pili preparations were titrated for hemagglutination in Mg^{2+}-free agglutination buffer, wild-type pili from pPAP5/HB101 agglutinated P_1-erythrocytes at a protein concentration of 200 μg/ml or higher. No other preparation agglutinated P_1-erythrocytes at the highest concentration tested (1600 μg/ml).

Neither the wild-type nor the mutant pili preparations agglutinated p̄-erythrocytes, which lack the digalactoside-containing globoside receptor. Addition of 100 mM $MgCl_2$ to the agglutination buffer aggregates pili and increases the sensivity of the hemagglutination assay for purified pili ~100-

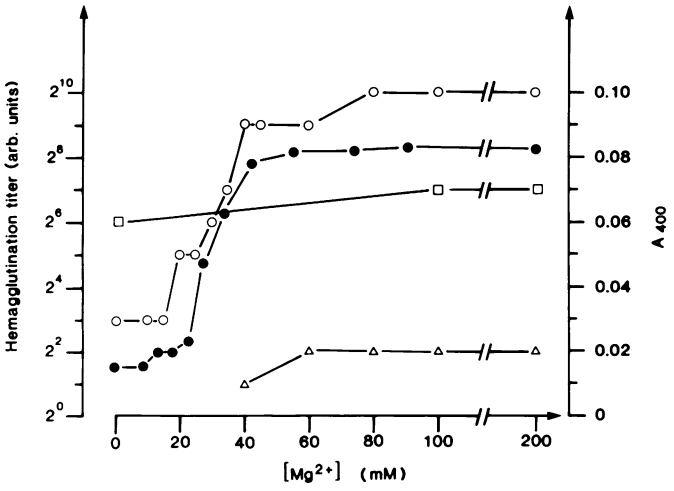

Fig. 4. Titration for hemagglutination was done in 150 mM NaCl-10 mM Tris-HCl (pH 7.5) containing increasing concentrations of $MgCl_2$. The figure demonstrates the dependence of the hemagglutination titer (open symbols) on the Mg^{2+} concentration for P_1-erythrocytes (○) or p̄-erythrocytes (△) using pili prepared from the wild-type pPAP5/HB101. A titer of 2° corresponds to a pilus-concentration of 1600 μg/ml. Also shown is the titration of whole cells with P_1-erythrocytes (□) where 2° corresponds to 5 x 10^9 cells/ml. Pilus aggregation was monitored by A_{400} of a 200 μg/ml-pilus solution at increasing $MgCl_2$ (●).

fold, without significantly affecting the hemagglutination titer of whole piliated cells or the specificity of the assay (see Materials and methods and Figure 4). To assay for digalactoside-specific agglutination, the agglutination titers for pili using P_1- and p̄-erythrocytes were compared (Table I). Only wild-type Pap pili gave specific agglutination (200-fold difference), whereas the pili preparations from the mutants showed no significant receptor-specific agglutination (1- to 4-fold difference). Although *E. coli* HB101 carrying the *papE1* derivative (pPAP15) expresses almost the same level of digalactose-specific agglutination of whole cells as the wild-type, purified pili from this mutant showed only a 4-fold difference in agglutination of P_1- to p̄-erythrocytes in the presence of 100 mM $MgCl_2$.

Discussion

The papA pilin is not required for digalactoside-specific agglutination

There is now firm evidence that the *papA* gene coding for the subunit polypeptide of Pap pili is not required for digalactoside-specific agglutination of human red blood cells. Here we show that a non-polar *papA* frameshift mutation does not quantitatively affect the specific agglutination, i.e., the titration of hemagglutination capacity was identical for the mutant and the wild-type. Thus it is evident that other gene products must be responsible for receptor-specific agglutination. We cannot, however, rule out the possibility that there are several hemagglutinins encoded by the pap clone. If so, they must all be digalactoside specific since this carbohydrate in solution totally inhibits hemagglutination by the clone.

