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Pap Pili as a Vector System for Surface Exposition of an Immunoglobulin G-Binding Domain of Protein A of *Staphylococcus aureus* in *Escherichia coli*

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Fusion genes between *papA*, the gene coding for the major Pap pilus subunit, and fragments coding for an immunoglobulin G-binding domain of the *Staphylococcus aureus* protein A were constructed in such a way that the *spa* fragments were inserted following either codon 7 or 68 of the coding sequence for the mature portion of PapA. Peptides in the area of amino acids 7 and 68 of PapA are localized at the external side of the pilus. A set of p_L expression plasmids containing *papA* and derivatives suitable for insertion were constructed. A *papA* gene carrying a *spa* insert following codon 68 was cloned back into the *pap* operon. The presence of this altered operon in a bacterial strain allowed the detection of immunoglobulin G-binding activity at the surfaces of the bacterial cells.

Pap pili are extracellular, hairlike proteinaceous appendages of the bacterial cell consisting of a limited set of different proteins (6, 9, 13). Hull et al. (10) cloned a chromosomal fragment from the uropathogenic *Escherichia coli* strain J96 which codes for Pap pilus formation. The formation of these pili involves the actions of different proteins encoded by the *pap* operon. Cells carrying this operon have about 300 pili. Each pilus contains around 1,000 PapA subunits and some minor compounds and extends roughly 1 μm into the growth medium (7). These structures are adhesins to glycoconjugate receptors on host epithelial tissues of the urinary tract. In view of their exceptional structure, we considered these to be possible vectors for the exposition of foreign adventitious protein domains toward the extracellular environment.

The bulk of the pilus consists of PapA, which is encoded by the *papA* gene (15). Schmidt et al. (24) prepared a set of different peptides corresponding to short sequences spanning the PapA protein. Immunization of mice with these peptides revealed that sera raised against regions corresponding to amino acids 7 to 12 and 65 to 75 reacted with intact Pap pili in an enzyme-linked immunosorbent assay. This observation suggests that these regions are directly exposed at the surface of the pilus. We made both regions accessible for the insertion of foreign protein domains. As a model system for insertion of a foreign protein segment, we chose an immunoglobulin G (IgG)-binding domain of the *Staphylococcus aureus* protein A (SPA) (16). This protein is localized at the surface of *S. aureus* (19) and binds strongly to the Fc part of various immunoglobulins (8). Protein A consists largely of seven functional entities (domains): S, E, D, A, B, C, and X (29). The S domain represents the signal sequence, whereas the X domain is the cell wall-anchoring domain (25). The regions E, D, A, B, and C are five strongly conserved, 58-amino-acid-long direct repeats. The whole of the latter five domains embodies the IgG-binding part of the protein. Abrahmsén et al. (1) showed that a single protein A domain is sufficient for binding IgG molecules.

By site-directed mutagenesis of the *spa* gene, we obtained information on the A domain flanked by two *Bam*HI restriction sites. This allowed us to construct two *papA-spa* fusion genes in which the *Bam*HI fragment was inserted either after codon 7 or after codon 68 of the *papA* gene. The PapA-SPA fusion proteins were overexpressed and shown to bind IgG molecules by a modified Western blot (immunoblot) immunodetection system. To obtain surface localization of one of these proteins, the fusion gene was cloned back into the *pap* operon. Bacteria carrying this mutant operon show surface-located IgG-binding activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. All plasmid constructions were performed in MC1061 [*hsdR mcrB araD139* Δ(*araABC-leu*)*7697* Δ*lacX74 galU galK rpsL thi*] (5). Initial expression studies to verify the correct orientation of the inserts were performed in MC1061(pCI857) (23). Studies implying the potential appearance of pili at the surfaces of the bacteria were carried out in HB101 [*supE44 hsdS20* ($r_B^- m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] (4). The plasmid pPAP5 (15) was a kind gift from B.-I. Marklund.

DNA manipulations. Restriction endonucleases and DNA polymerases were purchased from Boehringer, Mannheim, Germany, and Bethesda Research Laboratories, Bethesda, Md.; they were used according to the manufacturers' recommendations. T4 ligase was purified from K12ΔH1Δtrp (pPLc28lig8), a T4 ligase-overproducing strain (23). Ligation was performed as described by Kahn et al. (11).

