Authentic display of a cholera toxin epitope by chimeric type 1 fimbriae: effects of insert position and host background

Bodil Stentebjerg-Olesen,¹ Lars Pallesen,^{1†} Lars Bogø Jensen,^{1‡} Gunna Christiansen² and Per Klemm¹

Author for correspondence: Per Klemm. Tel: +45 45 25 25 06. Fax: +45 45 93 28 09.
e-mail: pk@im.dtu.dk

¹ Department of Microbiology, Building 301, Technical University of Denmark, DK-2800 Lyngby, Denmark

² Department of Medical Microbiology, University of Aarhus, DK-8000 Aarhus C, Denmark

The potential of the major structural protein of type 1 fimbriae as a display system for heterologous sequences was tested. As a reporter-epitope, a heterologous sequence mimicking a neutralizing epitope of the cholera toxin B chain was inserted, in one or two copies, into four different positions in the *fimA* gene. This was carried out by introduction of new restriction sites by PCR-mediated site-directed mutagenesis of *fimA* in positions predicted to correspond to optimally surface-located regions of the subunit protein. Subsequently, the synthetic cholera-toxin-encoding DNA segment was inserted. Several of the chosen positions seemed amenable even for large foreign inserts; the chimeric proteins were exposed on the bacterial surface and the cholera toxin epitope was authentically displayed, i.e. it was recognized on bacteria by specific antiserum. Display of chimeric fimbriae was tested with respect to host background in three different *Escherichia coli* strains, i.e. an isogenic set of K-12 strains, differing in the presence of an indigenous *fim* gene cluster, as well as a wild-type isolate. Immunization of rabbits with purified chimeric fimbriae resulted in serum which specifically recognized cholera toxin B chain, confirming the utility of the employed strategy.

Keywords: surface display of foreign epitopes, chimeric proteins, effect of host background, type 1 fimbriae

INTRODUCTION

Developments in biotechnology have led to a number of new strategies for designing and engineering vaccines. An attractive example is to fuse immunologically relevant sectors of an important protein antigen into a naturally occurring bacterial surface protein such as the structural proteins for flagella or fimbriae. Fimbriae are adhesive bacterial surface structures which enable bacteria to target and to colonize specific host tissues (for reviews see Duguid & Old, 1980; Klemm & Krogfelt, 1994; Krogfelt, 1991). These are long thread-like surface

organelles, found in up to about 500 copies per cell. A large variety of fimbriae are known, and have, where this aspect has been studied, turned out to be completely non-toxic proteins only serving as colonization factors. Vaccines based on wild-type fimbriae have been highly successful, for example in protecting against various diarrhoea-causing enterotoxigenic *Escherichia coli* strains (Levine *et al.*, 1994; Moon & Bunn, 1993). Such results have indicated that fimbriae normally are very good immunogens both in the context of live vaccines and as purified proteins.

Type 1 fimbriae are found on the majority of *E. coli* strains. A single type 1 fimbria is a thin, 7 nm wide and approximately 1 µm long, surface polymer. It consists of about 1000 subunits of a major building element, the FimA protein, stacked in a helical cylinder (Brinton, 1965). Additionally, a small percentage of minor components are also present as integral parts of the fimbriae (Krogfelt & Klemm, 1988). It has been shown that the minor components, FimF, FimG and FimH, are involved

[†] **Present address:** Department of Research and Development, Division of Microbiology, State Serum Institute, DK-2300 Copenhagen S, Denmark.

[‡] **Present address:** Danish Veterinary Laboratory, DK-1790, Copenhagen, Denmark.

Abbreviations: CTB, cholera toxin B chain; DICM, differential interference contrast microscopy; FITC, fluorescein isothiocyanate.

Fig. 1. Overview of plasmids (not drawn to scale). (a) *fim* gene cluster as present in plasmid pPKL4. Below is the replacement vector containing a *SacI* site into which cassettes of engineered *fimA* genes were inserted. (b) Expanded section of the *fimA* gene showing the engineering of the *fim* gene cluster to contain chimeric *fimA* with heterologous sequences of CTB. Hatched and black boxes indicate signal-peptide- and CTB-encoding sectors, respectively. Numbers above the gene boxes indicate insert positions. (c) Sequence of the synthetic DNA segment mimicking a sector of CTB.

in fimbrial-length regulation (Klemm & Christiansen, 1987; Russell & Orndorf, 1992). The FimH protein has been shown to be the actual receptor-binding molecule which recognizes D-mannose-containing structures (Krogfelt *et al.*, 1990). The FimF and FimG components seem to be required for integration of the FimH adhesin into the fimbriae. The minor components are *per se* not necessary for synthesis of the fimbriae, since recombinant bacteria entirely missing the corresponding genes are able to express pure FimA fimbriae (Klemm & Christiansen, 1987; Krogfelt & Klemm, 1988). However, such organelles are devoid of adherence activity.

Type 1 fimbriae are encoded by the *fim* gene cluster. This encompasses nine genes contained within a 9.5 kb DNA segment (Fig. 1). We have studied the *fim* genes responsible for type 1 fimbriation of *E. coli* K-12 strain PC31 for a number of years and have reached a detailed understanding of the biosynthesis of these adhesive organelles. The structural components of type 1 fimbriae are produced as precursors having an N-terminal signal sequence. This element is subsequently removed during export across the inner membrane. All evidence suggests that this translocation is dependent on the normal type II *E. coli* export system, viz. SecA, SecB, etc. (Dodd & Eisenstein, 1984; Pugsley, 1993). However, further export to the cell exterior, i.e. from the periplasm and across the outer membrane, is dependent on a fimbriae-specific export and assembly system constituted by the FimC and FimD proteins. FimC is a periplasmic located

chaperone that binds to nascently translocated organelle components and prevents them from making incorrect interactions that cause aggregation and proteolytic degradation (Jones *et al.*, 1993; Klemm, 1992; Pugsley, 1993). FimD, a so-called usher, is found in the outer membrane and is involved in organized polymerization of the structural components into fimbrial organelles (Hultgren *et al.*, 1993; Klemm & Christiansen, 1990; Pugsley, 1993).