Identification and function of cistrons required for digalactoside-specific agglutination

A region on *pap* DNA of roughly 2.5 kb in size and located ~5 kb from *papA* was recently shown to be essential for hemagglutination (Normark *et al.*, 1983a). However, genetic lesions in this region did not abolish pili formation (Norgren *et al.*, accompanying paper). We have shown that this region carries three separate cistrons, *papE, papF* and *papG* coding for polypeptides of 16.5 kd, 15 kd and 35 kd, respectively. In addition, a number of polypeptides were expressed from *papG* DNA. Since deletions of the 3′ end of the gene truncated all of these polypeptides to the same extent, they must contain the same carboxy terminus. This might implicate autoproteolytic cleavage of the the primer peptide in a similar way as has been demonstrated for the hemolysin of pathogenic *E. coli* (Goebel and Hedgpeth, 1982). It is equally possible that *papG* DNA codes for in-phase overlapping genes with different initiation sites. Such overlaps have been described for some morphogenetic genes in viruses (Shaw and Murialdo, 1980; Normark *et al.*, 1983b). The relevance of this phenomenon will have to be studied in a more physiological situation.

A cloned fragment carrying *papE, papF* and *papG* under *lac*UV5 transcription control did not mediate hemagglutination when harbored in *E. coli* HB101. This is in agreement with the fact that both hemagglutination and surface expression of Pap pili are completely abolished by deletions and Tn*5* insertions within *papC* and *papD* (Norgren *et al.*, accompanying paper). The gene products encoded by these genes, an 81-kd and a 28.5-kd protein, are therefore believed to be involved in the export and assembly of adhesin and Pap pilin. Also, B.-E. Uhlin (personal communication) has recently shown that plasmid derivatives carrying genes *papCDEFG* express hemagglutination, whereas constructs encoding *papBACD* give rise to Pap pili formation but not to hemagglutination.

A non-polar frameshift mutation created by a linker insertion of *papF* and a small deletion of the 3′ end of *papG* both abolished digalactoside-specific agglutination by whole cells, as well as for pili purified from these mutants. This shows that both the *papF* and *papG* gene products are essential for expression of this phenotype. In contrast, a frameshift mutation within *papE* did not affect hemagglutination of whole bacterial cells. When combining the *papA1* and *papE1* mutations in a double mutant, the capacity to hemagglutinate was lost. Since individual lesions of either *papA* or *papE* leave this phenotype intact, it is conceivable that the two gene products have partially overlapping functions. It could be that each of them can carry a putative adhesin composed of the *papF* and/or *papG* gene products, which would not be expressed without this carrier function. Alternatively, the *papF* and/or *papG* proteins modify both the *papA* and *papE* proteins to digalactoside-specific binding. It should be emphasized, however, that pili isolated from a *papE* mutant do not agglutinate specifically, which argues against the second hypothesis.

In the wild-type situation, purified Pap pili show all the binding characteristics of whole cells. Such preparations consist mainly of the *papA* pilin, although additional, very minor, protein components can be detected (Lindberg *et al.*, in preparation). In theory, only one adhesin molecule per cell could be sufficient for binding and two for agglutination. The male-specific filamentous phage M13 contains ~2700 copies of the major coat protein per virion (Newman *et al.*, 1977), whereas a minor coat protein which mediates adhesion to the F pilus (Pratt *et al.*, 1969) is present in ~5 copies only (Lin *et al.*, 1980) at one tip of the filament (Goldsmith and Konigsberg, 1977; Woolford *et al.*, 1977). A similar situation

could prevail in Pap pili as well as in other systems where pili have been correlated to bacterial adhesion and virulence. The individual role of Pap pili and digalactoside-binding adhesin for uropathogenicity has yet to be determined.

Materials and methods

Chemicals and enzymes

Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim GmbH or New England BioLabs, and used as recommended by the vendors. Filling in of 3'-recessive ends was done using the Klenow fragment (New England Biolabs) in ligation buffer to which 200 μM each of the required dNTPs had been added. The *Xho*I linker (5'-CCTCGAGG-3') and *Bam*HI linker (5'-CGGATCCG-3') were obtained from Collaborative Research and 5'-phosphorylated as described by the manufacturers using polynucleotide kinase from New England Biolabs. All chemicals were of the highest purity commercially available. p-erythrocytes were kindly supplied by Dr. B.Cedergren, Blood Bank, University Hospital, Umeå. Latex beads coated with receptor analogues were obtained from Chembiomed Ltd., Edmonton, Alberta, Canada.