For analytical purposes, plasmid DNA was extracted according to the method described by Birnboim and Doly (3). For preparative purposes, plasmid DNA was isolated according to the method described by Kahn et al. (11). Restriction fragments were prepared by the gene-clean method (28, 31). The required materials were purchased from Bio 101, La Jolla, Calif.

Purification of pili. Pili were purified from HB101(pPAP5) essentially according to the method described by Lindberg et al. (14).

Polyclonal antisera. Polyclonal rabbit antipilin (Rα-pilin) antiserum was raised essentially according to the method

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described by Maertens (17). The serum was purified on CL4B-protein A Sepharose (Pharmacia P-L Biochemicals, Uppsala, Sweden) and was shown to react with recombinant PapA in a Western blot.

Human placental alkaline phosphatase (hPLAP) was purchased from Sigma, Deisenhofen, Germany. Rabbit anti-hPLAP (Rα-hPLAP) was purchased from Dakopatts, Glostrup, Denmark. Goat anti-rabbit, alkaline phosphatase-conjugated antibody (Gα-RAP) was supplied by Organon Teknika, Cambridge, United Kingdom. Rabbit anti-β-lactamase (Rα-βLa) was a kind gift from R. Matthew (Glaxo Institute for Molecular Biology, Geneva, Switzerland).

Expression of recombinant proteins. Expression of proteins under control of the p_L promoter was performed by shifting a logarithmically growing culture from 28 to 42°C (21). A saturated preculture, grown in Luria-Bertani (LB) medium, was diluted 100-fold in M9 medium (18) supplemented with 0.2% Casamino Acids and grown for 5 h at 28°C under shaking. After switching to 42°C, it was incubated for another 3 h. The cultures were concentrated by centrifugation, resuspended in one-eighth volume of sample buffer and boiled for 3 min. A 20-μl volume was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12). Gels were fixed in 10% trichloroacetic acid and stained in 0.05% Serva Blue R (Serva Feinchemikalien, Heidelberg, Germany).

Western blot immunodetection. For immunological characterization, proteins were electrophoretically blotted from an SDS-PAGE gel onto a cellulose nitrate filter (pore diameter of 0.45 μm; Millipore, Bedford, Mass.) with a Consort dry blotting device. The filter was blocked for at least 2 h in a 2.5% solution of skim milk powder in NaCl-P_i (0.01 M P_i, 0.15 M NaCl, pH 7.5). Incubation with primary antibodies was performed at room temperature. Excess of unbound antibody was removed by washing. The antigen-antibody complex was detected by incubation with a secondary alkaline phosphatase-conjugated antibody with an appropriate specificity, and this was followed by incubation in 0.1 M Tris-HCl (pH 9.5)–0.1 M NaCl–0.05 M $MgCl_2$ containing 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT). Alternatively, the complex was detected by means of direct recognition between the primary antibody and hPLAP. After incubation with hPLAP, the complex was visualized as described above, with the aforementioned phosphatase substrate. The enzymatic reaction was terminated by rinsing the filter under flowing tap water.

Detection of surface-exposed protein domains. Single colonies were streaked on a cellulose nitrate filter (pore diameter, 0.45 μm; Millipore) which had been placed on a solid-agar LB plate. After overnight incubation at 37°C, the filter was washed in NaCl-P_i containing 2.5% skim milk powder until the bulk of the bacteria was removed. The filters were further incubated with 5 ml of a 5-mg/liter solution of the appropriate antibody in NaCl-P_i containing 2.5% skim milk powder. Detection of the different primary antibodies depended on the particular experiment (cf. Results). The final detection occurred as a result of localized alkaline phosphatase activity and was revealed by incubation of the filter in NBT-BCIP solution as described above. The reaction was stopped by rinsing the filter under flowing tap water.