The biogenesis machinery of type 1 fimbriae has been shown to be quite tolerant in accepting similar but heterologous structural components and still assemble such into fimbriae. Thus, components from F1C fimbriae exhibiting as little as 34% identity with their equivalents in type 1 fimbriae are readily integrated into type 1 fimbriae resulting in hybrid organelles (Klemm *et al.*, 1994). This kind of information suggested that type 1 fimbriae could be used as carriers of heterologous sequences representing foreign epitopes. Such fimbriae consist of chimeric FimA proteins resulting from in-frame fusion of the heterologous sequences into selected positions in the *fimA* gene. If insert positions are chosen which do not interfere with the bioassembly of the organelles, normal fimbriae, in which every subunit will carry a foreign peptide segment will result. There are several advantages of such an approach, one being the large amounts of major subunit protein and thereby foreign antigen produced, i.e. a single fimbriated cell has as many as half a million copies of FimA on its surface.

Another advantage is that type 1 fimbriae are excellent immunogens, which could boost the immune response against a passenger epitope.

In a previous study we inserted foreign sequences into FimA in positions corresponding to available unique restriction sites in the *fimA* sequence (Hedegaard & Klemm, 1989). Questions remaining open from this analysis included the best localities and permissive size of the inserts, and significantly, whether the foreign sequences were authentically displayed in the context of chimeric FimA proteins, and could evoke an immune response directed against the parental protein. Here we try to answer these questions.

METHODS

Bacterial strains and growth conditions. A list of *E. coli* strains used in this study is provided in Table 1. *E. coli* strain MS7, a Δ *fim*-derivative of PC31, was made essentially following the procedure of Schembri *et al.* (1996). In short, an *npt* cassette flanked by DNA segments containing the upstream and downstream regions of the *fim* gene cluster, respectively, was used for homologous double crossover recombination. Cells were grown on solid medium or liquid broth supplemented with the appropriate antibiotics.

Plasmids. Plasmids used in this study are listed in Table 1. Plasmid pLPA41 was made by inserting a 636 bp PCR fragment containing the *fimA* gene and the adjacent regions 70 bp upstream and 20 bp downstream and flanked by two *SacI* sites into the *SacI* site of plasmid pUC18. The insert was positioned downstream and in an expression-compatible orientation to the *lac* promoter residing on the vector part of the plasmid. Plasmids pLPA110, pLPA111, pLPA112 and pLPA119 were made by joining two individual PCR fragments in each case (see Fig. 2) at their *BglII* sites and inserting the resulting fragment into the *SacI* site of plasmid pUC18. Plasmids pLPA113, pLPA114, pLPA115 and pLPA125 were made by inserting a 51 bp synthetic dsDNA segment encoding amino acids 50–64 of the B subunit of cholera toxin and containing a *BglII* overhang at one end and a *BamHI* overhang at the other into the *BglII* site of plasmids pLPA110, pLPA111, pLPA112 and pLPA119, respectively (cf. Figs 1 and 2). Plasmids pLPA123, pLPA124 and pLPA126 were made by introducing a second cholera-epitope-encoding segment into the *BglII* sites of plasmids pLPA113, pLPA114 and pLPA125, respectively. Plasmid pLBJ312 was made as depicted in Fig. 3.

Prediction of surface-located positions in FimA. Positions with maximum probability of surface location were chosen according to concomitant occurrence of predicted hydrophilicity maxima and β -turns as described by Hedegaard & Klemm (1989).

DNA techniques. Isolation of plasmid DNA was carried out by the method of Birnboim & Doly (1979). Restriction endonucleases were used according to the manufacturer's specifications (New England Biolabs). DNA sequencing was by the dideoxy chain-termination technique (Sanger *et al.*, 1977) using a Sequenase version 2.0 kit from USB. Oligonucleotides were made at the department's core facilities.

PCR methodology. PCR were performed on a Perkin Elmer Cetus DNA Thermal Cycler 480. Reactions were set up as 100 μ l volumes containing 200 μ M each of dATP, dCTP,

dGTP and dTTP, 0.2–1.0 μ M of each of the two primers, 2 mM $MgCl_2$, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2.5 units of AmpliTaq DNA polymerase and 0.1–0.2 μ g plasmid template. The reactions were run for 25–30 cycles each consisting of 1 min at 94 °C, 1 min at 40 °C, and 1 min at 72 °C. The following primers, partially based on the *fimA* sequence (Klemm, 1984) were used for introduction of flanking *SacI* sites and an internal *BglII* site: P1: 5'-GTTCGAGCTC-GTTTACAAA-3'; P2: 5'-CAAGATCTGTTGATCAAAA-3'; P3: 5'-ACAGATCTTGCATCAACT-3'; P4: 5'-AAAGATC-TACCAGTTCTG-3'; P5: 5'-GTAGATCTTTCCTGTGCC-3'; P6: 5'-CGAGATCTACCAACGTTTC-3'; P7: 5'-GTAGA-TCTCGCATCAATC-3'; P8: 5'-ACAGATCTGGTGCTGC-GC-3'; P9: 5'-CCAGATCTGTCCAGGATC-3'; P10: 5'-AATGAGCTCCCTGAACCT-3'. PCR fragments were made, using primers P1–P10, as shown in Fig. 2 employing plasmid pLPA41 as template.