Construction of plasmid derivatives

Plasmid derivatives used for mapping and functional analysis are outlined in Table III. The 9.6-kb *Eco*RI-*Bam*HI fragment of pRHU30, containing all genes necessary for the expression of Pap pili and digalactoside-specific adhesion was cloned into *Eco*RI- and *Bam*HI-digested pBR322 (Bolivar *et al.*, 1977) giving pPAP5. To construct a derivative lacking the *Pvu*II site in the pBR322 part of the molecule, the vector was *Pvu*II digested and ligated to a 20-fold excess of *Bam*HI linker. This DNA was subsequently cut with *Eco*RI and *Bam*HI, and the largest fragment carrying nucleotides 2065–4360 of pBR322 (Sutcliffe, 1978) was isolated from a 0.7% agarose gel. This fragment, ligated to the *Eco*RI-*Bam*HI from pRHU30, was transformed (Mandel and Higa, 1970) into HB101 (Boyer and Roulland-Dussoix, 1969). The clone isolated was named pPAP22 and is identical to pPAP5, except that the clone lacks *Bam*HI-*Pvu*II segment. A derivative, pPAP23, carrying a frameshift mutation at the single *Pvu*II site in *pap*A was constructed by linearizing pPAP22 with *Pvu*II and ligating it to a 20-fold excess of *Xho*I linker. After digestion for 3 h using 20 units of *Xho*I/μg of DNA, the fragment was purified on a G150 column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 10 mM Tris-HCl pH 8.0 and 1 mM EDTA. After ligation and transformation into HB101, DNA from six clones was isolated (Birnboim and Doly, 1979) and analysed. Five of the clones had a new *Xho*I site at the former *Pvu*II site and one of these was called pPAP23 and used in further studies.

The following manipulations were done to construct plasmid pPAP16 (Figure 1) and derivatives of both this plasmid and pPAP5 with mutations in the *Sma*I$_1$-*Bam*HI region. Plasmid pPAP1 was constructed by linearizing 2 μg of pBR322 with *Cla*I, and blunt ends were created using 5 units of Klenow fragment and 200 μM each of dGTP and dCTP (15 min at 30°C in ligation buffer). This DNA was, after heat inactivation of the enzyme, ligated to the gel-purified *Sma*I$_1$-*Sma*I$_2$ fragment of pPAP5 (Figure 1), and by screening small-scale plasmid preparations, a plasmid carrying the fragment in the same orientation relative to the vector as on pPAP5, was isolated. The clone pPAP1 expressed the last polypeptide (35 kd) in a slightly truncated form (data not shown). Thus the gene for this polypeptide extends beyond the *Sma*I$_2$ site and is present in a truncated form in pPAP1. This mutation was isolated on a *Kpn*I-*Bam*HI fragment which was ligated into pPAP5 cut with these enzymes. The derivative, pPAP7 obtained in this way thus contains *pap* DNA up to the *Sma*I$_2$ site. Plasmid pPAP9, containing the whole *Sma*I$_1$-*Bam*HI region was constructed by ligating the *Kpn*I-*Bam*HI fragment of pPAP5 in excess to *Kpn*I-*Bam*HI digested pPAP1. To make frameshift mutations in the insert of pPAP9, this plasmid was partially digested with *Hinc*II in the presence of 150 μg/ml ethidium bromide (Greenfield *et al.*, 1975). Linearized plasmid was then isolated from a 0.7% agarose gel and ligated to an excess of *Xho*I linkers. After *Xho*I digestion, G150 gel chromatography, and ligation, the DNA was transformed into HB101, selecting for ampicillin resistance. The DNA purified from 23 clones was analysed by digestion with *Xho*I and *Sal*I. Of 15 mutants within the insert, 13 were linker insertions at *Hinc*II$_2$ and two at the *Hinc*II$_1$ site. No mutants at *Hinc*II$_3$ were obtained. pPAP5 derivatives carrying these mutations were constructed in a way analogous to pPAP7. These plasmids were named pPAP15 (*Hinc*II$_1$) and pPAP14 (*Hinc*II$_2$). pPAP19 was constructed by deleting the *Xho*I-*Sal*I fragment of pPAP14 by re-ligating an *Xho*I-*Sal*I digest of the latter plasmid. The construction of pPAP20 from pPAP15 was done in the same manner. To make plasmid pPAP26 (*papA1, papE1* double mutant), the large *Kpn*I-*Bam*HI fragment of pPAP23 (*papA1*) was ligated to the small *Kpn*I-*Bam*HI fragment of pPAP10 (*papE1*).