RESULTS

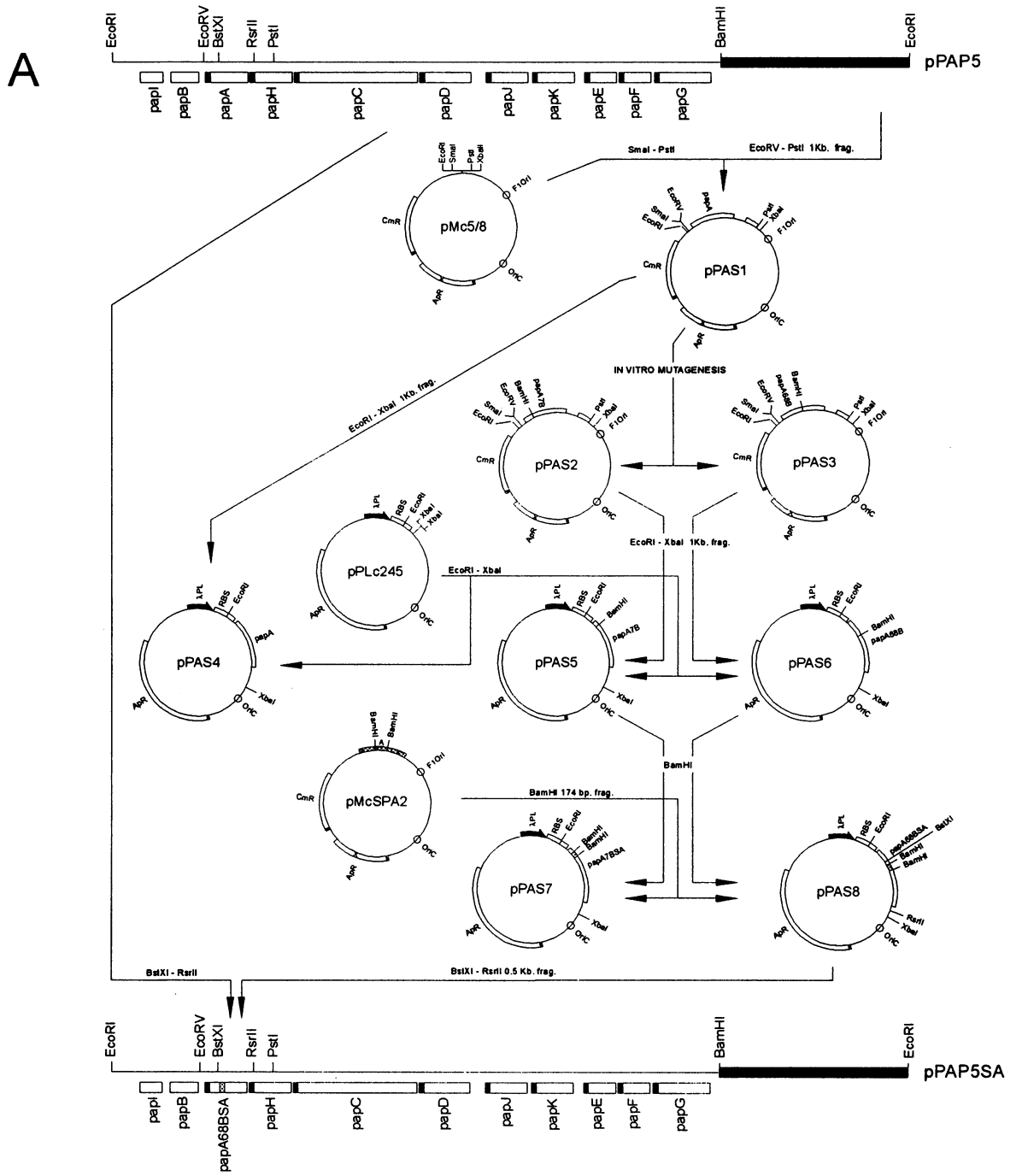
Construction of plasmids. The *papA* gene was subcloned from the plasmid pPAP5 (15) as a 1-kb *Eco*RV-*Pst*I fragment and ligated in the *Sma*I-*Pst*I-opened plasmid pMC58 (26). This plasmid was named pPAS1 and was subjected to site-specific mutagenesis according to the method described by Nakamaye and Eckstein (20). We created *Bam*HI restriction sites at locations in the *papA* gene coding for amino acids 7 and 68 of the mature part of PapA. The DNA sequence and data concerning the start of the mature protein were taken from Båga et al. (2). The positions of the new restriction sites relative to the reading frame were G.GAT.CC. The two plasmids containing the mutant genes were called pPAS2 and pPAS3, respectively. From these two plasmids, the modified *papA* genes were subcloned in the *Eco*RI-*Xba*I-opened p_L expression vector pPLc245 (22) as *Eco*RI-*Xba*I fragments. These plasmids, which contained the modified *papA* genes under control of the p_L promoter, were named pPAS5 and pPAS6, respectively. An analogous construct was made with the original *papA* gene and was named pPAS4.