Haemagglutination. The capacity of bacteria to express a D-mannose-binding phenotype was assayed by their ability to agglutinate guinea-pig erythrocytes on glass slides. Aliquots of liquid bacterial cultures at OD₅₅₀ 10.0 and 5% erythrocytes were mixed and the time until agglutination occurred was measured.

Antisera. Rabbit anti-type 1 fimbrial serum raised against purified type 1 fimbriae has previously been described (Krogfelt & Klemm, 1988). Hyperimmune serum against cholera toxin B chain (CTB) was obtained by immunizing a rabbit with CTB (Sigma). Aliquots of 20 μ l of a solution of 1 mg CTB ml⁻¹ in Freund's incomplete adjuvant were given intravenously every fifth day over a 7 week period, after which the animal was bled. Fluorescein isothiocyanate (FITC) conjugated anti-rabbit serum was supplemented by Dako, Denmark.

Western blot (immunoblot). Samples were subjected to sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis and transferred to PVDF microporous membrane filters using a semi-dry blotting apparatus. Filters were processed as previously described (Hedegaard & Klemm, 1989; Krogfelt & Klemm, 1988). Serum raised against either wild-type or chimeric fimbriae or CTB, respectively, was used as the primary serum and peroxidase-conjugated anti-rabbit serum was used as the secondary serum.

Fixation of bacterial cells and fluorescence labelling. Cells from overnight cultures were harvested and washed gently in PBS (0.145 M NaCl, 0.15 M sodium phosphate). Cells were fixed by mixing 250 μ l of cells with 750 μ l of a 4% (w/v) solution of paraformaldehyde in PBS. This mixture was incubated on ice for 20 min. To remove the fixative, cells were washed twice in PBS. Samples of 20 μ l were placed on a poly L-lysine coated slide and air dried. After washing in PBS, 16 μ l of a 1:100 dilution (anti-fimbriae) or 1:250 dilution (anti-CTB) of the primary antiserum was placed on top of each sample and left in a moist incubation chamber for 1 h. The slides were washed three times in PBS, and 16 μ l of a 1:25 dilution of FITC conjugated secondary antiserum was added. After 2 h in the dark the slides were washed three times in PBS and a drop of Citifluor (Citifluor) was placed on top of each sample.

Microscopy and image analysis. Differential interference contrast microscopy (DICM) and image analysis was carried out as described by Pallesen *et al.* (1995).

Electron microscopy. Electron microscopy and immuno-electron microscopy were carried out essentially as described previously (Klemm *et al.*, 1994; Pallesen *et al.*, 1995). In short

a 10 µl aliquot of bacterial suspension was placed on a carbon-coated, glow discharged grid for 30 s. Grids were washed in two drops PBS, dehydrated in increasing concentrations of ethanol, blotted dry, and shadowed with tungsten. For immunoelectron microscopy, a mixture of anti-CTB rabbit serum (diluted 1:100) and a monoclonal mouse anti-FimA serum (diluted 1:5) was used as the primary antiserum, and anti-rabbit and anti-mouse serum conjugated with colloidal gold particles, of 10 nm and 5 nm diameter, respectively (BioCell), were used as the secondary antibody. The monoclonal anti-FimA serum was a generous gift from Karen Krogfelt, State Serum Institute, Denmark.

Immunization with fimbriae. Fimbriae were purified essentially as previously described (Hedegaard & Klemm, 1989), the only change being that precipitation of fimbriae from the crude extract was performed in 20% (w/v) ammonium sulfate. Purified fimbriae were used to immunize rabbits. Two animals were immunized with purified chimeric (FimA-CTB) fimbriae, and one animal was immunized with wild-type type 1 fimbriae (control), respectively. Three doses of a suspension of 0.1 ml 0.5 mg fimbriae ml⁻¹ mixed with equal parts of Freund's incomplete adjuvant were administered subcutaneously at 2 week intervals. Blood was collected from animals before immunization and after 3, 6 and 9 weeks.

RESULTS

Research strategy

In a preliminary study (Hedegaard & Klemm, 1989) heterologous DNA segments, encoding consensus sequences for restriction enzymes or, in a few cases, mimicking foreign epitopes, were inserted into naturally available restriction sites in the *fimA* gene which were unique in the context of all or large parts of the *fim* gene cluster. Such an approach only permits insertion into a few positions which might not be optimal for harbouring heterologous sequences. The present work relies on a different strategy, viz. introduction of restriction sites by PCR technology. Such an approach offers the possibility to engineer any position of choice in the *fimA* gene to contain unique restriction sites which in turn can be used for insertion of heterologous sequences representing foreign epitopes. In this work, initial genetic manipulations of the *fimA* gene were carried out on plasmid vectors that uniquely contained this gene. To avoid cumbersome binary plasmid systems, all manipulated *fimA* chimeras were subsequently moved into the context of the total *fim* gene cluster on a single plasmid.