Table III. Characteristics of plasmids used for mapping and functional analyses of *papA, papE, papF* and *papG*

Plasmid	Relevant genotype	Phenotype	
		Pilus-antigen	Hemagglutination
pSN002	*papF*::Tn*5*-002	+	–
pSN021	*papF*::Tn*5*-021	+	–
pSN026	*papG*::Tn*5*-026	+	–
pSN042	*papG*::Tn*5*-042	+	–
pPAP5	wt	+	+
pPAP15	*papE1*	+	+
pPAP14	*papF1*	+	–
pPAP7	*papG1*	+	–
pPAP20	*papE1*,Δ*papF-G*	+	–
pPAP19	*papF1*,Δ*papG*	+	–
pPAP22	wt	+	+
pPAP23	*papA1*	–	+
pPAP26	*papA1*,*papE1*	–	–
pPAP9	Δ*papB-D*	–	–
pPAP1	Δ*papB-D*,*papG1*	–	–
pPAP16	Δ*papB-D*,*lacP*$_{UV5}$	–	–
pPAP18	Δ*papB-D*,*lacP*$_{UV5}$,*papE1*	–	–
pPAP17	Δ*papB-D*,*lacP*$_{UV5}$,*papF1*	–	–
pPAP4	Δ*papB-D*,*lacP*$_{UV5}$,*papG1*	–	–

pSN plasmids (Norgren *et al.*, accompanying paper) are pACYC184 (Chang and Cohen, 1978) derivatives, whereas pPAP plasmids (this study) are derivatives of pBR322. Pilus antigen was determined by slide agglutination of a cell suspension with antisera raised against Pap pili.

Plasmid pPAP9 did not complement Tn*5* insertions within the *Sma*I$_1$-*Bam*HI region. We suspected this to be due to insufficient transcription over the insert. Therefore the *Eco*RI fragment containing the *lac*UV5 promoter was isolated from pSKS106 (Casadaban *et al.*, 1983), and ligated in excess to EcoRI linearized pPAP9. A clone with the fragment in the correct orientation, pPAP16, was subsequently isolated by screening DNA preparations using *Pst*I digestion since the promoter fragment carries an asymmetrically placed site for this enzyme. The same procedure was applied to the other pPAP9 derivatives resulting in pPAP4 (*Sma*I$_2$-*Bam*HI deletion), pPAP18 (*Hinc*II$_1$ mutation) and pPAP17 (*Hinc*II$_2$ mutation).

Purification of pili

Pili were purified according to a modification of the method described by Brinton *et al.* (1978). Cells were grown for 22 h at 37°C on five trays (400 x 250 mm) containing L-agar without glucose. The cells were scraped off the trays, suspended in 340 ml ice-cold 5 mM Tris-HCl (pH 8.0) and blended for 10 min on ice in a Sorvall Omnimixer at setting 4. After pelletation of the cells and cellular debris (twice for 30 min at 20 000 *g*), ammonium sulfate was added to the supernatant to 55% saturation and pili were allowed to precipitate on ice overnight. The precipitate was collected by centrifugation and resuspended in 5 mM Tris (pH 8.0). After dialysis overnight against the same buffer at 4°C, non-dissolved material was removed by centrifugation for 30 min at 40 000 *g*. The precipitation-dialysis procedure was repeated an additional three times (precipitation for 3 h) after which pili were precipitated by addition of 0.2 volumes of 1 M $MgCl_2$-1.5 M NaCl-100 mM Tris-HCl (pH 7.5). The precipitate was dissolved to a protein concentration of 2 mg/ml as measured according to Lowry *et al.* (1951). The yield was ~15 mg for the wild-type and 2–30 mg for the mutants.