A 174-bp *Bam*HI fragment coding for the A domain of SPA was isolated from pMCSPA2 (27) and was ligated in the *Bam*HI-opened plasmids pPAS5 and pPAS6. The correct orientations of the insertions were verified by expression of the fusion proteins. The resulting plasmids containing the fusion genes of *papA* and *spa* were named pPAS7 and pPAS8, respectively, while the fusion genes themselves are referred to as *papA7BSA* and *papA68BSA*, respectively.

The region holding the *spa* fragment in the *papA68BSA* gene was isolated as a 0.7-kb *Bst*XI-*Rsr*II fragment from pPAS8 and ligated in the corresponding sites in the plasmid pPAP5. This plasmid, now coding for a fusion gene between *papA* and *spa* in the natural context of the *papA* gene, was named pPAP5SA. The flow scheme of these constructions is given in Fig. 1.

Detection of recombinant proteins. The expression of the different recombinant proteins is shown in Fig. 2. The protein patterns of the induced cultures show prominent extra bands with apparent M_rs corresponding to the expected calculated sizes. All induced proteins react with Rα-pilin serum in Western blot immunodetection, as revealed by Gα-RAP and BCIP-NBT (data not shown). The IgG-binding activities of the fusion proteins PapA7BSA and PapA68BSA were assessed in a modified Western blot. In this system, the primary antibody was Rα-hPLAP, which should bind with its Fc domain to IgG-binding proteins. The membrane was then incubated in an appropriate volume of 80-μg/ml hPLAP solution in NaCl-P_i containing 2.5% skim milk powder. The enzyme hPLAP could be detected on the basis of its phosphatase activity. The results are shown in Fig. 2. The appearance of a band of the correct size in the lane corresponding to the protein pattern of induced cultures of MC1061(pCI857)(pPAS7) and MC1061(pCI857)(pPAS8) proves that these fusion proteins had IgG-binding capacity. The slightly higher molecular weight of PapA7BSA, compared with that of Pap68BSA, may be due to an inability of the PapA7BSA protein to be processed by the cell. For this reason, we did not further investigate the latter fusion gene system.

Detection of surface-exposed protein domains. Surface expression of HB101 cells harboring the plasmid pPAP5SA compared with that for the same strain carrying pPAP5 was evaluated as described. Three different primary sera were used, i.e., Rα-hPLAP, Rα-pilin, and Rα-βLa. Rα-hPLAP was used in combination with hPLAP to monitor the presence of IgG-binding activity, as described above. Rα-pilin should, in combination with Gα-RAP, disclose the presence or absence of pilin antigen. Rα-βLa should indicate the appearance of signals derived from proteins normally present in the periplasm. Figure 3 indicates that HB101(pPAP5) reacted with Rα-pilin and not with the other antibodies; therefore, the test



B

6 7 8 Gln Gly Gln Gly Lys Val Thr CAG GGG CAG GGT AAA GTA ACT	Wild Type [pPAS1]	68 69 70 Phe Lys Gly Gly Asn Gly Ala TTT AAA GGT GGT AAT GGC GGC	Wild Type [pPAS1]
CAG GGG GAT CCT AAA GTA ACT Gln Gly Asp Pro Lys Val Thr	Mutant [pPAS2]	TTT AAA GGG GAT CCT GGC GGC Phe Lys Gly Asp Pro Gly Ala	Mutant [pPAS3]

FIG. 1. (A) Flow scheme of the plasmid constructions; (B) mutations in *papA* and their effects on the amino acid sequence.

used was not subject to artifacts originating from proteins present in the periplasm. HB101(pPAP5SA) reacted with all three sera. This indicates at least the presence of IgG-binding properties at the surfaces of these cells. However, the IgG-binding properties appeared to be very unstable. They were observed only on colonies which had not been passed through liquid cultures. For this reason, tests on surface exposition were performed on streaks of freshly obtained transformants.