Engineering new restriction sites into *fimA*

Based on algorithms for prediction of such parameters as hydrophilicity and secondary structure, five positions, corresponding to 25, 45, 60, 80 and 105 in the mature FimA protein were predicted to have maximum probability of surface-exposure in the protein. One of these, position 60, is proximal to a cysteine residue known to be involved in the intramolecular S₂₁-S₆₁ bridge (McMichael & Ou, 1979). To avoid interfering with this structural feature we decided to concentrate on the four other positions, viz. 25, 45, 80 and 105, as insertion sites

for heterologous sequences. To facilitate later manipulations the *fimA* gene was subcloned into the *SacI* site of the pUC18 vector by PCR technology (cf. Methods) resulting in plasmid pLPA41. Subsequently, *BglII* sites were introduced into the *fimA* gene in locations corresponding to the previously mentioned positions in the mature FimA protein. This was carried out by site-directed mutagenesis employing synthetic oligonucleotides and PCR amplification resulting in plasmids pLPA110, pLPA119, pLPA111 and pLPA112 (Fig. 2). The introduced *BglII* sites did not result in any change in the number of amino acids, but did cause a few codon changes. Sequence analysis of the entire modified *fimA* genes in plasmids pLPA110, pLPA119, pLPA111 and pLPA112 confirmed that no other changes had occurred.

Engineering heterologous DNA sequences encoding a cholera toxin epitope into four sites in *fimA*

As a relevant heterologous reporter-epitope we have chosen a well-characterized region of the CTB. This segment consists of residues 50–64 of CTB and has previously been shown to elicit antibodies that bind to and neutralize cholera toxin (Jacob *et al.*, 1983). Residues 50–64 comprise a conformational loop on the surface of CTB that is entirely conserved between CTB and the closely related heat-labile toxin of *E. coli* (Merritt *et al.*, 1994; Shoham *et al.*, 1995). This loop is directly involved in receptor-binding of the toxin to epithelial cells (Merritt *et al.*, 1994). A synthetic DNA segment encoding the cholera epitope was made by annealing two complementary 51 bp oligonucleotides, which were designed, after annealing, to result in a dsDNA segment with a *BglII* overhang in one end, and a *BamHI* overhang in the other. A single copy of the epitope-encoding segment was inserted into the *BglII* sites in the *fimA* gene in plasmids pLPA110, pLPA119, pLPA111 and pLPA112, respectively, resulting in plasmids pLPA113, pLPA125, pLPA114 and pLPA115 (Fig. 2). Insertion of the cholera-toxin-encoding oligonucleotide resulted in regeneration of a *BglII* site at only one end of the insert. This feature was used to identify plasmids with correct orientation of the insert. Furthermore, this feature also permitted creation of a number of chimeras containing tandem repeats of the cholera toxin insert, i.e. plasmids pLPA123, pLPA126 and pLPA124 (Table 1; Fig. 2). Restriction enzyme and DNA sequence analysis of the chimeric plasmids, harbouring either a single or double cholera insert, confirmed the orientation and conservation of the reading frame in the chimeric *fimA*-cholera gene.

Construction of single plasmid systems containing *fim* gene clusters with chimeric versions of *fimA*

To create a cassette into which chimeric versions of the *fimA* gene could be easily inserted into a background of the rest of the *fim* genes, the *fimA* gene was replaced by a unique *SacI* restriction site resulting in plasmid pLB312 (Fig. 3). A *fim* host containing this plasmid is

Fig. 2. Flow diagram showing the pedigree of the engineering of the *fimA* gene to contain *Bgl*II handles, and segments of CTB. Numbers correspond to manipulated positions; numbers in boxes refer to the employed primers (cf. Methods).

unable to produce fimbriae. To test the replacement system a *Sac*I fragment from plasmid pLPA41, containing the wild-type version of the *fimA* gene, was introduced into the *Sac*I site of pLBJ312 resulting in pLBJ318 (Fig. 1). Host cells that harboured this plasmid produced type 1 fimbriae which in quality and quantity were indistinguishable from those produced by hosts containing a control plasmid (pPKL4) that harboured the non-engineered *fim* gene cluster (data not shown). Since all chimeric *fimA* genes in plasmids pLPA113–pLPA115 and pLPA123–pLPA126 were designed such that they were flanked by *Sac*I sites, all of these cassettes were subsequently inserted into the unique *Sac*I site of pLBJ312, resulting in plasmids pBSO1–pBSO 7, respectively (Table 1, Fig. 1).

Expression of chimeric FimA and chimeric type 1 fimbriae

The ability of *E. coli* hosts, which contained plasmids encoding chimeric *fimA* genes, to express fimbriae was assayed by such criteria as immunoblotting, haemagglutination, electron and immunofluorescence microscopy. Immunoblotting employing anti-fimbrial serum of bacterial hosts that harboured the recombinant plasmids in most cases revealed the presence of chimeric proteins with the expected molecular masses (Fig. 4). The most notable examples were pBSO1 and pBSO2, encoding *fimA* genes with one or two heterologous inserts in position 25, respectively, which resulted in protein levels comparable to non-chimeric controls (pLBJ318). At the other end of the spectrum were strains from which very little or no recombinant protein could be observed, e.g. hosts containing plasmid pBSO7 with a single insert in position 105. The haemagglutination titres of host cells containing chimeric *fimA*–cholera genes were generally lower than those of comparable wild-type *fimA* controls (Table 2).