Receptor binding assays

For slide agglutination, bacterial cells, grown for 22 h on glucose-free L-agar, were suspended to ~10^{10} cells/ml in agglutination buffer (150 mM NaCl; 10 mM Tris-HCl pH 7.5) containing 3% heparinized and washed human erythrocytes. The reaction, when positive, was usually apparent within 60 s. The positive reaction was a macroscopically visible aggregation of erythrocytes. For receptor-coated latex beads, the same procedure was applied, except that the suspension contained 1% latex beads instead of erythroctes. In the semiquantitative assay, cells grown as above were resuspended to an A_{600} = 20. They were then serially 2-fold diluted in 50 μl agglutination buffer using microtiter plates with conical-bottom wells (Linbro/Titertek, cat. no. 76-321-05, CT, USA). To this was added 10 μl of a 3% erythrocyte suspension in the same buffer. The dilution in the last well giving a positive ag-

glutination after 2 h at 4°C was taken as the agglutination titer. The cell count of the original suspension was used together with the titer, to calculate the minimum bacterial concentration required for agglutination.

The agglutination titer of purified pili was determined essentially in the same manner as used for whole cells. Using agglutination buffer, however, the pilus concentration required for agglutination was very high and various means of increasing the sensitivity of the assay were therefore tried. Since pili are negatively charged at physiological pH (data not shown) and aggregate in the presence of 167 mM $MgCl_2$ (see purification of pili), we titrated a wild-type pilus preparation at increasing $MgCl_2$ concentration, using P_1-erythrocytes containing the globoside receptor, and $\bar{p}$-erythrocytes which lack this carbohydrate. As seen in Figure 4, the agglutination titer increased 128-fold as the $MgCl_2$ concentration was increased to 100 mM. This was paralleled by an increase of A_{400} of a 200 μg/ml pilus solution in the same buffers. With $CaCl_2$ and 10-fold higher concentrations of NH_4Cl (data not shown), the same results are obtained, suggesting that the effect is on pilus-pilus interaction and not on specific receptor binding. In addition, the agglutination titer of whole piliated cells is not significantly affected by the addition of $MgCl_2$ up to 200 mM. Also the increase in agglutination titer using $\bar{p}$-erythrocytes shows that unspecific pilus-erythrocyte aggregation is favored by the addition of Mg^{2+} ions, although, the specificity of the assay (P_1-titer over $\bar{p}$-titer) seems to be unaffected. All titrations of pilus preparations shown in Table I are therefore done in agglutination buffer with 100 mM $MgCl_2$ to give a semiquantitative value for specific agglutination.

Pilus antigen assay

For slide agglutination, bacteria were grown and prepared as described for the hemagglutination assays. Agglutination test of whole cells was performed with 500-fold-diluted (PBS pH 7.5) antiserum raised against purified Pap pili (Normark *et al.*, 1983a). The positive reaction was determined as a macroscopically visible aggregation of bacteria appeared within 60 s.

Protein expression in minicells

Plasmid pPAP5 and its derivatives were transformed into the minicell-producing strain P678-54 (Adler *et al.*, 1967). Preparation and labelling of plasmid containing minicells with [^{35}S]methionine were as described by Thompson and Achtman (1978). The radioactive samples were subjected to SDS-polyacrylamide electrophoresis (Laemmli, 1970). The gels were subsequently fixed, stained, enhanced (Enhance, New England Nuclear) and autoradiographed. Mol. wt. standards (Pharmacia Fine Chemicals, Uppsala, Sweden) and purified pilin were electrophoresed in parallel.

Acknowledgements

We thank Monica Persson for technical assistance. We are indebted to Bernt-Eric Uhlin for valuable suggestions. The inspiring discussions and ideas provided by Per Hagblom are gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (Dnr 5428), Swedish Natural Science Research Council (Dnr 3373), and the Board for Technological Development (Dnr 81-3384).

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Received on 21 February 1984