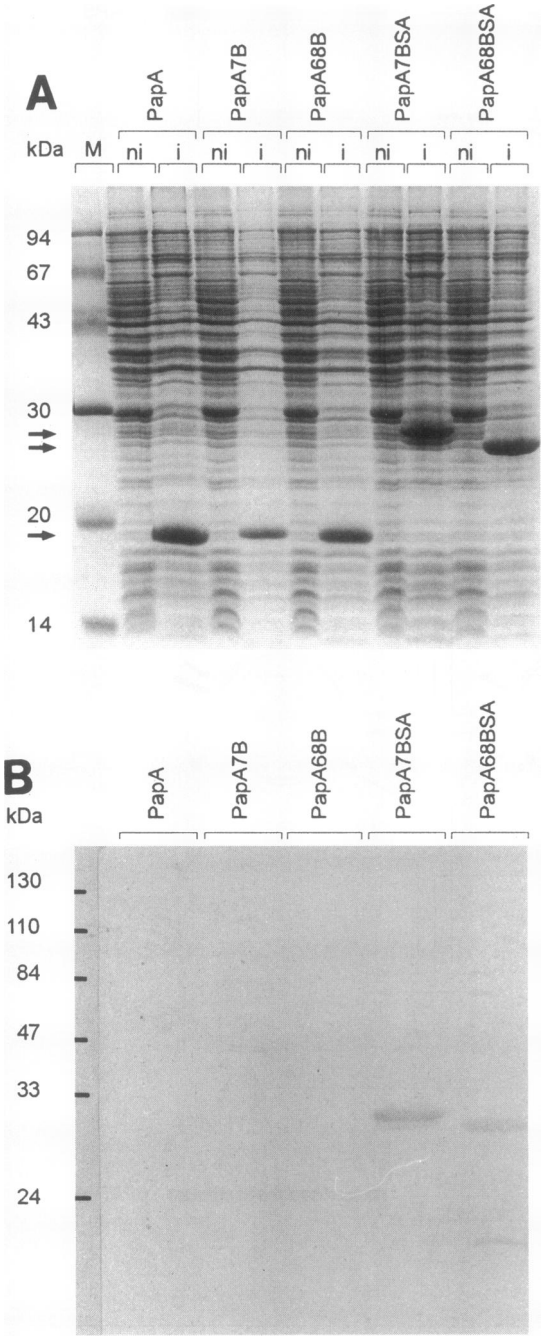


FIG. 2. Expression of the different recombinant *papA* derivatives and assessment of their IgG-binding capacities. (A) Total protein pattern, analyzed on an SDS-15% PAGE gel, which was revealed by Coomassie brilliant blue staining, of noninduced (ni) and induced (i) cultures carrying the information for the proteins indicated. M, M_r markers (Pharmacia P-L Biochemicals). Arrows indicate the induced proteins. (B) Total protein extracts, analyzed on an SDS-15% PAGE gel, which was revealed in a modified Western blot to show IgG-binding capacity (see Results), of induced cultures expressing the PapA derivatives. Prestained M_r standards (Bio-Rad, Richmond, Calif.) were used as markers.

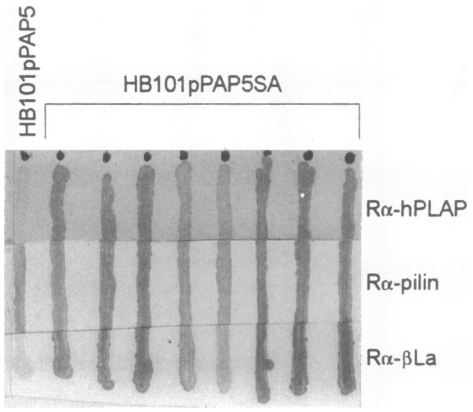


FIG. 3. Surface exposition assay to reveal the presence of pilin antigen and IgG-binding activity, as well as to exclude artifacts due to detection of periplasmic proteins. Colonies of the strains indicated were streaked on a nitrocellulose membrane. Excess bacteria were washed off, and the filter was cut transversally. The parts were incubated with the antibodies indicated and were detected with an appropriate secondary antibody (see Results).