Immunological detection of the cholera toxin epitope in the context of chimeric type 1 fimbriae

In Western blots of total lysates of host cells harbouring plasmids pBSO1–pBSO7 chimeric FimA–CTB fimbriae were not recognized by specific anti-CTB serum. In the light of the fact that residues 50–64 form a conformational loop it is perhaps not surprising that this

feature is not correctly re-folded in chimeric FimA–CTB proteins after extensive denaturation. It should be noted that this loop is indeed re-folded in the context of the parental protein, CTB (see later), however, the molecular environment of the loop present in the chimeras or in the parental molecular background is obviously quite different. Therefore, we investigated if the heterologous segments were authentically displayed directly on the bacteria. This was studied by CCD (charge-coupled device) microscopy in combination with fluorescent antibody methodology. By this technique *E. coli* hosts expressing chimeric FimA–CTB fimbriae were specifically recognized by serum raised against cholera toxin B subunit. Fig. 5 shows two representative examples of surface display of chimeric FimA proteins, harbouring a single and a tandem repeat of the heterologous sequence, respectively. In both cases the chimeric proteins are recognized by insert-specific anti-serum. This demonstrates that the foreign epitopes are exposed on the surface of the host cells and, notably, in a conformation which mimics the natural conformation of the epitope in the context of the cholera toxin. We also investigated surface-localization and organelle assembly of the chimeric proteins by electron and immunoelectron microscopy (Fig. 6). The data indicate that the chimeric proteins were specifically recognized on the surface of bacterial hosts by anti-CTB serum. The chimeric proteins were also, at least to some extent, assembled into fimbriae (Fig. 6c, e and g).

Expression of chimeric fimbriae is dependent on the host background

In the present study we have investigated the expression of chimeric FimA and fimbriae in three different strains. One, MS7, is Δ *fim*, permitting us to investigate the ability of the introduced plasmids to confer fimbrial synthesis without interference from a chromosomally located *fim* gene cluster. The isogenic *fim*⁺ parent strain PC31, was used to provide information about the influence of a resident *fim* gene cluster on the plasmid-encoded systems. Additionally, we employed a commensal isolate, DSM 6601, which is also *fim*⁺ (Blum *et al.*, 1995). Comparing the MS7 and PC31 strains there seemed to be a tendency that a resident *fim* gene cluster provided a better background for expression of chimeric FimA proteins and fimbriae. For example, in the case of plasmid pBSO2 a high level of chimeric protein was seen in immunoblotting in a PC31 background, whereas

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant genotype	Reference or construction
PC31	<i>gal, tonA, phx, argF, rel</i>	Klemm <i>et al.</i> (1985)
MS7	PC31Δ <i>fim</i>	<i>fim</i> gene cluster replaced by a <i>npt</i> cassette (cf. Methods)
DSM 6601	<i>fim</i> ⁺	Blum <i>et al.</i> (1995)
pPKL4	All <i>fim</i> genes	Klemm <i>et al.</i> (1985)
pLBJ312	<i>fimA</i>	Contains a <i>fim</i> gene cluster in which the <i>fimA</i> gene is replaced by a <i>SacI</i> site
pLBJ318	All <i>fim</i> genes	<i>fimA</i> gene inserted in the <i>SacI</i> site of pLBJ312
pLPA41	<i>fimA</i> ⁺	PCR fragment containing the <i>fimA</i> gene inserted in the <i>SacI</i> site of pUC18
pLPA110	<i>fimA</i> - <i>Bgl</i> III (25)	<i>Bgl</i> III linker inserted in-frame in a position corresponding to aa 25 in the <i>fimA</i> gene of pLPA41
pLPA119	<i>fimA</i> - <i>Bgl</i> III (45)	<i>Bgl</i> III linker inserted in-frame in a position corresponding to aa 45 in the <i>fimA</i> gene of pLPA41
pLPA111	<i>fimA</i> - <i>Bgl</i> III (80)	<i>Bgl</i> III linker inserted in-frame in a position corresponding to aa 80 in the <i>fimA</i> gene of pLPA41
pLPA112	<i>fimA</i> - <i>Bgl</i> III (105)	<i>Bgl</i> III linker inserted in-frame in a position corresponding to aa 105 in the <i>fimA</i> gene of pLPA41
pLPA113	<i>fimA</i> -cholera (25)	DNA segment encoding 15 aa of CTB inserted in the <i>Bgl</i> III site of pLPA110
pLPA125	<i>fimA</i> -cholera (45)	DNA segment encoding 15 aa of CTB inserted in the <i>Bgl</i> III site of pLPA119
pLPA114	<i>fimA</i> -cholera (80)	DNA segment encoding 15 aa of CTB inserted in the <i>Bgl</i> III site of pLPA111
pLPA115	<i>fimA</i> -cholera (105)	DNA segment encoding 15 aa of CTB inserted in the <i>Bgl</i> III site of pLPA112
pLPA123	<i>fimA</i> -2 × cholera (25)	pLPA110 containing a tandem CTB segment inserted in the <i>Bgl</i> III site
pLPA126	<i>fimA</i> -2 × cholera (45)	pLPA119 containing a tandem CTB segment inserted in the <i>Bgl</i> III site
pLPA124	<i>fimA</i> -2 × cholera (80)	pLPA111 containing a tandem CTB segment inserted in the <i>Bgl</i> III site
pBSO1	<i>fimA</i> -cholera (25)	<i>SacI</i> fragment containing the chimeric <i>fimA</i> -cholera gene from pLPA113 inserted in the <i>SacI</i> site of pLBJ312
pBSO2	<i>fimA</i> -2 × cholera (25)	<i>SacI</i> fragment containing the chimeric <i>fimA</i> -cholera gene from pLPA123 inserted in the <i>SacI</i> site of pLBJ312
pBSO3	<i>fimA</i> -cholera (45)	<i>SacI</i> fragment containing the chimeric <i>fimA</i> -cholera gene from pLPA125 inserted in the <i>SacI</i> site of pLBJ312
pBSO4	<i>fimA</i> -2 × cholera (45)	<i>SacI</i> fragment containing the chimeric <i>fimA</i> -cholera gene from pLPA126 inserted in the <i>SacI</i> site of pLBJ312
pBSO5	<i>fimA</i> -cholera (80)	<i>SacI</i> fragment containing the chimeric <i>fimA</i> -cholera gene from pLPA114 inserted in the <i>SacI</i> site of pLBJ312
pBSO6	<i>fimA</i> -2 × cholera (80)	<i>SacI</i> fragment containing the chimeric <i>fimA</i> -cholera gene from pLPA124 inserted in the <i>SacI</i> site of pLBJ312
pBSO7	<i>fimA</i> -cholera (105)	<i>SacI</i> fragment containing the chimeric <i>fimA</i> -cholera gene from pLPA115 inserted in the <i>SacI</i> site of pLBJ312

* Numbers in brackets indicate insert positions.

Fig. 3. Construction of the replacement vector, pLBJ312, harbouring a unique *SacI* site for integration of engineered *fimA* genes into the *fim* gene cluster. Relevant restriction sites are indicated: B, *BsaBI*; H2, *HindII*; H3, *HindIII*; E, *EcoRI*; N, *NruI*; S, *Sall*; S1, *SacI*; St, *StuI*.

Fig. 4. Production of fimbriae from recombinant strains assayed by Western blot of total cell lysates of *E. coli* DSM 6601 (D), MS7 (M) and PC31 (P) hosts containing plasmid pBSO1–pBSO7, pLBJ312, pLBJ318 and pBR322; 'pwf' indicates purified wild-type fimbriae. Rabbit anti-fimbrial serum was used as primary serum and peroxidase-coupled anti-rabbit serum was employed as secondary serum.

none was seen in the case of MS7 (Fig. 4). Meanwhile, judging from all the criteria we have used, strain DSM 6601 is apparently the best host for expression of

chimeric FimA proteins. With the exception of plasmid pBSO7 all chimeric FimA proteins were expressed and exposed on the surface of DSM 6601.

Table 2. Fimbriation level of various strains assayed by haemagglutination

Values are time (s) before reaction occurred (means of three measurements). Subscript numbers indicate the corresponding positions in the mature FimA protein after which the heterologous sequence was inserted.

Plasmid	Relevant plasmid genotype	Strain		
		DSM 6601	PC31	MS7
None		> 300	16	> 300
pBR322		> 300	17	> 300
pLBJ318	All <i>fim</i> genes	6	5	5
pLBJ312	<i>fimA</i>	8	5	> 300
pBSO1	<i>fimA</i> -1 cholera ₂₅	20	65	50
pBSO2	<i>fimA</i> -2 cholera ₂₅	19	43	> 300
pBSO3	<i>fimA</i> -1 cholera ₄₅	17	33	60
pBSO4	<i>fimA</i> -2 cholera ₄₅	22	47	> 300
pBSO5	<i>fimA</i> -1 cholera ₈₀	9	12	15
pBSO6	<i>fimA</i> -2 cholera ₈₀	15	50	140
pBSO7	<i>fimA</i> -1 cholera ₁₀₅	16	32	> 300

Immunogenicity of chimeric fimbriae

Chimeric FimA-CTB fimbriae were purified from hosts containing plasmid pBSO1 (a single CTB segment in position 25), and used to immunize rabbits. As control one rabbit was immunized with wild-type fimbriae. Serum from animals immunized with the chimeric fimbriae was specifically able to recognize CTB in immunoblots, whereas serum from the control, immunized with wild-type fimbriae, was not (Fig. 7).

DISCUSSION

In this study we have expressed the sequence of an important neutralizing epitope of CTB in localities of the FimA protein predicted to be surface-located. Our data demonstrate that this was indeed the case: the inserted cholera toxin epitope was presented in an antigenic configuration mimicking that in the toxin, and was exposed on the surface of the bacterial hosts. The CTB segment was recognized in the context of chimeric FimA proteins by two independent methods, i.e. fluorescence microscopy and immunoelectron microscopy, on the surface of bacterial hosts (Figs 5 and 6). Also, the chimeric proteins were to some extent polymerized into fimbriae (Fig. 6). In one of the constructs, harbouring the heterologous insert in position 80, the chimeric protein was recognized by anti-CTB serum but not by anti-FimA monoclonal antibodies (mAbs) (Fig. 6c, e and g). However, the mAbs were able to recognize wild-type FimA as well as chimeric FimA with an insert in position 25 (Fig. 6a and h). Consequently, the CTB insert in position 80 must either destroy or shield the specific epitope recognized by the mAbs. The CTB epitope present in the context of chimeric fimbriae also elicited a specific immune response in rabbits (Fig. 7).

Of the four positions in FimA (25, 45, 80 and 105) that we have tested for display of the reporter epitope, only position 105 proved incompatible with the approach, whereas inserts in the other positions resulted in production of chimeric FimA. Large heterologous inserts seemed to be tolerated in FimA, provided the insert position was amenable to engineering. Insertion of 34 foreign amino acids (a tandem repeat of the CTB segment) in positions 25 and 45 of the FimA sequence, respectively, in both cases resulted in synthesis and surface-presentation of the chimeric proteins. Several factors can influence the tolerance for heterologous inserts in the FimA protein. Insertion into domains critical for the structural integrity of the protein and those involved in subunit-subunit interaction, and in interactions with the fimbrial biogenesis machinery, i.e. the FimC chaperone and the FimD usher protein, would probably not be tolerated. However, since the three-dimensional structure of FimA is unknown, a strategy based on computer algorithms for prediction of surface-exposed sectors for insertion of heterologous inserts seems to work reasonably well, judging from the fact that three out of the four positions were in fact compatible with the strategy.

The presence of an indigenous wild-type *fim* gene cluster apparently had a positive effect on the production and surface exposure of the chimeric proteins. The *fimA*⁺ strains PC31 and DSM 6601 proved to provide a better background for presentation of chimeric FimA proteins than did the MS7 strain (Δ *fim*). This could be due to co-assembly of wild-type and chimeric FimA proteins into fimbrial organelles. However, a positive influence of a chromosomally located wild-type *fim* gene cluster on the plasmid-encoded *fim* genes was apparently not the only criterion for a better display, since when comparing PC31 and DSM 6601, both of which have a resident

Fig. 5. Surface display of chimeric fimbriae assessed by fluorescence microscopy (FM) and DICM of *E. coli* DSM 6601 cells. In all cases fluorescence-labelled secondary antisera were used, the type of primary serum employed is indicated. Bacteria were fixed, reacted with primary antiserum, washed, reacted with FITC-labelled secondary antiserum, washed and subjected to microscopy. Images were recorded with CCD technology (see Methods). (a) FM and (b) DICM of cells harbouring plasmid pBSO5 (encoding a *fimA* gene with a single cholera insert) reacted with anti-type 1 serum (primary serum). (c) FM and (d) DICM of cells harbouring plasmid pBSO5 (encoding a *fimA* gene with a single cholera insert) reacted with anti-CTB serum (primary serum). (e) FM and (f) DICM of cells harbouring plasmid pBSO4 (encoding a *fimA* gene with a tandem cholera insert) reacted with anti-type 1 serum (primary serum). (g) FM and (h) DICM of cells harbouring plasmid pBSO4 (encoding a *fimA* gene with a tandem cholera insert) reacted with anti-CTB serum (primary serum). (i) FM and (j) DICM of cells harbouring plasmid pLBJ312 (*fimA*) reacted with anti-type 1 serum (primary serum). (k) FM and (l) DICM of cells harbouring plasmid pLBJ312 (*fimA*) reacted with anti-CTB serum (primary serum). Bar, 10 μ m.

wild-type *fim* gene cluster, the latter was clearly a better presenter of engineered versions of FimA. A multitude of factors, such as higher levels of chaperones and members of the general secretory pathway and lower levels of scavenging proteinases, might cause the DSM 6601 strain to be such an amenable host in this respect.

We believe that the fimbrial presentation system has a considerable potential, firstly because of the antigen-amplification effect inherent in the fact that the major structural protein, and thereby the heterologous sequence, is present in large amounts predominantly on the bacterial surface (up to 500 000 copies per cell in the wild-type situation). Surface presentation seems to be important for a strong immune-response in the context of live bacterial vaccines, e.g. in attenuated *Salmonella*

strains it has been shown that a stronger immune response was elicited when the heterologous antigen was exposed on the bacterial surface (Hormaeche *et al.*, 1992; Leclerc *et al.*, 1991). Secondly, fimbriae are easy to purify, a fact which might be relevant for a component vaccine approach.

The present system is an example of a strategy that has been pursued by several investigators in recent years, namely to use naturally occurring bacterial surface organelles for presentation of foreign epitopes. Two major approaches, employing fimbriae and flagella, respectively, have been used to this end (Newton *et al.*, 1989; Pallesen & Klemm, 1994; Wu *et al.*, 1989). Previous studies employing the major subunit protein of various fimbriae have succeeded in displaying hetero-

Fig. 6. Double-labelled immunoelectron micrographs (a, c, e, g and h), showing labelling of FimA (5 nm) and the CTB segment on chimeric FimA (10 nm). Bacteria were adsorbed to the surface of the grid and incubated with a mixture of anti-CTB rabbit serum (1:100) and anti-FimA monoclonal antibody (1:5). As secondary antibodies a mixture of goat anti-mouse serum conjugated with 5 nm colloidal gold particles and goat anti-rabbit serum conjugated with 10 nm colloidal gold particles were used in dilution 1:20. Surface structures of the bacteria were analysed by shadow casting with tungsten at an angle of 30° (b, d and f). (a, b) pLBJ318/MS7; (c, d), pBSO5/MS7; (e, f) pBSO5/DSM 6601; (g) pBSO5/PC31; (h) pBSO1/MS7. Black bars (a, c, e, g and h), 100 nm. White bars (b, d and f), 200 nm.

logous segments of sizes up to, but not beyond, 20 amino acid residues (for review see Pallesen & Klemm, 1994). In the present study we have succeeded in the authentic

display of segments of 34 foreign amino acid residues, a reasonable improvement. We have recently studied the potential of the FimH minor component of type 1

Fig. 7. Immunoblots showing the immune response of rabbits immunized with (a) wild-type FimA (serum dilution 1:1000), (b) chimeric FimA harbouring a CTB-segment in position 25 (serum dilution 1:250) and (c) CTB (serum dilution 1:40000). Three identical runs of CTB, chimeric FimA and wild-type FimA are shown in (a), (b) and (c).

fimbriae for display of heterologous sequences (Pallesen *et al.*, 1995), and have, in this background succeeded in displaying even larger segments (58 amino acids). It might be that the FimH protein is able to accept larger heterologous grafts than FimA. However, one drawback of FimH is the lower ratio of this protein when integrated into the fimbriae; about a hundredfold less than FimA. The high valency display format provided by the FimA presentation system may be a significant advantage in vaccine design.

Future research will reveal whether a new generation of vaccines can be based on chimeric fimbriae or flagella, which fulfil the requirements for successful exposure and antigenicity of the heterologous epitopes while at the same time maintaining the functionality of the parental proteins.

ACKNOWLEDGEMENTS

The authors wish to thank Pernille Olsson for expert technical assistance. *E. coli* strain MS7 was generously made by Mark Schembri. This work was supported by The Danish Medical and Technical Research Councils and the WHO/UNDP Programme for Vaccine Development (ref. no. MIM/I5/181/171).

REFERENCES

- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res* 7, 1513–1523.
- Blum, G., Marre, R. & Hacker, J. (1995). Properties of *Escherichia coli* strains of serotype O6. *Infection* 23, 234–236.
- Brinton, C. C., Jr (1965). The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in Gram negative bacteria. *Trans NY Acad Sci* 27, 1003–1054.
- Dodd, D. C. & Eisenstein, B. I. (1984). Dependence of secretion and assembly of type 1 fimbrial subunits on normal protein export. *J Bacteriol* 159, 1077–1079.
- Duguid, J. P. & Old, D. C. (1980). Adhesive properties of *Enterobacteriaceae*. In *Receptors and Recognition*, series B, vol. 6, pp. 186–217. Edited by E. Beachey. London: Chapman & Hall.
- Hedegaard, L. & Klemm, P. (1989). Type 1 fimbriae of *Escherichia coli* as carriers of heterologous antigenic sequences. *Gene* 85, 115–124.
- Hormaeche, C. E., Dougan, G. & Chatfield, S. N. (1992). Live attenuated salmonella: oral vaccines for salmonellosis and combined vaccines carrying heterologous antigens. In *The Release of Genetically Modified Microorganisms*, pp. 71–83. Edited by D. E. S. Stewart-Tull & M. Sussmann. New York: Plenum Press.
- Hultgren, S. J., Abraham, S. N., Caparon, M., Falk, P., St Geme, J. W., III & Normark, S. (1993). Pilus and non pilus bacterial adhesins: assembly and function in cell recognition. *Cell* 73, 887–901.
- Jacob, O. C., Sela, M. & Arnon, R. (1983). Antibodies against synthetic peptides of the B subunit of cholera toxin: crossreaction and neutralization of the toxin. *Proc Natl Acad Sci USA* 80, 7611–7615.
- Jones, C. H., Pinkner, J. S., Nicholes, A. V., Slonim, L. N., Abraham, S. N. & Hultgren, S. J. (1993). FimC is periplasmic PapD-like chaperone that directs assembly of type 1 pili in bacteria. *Proc Natl Acad Sci USA* 90, 8397–8401.
- Klemm, P. (1984). The *fimA* gene encoding the type 1 fimbrial subunit of *Escherichia coli*, nucleotide sequence and primary structure of the protein. *Eur J Biochem* 143, 395–399.
- Klemm, P. (1992). FimC, a chaperone-like periplasmic protein of *Escherichia coli* involved in biogenesis of type 1 fimbriae. *Res Microbiol* 143, 831–838.
- Klemm, P. & Christiansen, G. (1987). Three *fim* genes required for the regulation of length and mediation of adhesion of *Escherichia coli* type 1 fimbriae. *Mol Gen Genet* 208, 439–445.
- Klemm, P. & Christiansen, G. (1990). The *fimD* gene required for cell surface localization of *Escherichia coli* type 1 fimbriae. *Mol Gen Genet* 220, 334–338.
- Klemm, P. & Krogfelt, K. A. (1994). Type 1 fimbriae of *Escherichia coli*. In *Fimbriae, Adhesion, Genetics, Biogenesis and Vaccines*, pp. 9–28. Edited by P. Klemm. Boca Raton, FL: CRC Press.
- Klemm, P., Jørgensen, B. J., van Die, I., de Ree, H. & Bergmans, H. (1985). The *fim* genes responsible for synthesis of type 1 fimbriae in *Escherichia coli*. *Mol Gen Genet* 199, 410–414.
- Klemm, P., Christiansen, G., Kreft, B., Marre, R. & Bergmans, H. (1994). Reciprocal exchange of minor components of type 1 and F1C fimbriae results in hybrid organelles with changed receptor specificity. *J Bacteriol* 176, 2227–2234.
- Krogfelt, K. A. (1991). Bacterial adhesion: genetics, biogenesis, and role in pathogenesis of fimbrial adhesins of *Escherichia coli*. *Rev Infect Dis* 13, 721–735.
- Krogfelt, K. A. & Klemm, P. (1988). Investigation of minor components of *Escherichia coli* type 1 fimbriae: protein chemical and immunological aspects. *Microb Pathog* 4, 231–238.
- Krogfelt, K. A., Bergmans, H. & Klemm, P. (1990). Direct evidence that the FimH protein is the mannose specific adhesin of *Escherichia coli* type 1 fimbriae. *Infect Immun* 58, 1995–1998.
- Leclerc, C., Charbit, A., Martineau, P., Deriaud, E. & Hofnung, M. (1991). The cellular location of a foreign B cell epitope expressed by recombinant bacteria determines its T cell-dependent characteristics. *J Immunol* 147, 3545–3550.
- Levine, M. M., Giron, J. & Noriega, F. (1994). Fimbrial vaccines. In *Fimbriae, Adhesion, Genetics, Biogenesis and Vaccines*, pp. 271–286. