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Expression of foreign epitopes in P-fimbriae of *Escherichia coli*

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Summary. Hypervariable regions (HRs) of the major subunit of F11 fimbriae were exploited for insertion of foreign epitopes. Two insertion vectors were created that contain a unique cloning site in HR1 or HR4 respectively. Several oligonucleotides, coding for antigenic determinants derived from different pathogens, were cloned in both insertion vectors. Hybrid fimbrial subunits were generally shown to be assembled in fimbriae when the length of the inserted peptide did not exceed 14 amino acids. The inserted peptides appeared to be exposed in the fimbrial filament. One hybrid fimbrial protein induced detectable levels of antibodies against the inserted epitope if injected into mice.

Key words: Recombinant DNA – Vaccine development – Carrier protein

Introduction

Fimbriae are long polymeric structures that consist of about a thousand protein subunits. P-fimbriae, found on uropathogenic *Escherichia coli* strains, mediate adherence of the bacteria to the uroepithelium where they recognize specifically the α-D-galactose (1,4) β D-galactose moiety of the antigens of the P blood group (Hagberg et al. 1981; Källenius et al. 1981; Leffler and Svanborg-Edèn 1980; Svanborg-Edèn et al. 1976; Vaïsänen et al. 1981). P-fimbriae have been widely studied and the organization of the subunits in these fimbriae has been established. The fimbrial filament consists mainly of one type of subunit, the major subunit, while at the tip some different subunits are found, among them the P-lectin. A gene cluster consisting of seven to nine genes is responsible for biosynthesis of P-fimbriae. (Lindberg et al. 1987; Normark et al. 1983; Riegman et al. in press; Van Die et al. 1985, 1986a).

Like other fimbriae, P-fimbriae are very immunogenic structures (Gyles and Maas 1987; Korhonen and Rhen 1982). The major subunit mainly determines the antigenic properties of the fimbriae, and the serotypes F7–F13 are allotted to P-fimbriae (De Ree et al. 1985b; Ørskov and Ørskov 1985; Van Die et al. 1988a).

As fimbriae are easy to purify, they are potentially suitable as carriers of foreign synthetic peptides. Synthetic peptides generally are poorly immunogenic. It might be possible to enhance the immunogenicity of an epitope by incorporating it into the fimbrial structure.

In the P-fimbrial major subunit gene we have located five segments that code for hypervariable regions (HRs 1–5), containing the natural epitopes of the P-fimbriae (Van Die et al. 1987, 1988a). The HRs of the major subunit of F11 fimbriae appeared to be particularly suitable for genetic manipulation. We have shown that one of these HRs can be exchanged with an epitope of foot and mouth disease virus (FMDV) without losing the capacity to assemble the hybrid subunits into fimbriae; furthermore, the foreign protein sequence was recognized by an FMDV-specific monoclonal antibody in the fimbrial structure (Van Die et al. 1988b). In this paper we describe experiments that study more extensively the possibilities and limitations of this system.

Materials and methods

Bacterial strains, growth conditions and plasmids. The *E. coli* K12 strain used in this study for fimbriae expression was HB101 (Boyer and Roulland-Dussoix 1969) which is deficient in the production of type 1 fimbriae. *E. coli* JM 101 (*lac, leu, pro*, F*tra*D36) was used as recipient for M13mp8 derivatives (Messing and Vieira 1982). HB2154 (Carter et al. 1985) was used as the host strain

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for M13mp18 (Norrander et al. 1983) in the site-directed mutagenesis experiments. The mutant plasmids described were derived from pPIL291-15, carrying the *fel* (F-eleven) gene cluster (De Ree et al. 1985a; Van Die et al. 1986b). Bacteria were cultivated in L broth, or Brain Heart Infusion (BHI) agar (Oxoid). For analysis of expression of P-fimbriae, strains were grown overnight on BHI agar. Selective pressure against loss of plasmids was imposed by adding 50 μg/ml ampicillin.

DNA techniques. Isolation of plasmid DNA was carried out by the mini-lysate method, essentially as described by Holmes and Quigley (1981). Restriction endonucleases and ligase (Pharmacia, Uppsala, Sweden) were used according to the manufacturers specifications. Site-directed mutagenesis was performed by the gapped-duplex method (Kramer et al. 1984) essentially as described before (Van Die et al. 1988b). Transformation of *E. coli* K12 strains was carried out as described by Kushner (1978). The chain termination method of Sanger et al. (1977) was used to sequence the mutations after cloning.

Electron microscopy. Negative staining of whole cells was performed as described previously (Van Die et al. 1988b).

Purification of fimbriae and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fimbriae were purified essentially as described before (Riegman et al. 1990). Purified fimbriae were analysed by SDS-PAGE on 14% acrylamide gels as described by Lugtenberg et al. (1975). Protein staining was performed with Fast Green.

Antisera and enzyme-linked immunosorbent assay (ELISA). The F11-specific monoclonal antibodies (Mabs) M7-4, M7-6, M7-7 and M7-13 were isolated by De Ree et al. (1985b) and characterized by Van Die et al. (1988a). The FMDV-specific Mabs MA11 and MA18 were described by Meloen et al. (1987) and Thomas et al. (1988). Mab 32F81, specific for the *Plasmodium falciparum* epitope was described by Van Amerongen et al. (1989). ELISA was performed with whole cells essentially as described by Van der Ley et al. (1985). Orthophenylenediamine (0.4 mg/ml in 25 mM citric acid, 51 mM disodium hydrogen phosphate, pH 5) was used as a substrate for the peroxidase reaction. The A_{492} was read with the Easy Reader EAR 400.

Immunization experiments. Three BALB/C mice were immunized by intraperitoneal injection. On day 0, each mouse was injected with 10 μg fimbriae in complete Freund's adjuvant (FA). On day 14 the mice were each injected with 10 μg fimbriae in incomplete FA. On day 28 the mice were bled. To screen the sera, total proteins from inactivated FMDV virus type A_{10}-Holland were separated by SDS-PAGE, transferred to nitrocellulose and tested in Western immunoblots as described previously (Van Die et al. 1988b). The virus had been inactivated by an azividin derivative that reacts with the virus RNA and not with the viral coat proteins (Barteling and Anemaet 1987).

Results

Construction of insertion vectors

Foreign peptides were inserted into the major subunit of F11 serotype P-fimbriae by genetic manipulation of the *fel*A gene that encodes this subunit (De Ree et al. 1985a; Van Die et al. 1986b). Synthetic oligonucleotides encoding known specific epitopes were introduced into HR1 and HR4 of the *fel*A gene. To perform these experiments, two insertion vectors, pPIL291-1519 and pPIL291-1510, were constructed that contain the *fel*A gene with unique cloning sites in HR4 and HR1 respectively (Fig. 1A–C). Plasmid pPIL291-1519 was constructed by cloning the 3.0 kb *Cla*I-*Bam*HI fragment of pPIL291-1529 (Van Die et al. 1988b) into pBR322 (Bolivar et al. 1977). In plasmid pPIL291–1519 the nucleo-

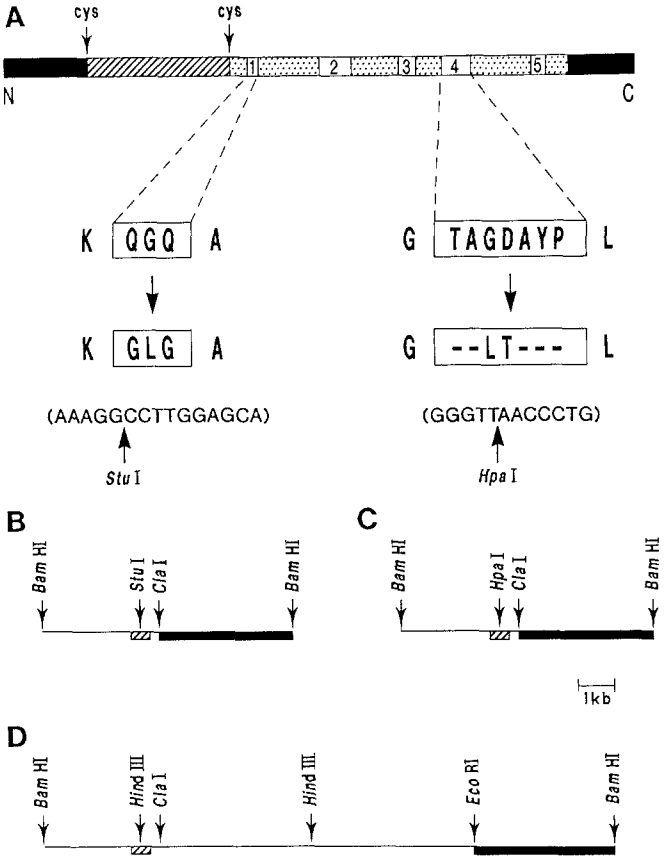

Fig. 1A–D. Schematic representation of the FelA major subunit and physical maps of plasmids encoding the *fel*A gene and the *fel* gene cluster. **A** Model of the FelA subunit (Van Die et al. 1987). The *degree of shading* indicates the levels of homology between different serotypes in different regions of the subunit; from *black* (high homology) to *white* [hypervariable (HR) regions 1–5]. The mutated regions in HRs 1 and 4 and details of the site-directed mutations, introduced during construction of the insertion vectors are indicated. **B** Physical map of the insertion vector pPIL291-1510, with a unique *Stu*I cloning site in HR1. **C** Physical map of the insertion vector pPIL291-1519, with a unique *Hpa*I cloning site in HR4. **D** Physical map of pPIL291-15, carrying the complete *fel* gene cluster. The *shadowed boxes* in **B–D** represent the *fel*A gene; the *black boxes* represent pBR322 vector DNA (Bolivar et al. 1977)

tides encoding HR4 have been deleted and replaced by an oligonucleotide containing an *Hpa*I recognition sequence. Plasmid pPIL291-1510 contains a unique *Stu*I restriction site in the region encoding HR1. This *Stu*I site was obtained by site-directed mutagenesis of the *felA* gene with the aid of a 30 bp primer (Figs. 1C and 2H). Both cloning sites were constructed in the same reading frame, so that various synthetic oligonucleotides could easily be cloned in both vectors.

Introduction of foreign sequences into the insertion vectors

Several oligonucleoties of varying lengths were inserted into pPIL291-1510 and/or pPIL291-1519. They code for

A FMDV A TAT AAA CAG AAG ATC ATC GCC CCG GG
epitope Y K Q K I I A P

B M.leprae A ACG TTC GGA CTA CAG CTG GAG CTT ACG TC
epitope T P G L Q L E L T

C P.falc. A ATA TTA GAT ACA AGC AAT CCT GTT AAA ACT GG
epitope I L D T S N P V K T

D FMDV A TCG CGC CGT GGT GAT CTG GGA TCC TTA GCA CCA CGT GTT AA
epitope S R R G D L G S L A P R V

E HIV T AAA CAG TTC ATC AAC ATG TGG CAG GAA GTA GGC AAA GCA ATG TAC GC
epitope K Q F I N M W Q E V G K A M Y

F FMDV C GCT GTT CCA AAC CTG CGT GGT GAC CTG CAA GTG CTG GCT CAA AAG GTT GCG CTG ACT CTG CC
epitope A V P N L R G D L Q V L A Q K V A R T L

G HIV A ACC ATC ACT CTG CCA TGC CGT ATT AAA CAG TTC ATC AAC ATG TGG CAG GAA GTA GGC AAA GCA ATG TAC GCA CCC CCG GG
epitope T I T L P C R I K Q F I N M W Q E V G K A M Y A P P

H primer HR1 CAG CTT TTA AAG GCC TTG GAG CAG CTA AAA

Fig. 2A–H. Sequences of synthetic oligonucleotides. **A–G** Sequences of synthetic oligonucleotides that encode epitopes of foot and mouth disease virus (FMDV), *Mycobacterium leprae*, *Plasmodium falciparum* or human immunodeficiency virus (HIV). **A** C-terminal epitope (amino acids 200–207) of vp1 protein of FMDV type A_{10}-Holland reacting with monoclonal antibody (MAb) MA18 (Meloen et al. 1987); **B** T-cell epitope (180–188) of the 65 kDa protein of *M. leprae* (Van Eden et al. 1988); **C** epitope (122–130) of the 25 kDa surface protein of *P. falciparum*, reacting with Mab32F81 (Van Amerongen et al. 1989); **D** epitope (141–153) of vp1 protein of FMDV type A_{10}-Holland reacting with Mab MA11 (Meloen et al. 1987); **E** epitope (426–441) of the gp120 coat protein of HIV (Kowalski et al. 1987); **F** epitope (140–160) of the vp1 protein of FMDV type O (Makoff et al. 1982); **G** epitope (418–443) of the gp120 coat protein of HIV (Kowalski et al. 1987). **H** Primer used for site-directed mutagenesis of HR1

Table 1. Characteristics of insertion-mutant plasmids carrying sequences coding for foreign epitopes

Plasmid	Mutated HR of FelA	Inserted oligonucleotide[a]	Length of resultant insert (aminoacids)	Formation of fimbriae in HB101[b]
pPIL291-15102	1	FMDV (A)	9	++
pPIL291-1569	4	FMDV (A)	9	++
pPIL291-1566	4	*Mycobacterium leprae* (B)	10	–
pPIL291-15303	1	*Plasmodium falciparum* (C)	11	+++
pPIL291-15301	4	*Plasmodium faliparum* (C)	11	++
pPIL291-15104	1	FMDV (D)	14	++
pPIL291-1567	4	FMDV (D)	14	+
pPIL291-15206	1	HIV (E)	16	–
pPIL291-15204	4	HIV (E)	16	–
pPIL291-1558	4	FMDV (F)	21	–
pPIL291-15202	1	HIV (G)	27	–
pPIL291-15 (w.t.)	–	–	0	++++

[a] Details of oligonucleotide are shown in Fig. 2A–G
[b] Fimbriae formation as judged by electron microscopy

antigenic determinants of FMDV, human immunodeficiency virus (HIV), *P. falciparum* or *Mycobacterium leprae* (Fig. 2A–G). The plasmids were digested with *Stu*I or *Hpa*I, ligated to the respective oligonucleotides, and the ligation mixtures used to transform HB 101 cells. Plasmids were isolated from individual transformants and tested for the presence of the oligonucleotide in the proper orientation by restriction enzyme analysis and nucleotide sequencing.

To reconstitute the entire *fel* gene cluster, an 8.5 kb *Eco*RI-*Cla*I fragment of plasmid pPIL291-15, lacking the *fel*A structural gene coding for the major subunit, was introduced into the mutant plasmids obtained (Fig. 1D). Details of the constructed plasmids and their characteristics are summarized in Table 1.

Formation of fimbriae

The effect of the insertion of antigenic sequences into the major subunit on formation of fimbriae was studied by electron microscopic examination of negatively stained HB101 cells carrying the plasmids listed in Table 1. Formation of fimbriae was generally observed when the length of the inserted peptide did not exceed 14 amino acids (Table 1). However, fimbriae were often generated in smaller amounts than normal and reduced in size. In some cases they appeared to be more fragile, as numerous fragmented fimbriae were observed by electron microscopy (i.e. fimbriae encoded by pPIL291-15104 and pPIL291-15102, Fig. 3B). Insertion of protein sequences exceeding 14 amino acids in the major subunit apparently disturbed the formation of fimbriae (Table 1).

In an ELISA, the binding of four different F11-specific Mabs to mutant strains that formed fimbriae was studied (Table 2). The results indicated that fimbriae containing an insertion in HR1 all showed efficient binding to Mab M7-13 and did not bind M7-6; binding of M7-4 was reduced in all cases, but the degree of reduction varied; binding of M7-7 was eliminated in the case of two strains, while one strain showed a normal binding of M7-7. These results suggest that, in normal F11 fimbriae, HR1 contains (part of) an epitope that is recognized by M7-6. Insertion of foreign sequences into HR1 apparently affects the conformation of the major subunit in different ways or causes steric hindrance, resulting in reduction or loss of binding of M7-4 and M7-7.

Fimbriae that contain an insertion in HR4 all showed slightly reduced binding of M7-6 and M7-4 compared with F11 fimbriae and did not bind M7-7 or M7-13

We have shown previously that fimbriae containing an insertion of two amino acids in HR4 still react with M7-13 (Van Die et al. 1988a). Apparently larger inserts may cause distortion of the fimbrial structure or cause steric hindrance, resulting in loss of the M7-13 antigenic determinant, as was also shown for insertions in HR1.

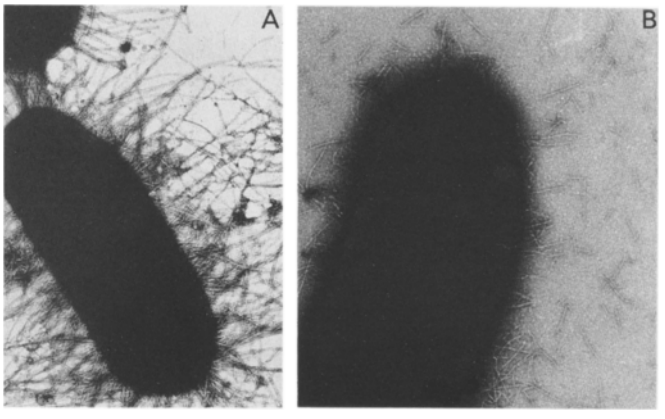

Fig. 3A and B. Electron micrographs of negatively stained HB101 cells carrying (**A**) pPIL291-15 and (**B**) pPIL291-15104

Table 2. Whole cell ELISA using F11-specific Mabs M7-4, M7-6, M7-7 and M7-13

HB101 cells[a] carrying plasmids	A_{492}				Insertion in HR
	M7-4	M7-6	M7-7	M7-13	
No plasmid	0	0	0	0	–
pPIL291-15	1.2	1.7	0.6	1.8	–
pPIL291-15102	0.1	0	0	1.3	1
pPIL291-15104	0.3	0	0.6	1.5	1
pPIL291-15303	0.7	0	0	1.4	1
pPIL291-1569	0.9	1.2	0	0	4
pPIL291-1567	0.7	1.0	0	0.1	4
pPIL291-15301	1.1	1.4	0	0	4

[a] Cell suspension used to coat microtiter plates had an optical density at 660 nm of 0.2
[b] Mabs were used at a dilution of 1/1000

Exposure of the inserted epitopes in the fimbriae

To determine whether the inserted foreign epitopes, derived from FMDV or *P. falciparum*, were exposed in

Table 3. Whole cell ELISA using the FMDV-specific Mabs MA11 and M18, and the *Plasmodium falciparum*-specific Mab 32F81

HB101 cells[a] carrying plasmids	A_{492}			Insertion in HR
	MA11[b]	MA18	Mab32F81	
pPIL291-15102	–[c]	1.6	–	1
pPIL291-15104	1.4	–	–	1
pPIL291-15303	–	–	1.4	1
pPIL291-1569	–	0.5	–	4
pPIL291-1567	0.1	–	–	4
pPIL291-15301	–	–	0	4
pPIL291-15 (F11)	0	0	0	–

[a] Optical density of the cell suspensions at 660 nm was 0.2
[b] Mabs were used at a dilution of 1/1000. Mab MA11 recognizes epitope D, Mab MA18 recognizes epitope A and Mab 32F81 recognizes epitope C (Fig. 2)
[c] Not determined

the fimbriae, the mutant strains that formed fimbriae were analysed in ELISA using specific Mabs. These Mabs have been shown to bind to the FMDV or *P. falciparum* epitopes respectively in a PEPSCAN (Meloen et al. 1987; Van Amerongen et al. 1989).

The results in Table 3 show that the Mabs MA11 and M18 bound efficiently to the hybrid fimbriae containing the corresponding FMDV epitopes when they were inserted in HR1, whereas lower binding was observed when the epitopes were inserted in HR4. Mab32F81 bound very well to fimbriae that contained the *P. falciparum* epitope in HR1, whereas no binding was observed to fimbriae that contained the epitopes in HR4. F11 fimbriae did not show any binding to the Mabs in the same experiment.

The results indicate that the foreign epitopes are exposed correctly in the fimbrial filaments when they are inserted in HR1. The exposure of these epitopes seems to be less favourable when they are inserted in HR4.

Fimbriae were isolated from HB101 cells carrying pPIL291-15104. Three mice were injected with the purified fimbriae, and sera, taken on day 28, were tested in Western blots for the presence of virus-specific antibodies. All sera contained high levels of anti-fimbriae antibodies, while the sera of two mice also reacted with vp1 (data not shown). These results indicate that the FMDV/F11 hybrid fimbriae encoded by pPIL291-15104 can elicit a vp1-specific antibody response in mice.

Discussion

The HRs of the FelA subunit, the major subunit of F11 fimbriae, have been examined for their ability to accept insertions of foreign epitopes without affecting the capacity to form fimbriae. In general, the ability to form fimbriae was not abolished when sequences composed of maximally 14 amino acids were inserted into HR1 or HR4. The *M. leprae* epitope was an exception: insertion of this peptide in HR4 resulted in non-fimbriated cells. The presence of this sequence either prevents the subunits from assuming the conformation needed for interaction with accessory proteins, or it prevents polymerization. Insertion of larger peptides in the F11 fimbriae was not successful. In contrast, some of the other bacterial carrier systems described (e.g. the outer membrane proteins LamB and PhoE) accept insertions of 30–50 amino acids (Agterberg et al. in press; Charbit et al. 1988) and have been shown to be very attractive and flexible carrier systems.

Both HR1 and HR4 encompass natural epitopes of the F11 fimbriae; the FelA-specific Mab M7-6 most likely recognizes an epitope that is located in HR1 and M7-7 binds to an epitope in HR4 (Van Die et al. 1988a; this report). As expected, insertion of foreign epitopes in these regions destroyed the binding of these Mabs to the hybrid fimbriae. However, it was observed that, depending on the sequence inserted, binding of one or more of the other F11-specific Mabs also appeared to be affected after insertion. These results suggest that the inserted sequence causes steric hindrance, such that the Mabs cannot interact properly with their own epitopes, or that the conformatian of the major subunit has been changed as a result of insertion.

We have shwon that insertion of epitopes in HR1 or HR4 affects binding of F11-specific Mabs to the hybrid fimbriae in a rather unpredictable manner. In the whole cell ELISA used, binding of the Mabs is dependent on the numbers and lengths of fimbriae expressed per cell, and may be affected by a conformational change in the subunit. Therefore, quantitation of the efficiency of fimbrial production in the different recombinant strains is very difficult and a valid judgement can only be made by the combination of electron microscopic examination and ELISA tests with several F11-specific Mabs.

In principle, hybrid fimbriae as described here can be used for diagnostic purposes, or as components of a subunit vaccine. The restriction in length of the sequence that can be inserted in the P-fimbrial subunit however could be a major drawback for general application of this carrier system. HR1 accepts the insertion of up to 14 amino acids, while HR4 accepts the replacement of 7 amino acids and an additional insertion of 4-7 amino acids. However, the comparison of different epitopes from several pathogens, that differ not only in length but also in other respects (e.g. hydrophilicity), complicates general conclusions. Our results suggest that HR1 is the most promising HR for manipulation. All epitopes tested here were recognized better by the corresponding Mabs when inserted in HR1 than when they were inserted in HR4 of the fimbriae. Initial immunization experiments showed that fimbriae encoded by pPIL291-15104, which carry an FMDV epitope in HR1, can elicit a vp1-specific antibody response in mice. Very recent data however have shown that some epitopes are expressed in an antigenic form only when inserted in HR4 of the F11 fimbriae (A. Van der Zee and I. Van Die, manuscript in preparation). The availability of two regions for the insertion of a specific epitope clearly enhances the chances of obtaining hybrid fimbriae with the desired antigenic properties.

Other fimbrial carrier systems have recently been examined that show possibilities and restrictions similar to those of the P-fimbrial system. Thiry et al. (1989) have exploited HRs of K88 fimbriae for presentation of epitopes. Interestingly, fimbriae of *Bacteroides nodosus* did accept insertions in HRs, but epitopes were antigenic only when they were fused to the C-terminal part of the fimbrial subunit (Jennings et al. 1989). We have used a similar approach for P-fimbriae, but without success; fusion of even a few amino acids to the C-terminal Gln residue of the FelA subunit interfered with formation of fimbriae (unpublished results).

In summary, we expect that application of a fimbrial carrier system will be restricted to some specific sequential epitopes that are antigenic when incorporated in the fimbriae. However when these requirements are fulfilled, fimbriae are very attractive carriers. Fimbriated bacteria can be cultivated at low cost and fimbriae are easy to purify. Due to the polymeric structure of the fimbrial filament, the foreign antigen is presented as repeating

units which have been found to be advantageous for immunogenicity (Broekhuysen et al. 1987). More immunological data however are needed to establish the ultimate value of this system.

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