The disappearance of IgG-binding activity did not correlate with loss of the plasmid.

DISCUSSION

We made the *papA* gene accessible for insertion of foreign genetic material in two regions which code for surface-exposed parts of the protein. To this end, we created two mutant genes carrying extra *Bam*HI sites after codons 7 and 68, respectively, of the mature protein. To provide rapid screening for and evaluation of the fusion proteins, we constructed a set of plasmids coding for the modified and natural *papA* gene under control of the inducible p_L of phage λ. Expression of the proteins from these plasmids proved to be very convenient for their characterization.

As a model system for insertion of a foreign sequence, we chose a fragment of the *spa* gene. This gene codes for SPA and was modified in such a way that a part of the gene coding for one of the IgG-binding domains of the protein, namely, the A domain, became flanked by two *Bam*HI sites. These sites were introduced in such a way that an in-frame fusion was obtained when the fragment was ligated in the mutated *papA* genes. The fusion between the *papA* and *spaA* genes was carried out in the expression vectors described above. Thus, it was possible to verify the correct lengths of the expressed products as well as their IgG-binding capacities. Insertion of the *spaA* fragment after codon 7 gave rise to a gene product, Pap7BSA, which was slightly larger than Pap68BSA derived from the gene carrying the fragment after codon 68. The reason for this discrepancy was probably poor secretion and/or processing of the former gene product, which we presume to be caused by the position of the inserted domain at only 7 amino acids from the signal-processing site.

To obtain surface exposition with the *pap* system, we subcloned the *papA68BSA* gene back into the *pap* operon. The presence of the mutant *pap* operon, *pap5SA*, conferred IgG-binding capacity to the cell. In view of the comparison with the original *pap* operon, we can conclude that the test used could distinguish between exposition at the surface of the bacteria and intracellular presence. In view of the Fc-dependent IgG-

binding capacity of the cells, we could no longer test for potential antipilin-specific immunoreactivity. The same argument holds for detection with Rα-βLa; hence, it is not strictly possible to conclude that the signals were confined to the surface of HB101(pPAP5SA). Nevertheless, in view of the results with HB101(pPAP5), we do not believe that proteins present in the periplasm were revealed in these tests.

In summary, we show that adventitiously coding sequences can be functionally incorporated in the PapA subunit of pili and can become exposed at the surfaces of the bacteria, presumably as a part of a genuine pilus structure. This conclusion is consistent with the observations of Van Die et al. (30), who incorporated an 8-amino-acid foot-and-mouth disease virus epitope in the F11 major pilus subunit and could observe the presence of the epitope at the cell surface, incorporated in a pilus structure. Our observations show that a stretch of at least 58 amino acids can be inserted into PapA and can be functionally exposed at the surface of the bacterium.

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REFERENCES

1. **Abrahmsén, L., T. Moks, B. Nilsson, U. Hellman, and M. Uhlén.** 1985. Analysis of signals for secretion in the staphylococcal protein A gene. EMBO J. **4**:3901–3906.
2. **Båga, M., S. Normark, J. Hardy, P. O'Hanley, D. Lark, O. Olsson, 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**:330–333.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
5. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
6. **Dougan, G., G. Dowd, and M. Kehoe.** 1983. Organization of K88ac-encoded polypeptides in the *Escherichia coli* cell envelope: use of minicells and outer membrane protein mutants for studying assembly of pili. J. Bacteriol. **153**:364–370.
7. **Duguid, J. P., and D. C. Old.** 1980. Adhesive properties of *Enterobacteriaceae*, p. 185–217. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman and Hall, London.
8. **Forsgren, A., and J. Sjöquist.** 1966. Protein A from *Staphylococcus aureus*. I. Pseudo-immune reaction with human gamma-globulin. J. Immunol. **97**:822–827.
9. **Hanson, M. S., J. Hempel, and C. C. Brinton, Jr.** 1988. Purification of the *Escherichia coli* type 1 pilin and minor pilus proteins and partial characterization of the adhesin protein. J. Bacteriol. **170**: 3350–3358.
10. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
11. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K and RK2. Methods Enzymol. **68**:268–280.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Lindberg, F., B. Lund, L. Johansson, and S. Normark.** 1987. Localization of the receptor-binding protein adhesin at the tip of the bacterial pilus. Nature (London) **328**:84–87.
14. **Lindberg, F., B. Lund, and S. Normark.** 1986. Gene products specifying adhesion of uropathogenic *Escherichia coli* are minor components of pili. Proc. Natl. Acad. Sci. USA **83**:1891–1895.
15. **Lindberg, F. P., B. Lund, and S. Normark.** 1984. Genes of pyelonephritogenic *E. coli* required for digalactoside-specific agglutination of human cells. EMBO J. **3**:1167–1173.
16. **Löfdahl, S., B. Guss, M. Uhlén, L. Philipson, and M. Lindberg.** 1983. Gene for staphylococcal protein A. Proc. Natl. Acad. Sci. USA **80**:697–701.
17. **Maertens, G.** 1990. Ph.D. thesis. State University, Ghent, Belgium.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Movitz, J.** 1976. Formation of extracellular protein A by *Staphylococcus aureus*. Eur. J. Biochem. **68**:291–299.
20. **Nakamaye, K. L., and F. Eckstein.** 1986. Inhibition of restriction endonuclease Nci I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. Nucleic Acids Res. **14**:9679–9698.
21. **Remaut, E., P. Stanssens, and W. Fiers.** 1981. Plasmid vectors for high-efficiency expression controlled by the p_L promoter of coliphage lambda. Gene **15**:81–93.
22. **Remaut, E., P. Stanssens, and W. Fiers.** 1983. Inducible high level synthesis of mature human fibroblast interferon in *Escherichia coli*. Nucleic Acids Res. **11**:4677–4688.
23. **Remaut, E., H. Tsao, and W. Fiers.** 1983. Improved plasmid vectors with a thermoinducible expression and temperature-regulated runaway replication. Gene **22**:103–113.
24. **Schmidt, M. A., P. O'Hanley, D. Lark, and G. K. Schoolnik.** 1988. Synthetic peptides corresponding to protective epitopes of *Escherichia coli* digalactoside-binding pilin prevent infection in a murine pyelonephritis model. Proc. Natl. Acad. Sci. USA **85**:1247–1251.
25. **Sjödahl, J.** 1977. Repetitive sequences in protein A from *Staphylococcus aureus*. Arrangement of five regions within the protein, four being highly homologous and Fc-binding. Eur. J. Biochem. **73**:343–351.
26. **Stanssens, P., C. Opsomer, Y. M. McKeown, W. Kramer, M. Zabeau, and H.-J. Fritz.** 1989. Efficient oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternating selectable markers. Nucleic Acids Res. **17**:4441–4453.
27. **Steidler, L., E. Remaut, and W. Fiers.** 1993. LamB as a carrier molecule for the functional exposition of IgG-binding domains of the *Staphylococcus aureus* protein A at the surface of *Escherichia coli* K12. Mol. Gen. Genet. **236**:187–192.
28. **Struhl, K.** 1985. A rapid method for creating recombinant DNA molecules. BioTechniques **3**:452–453.
29. **Uhlén, M., B. Guss, B. Nilsson, S. Gatenbeck, L. Philipson, and M. Lindberg.** 1984. Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. J. Biol. Chem. **259**:1695–1702.
30. **Van Die, I., M. Wauben, I. Van Megen, H. Bergmans, N. Riegman, W. Hoekstra, P. Pouwels, and B. Enger-Valk.** 1988. Genetic manipulation of major P-fimbrial subunits and consequences for formation of fimbriae. J. Bacteriol. **170**:5870–5876.
31. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76**:615–619.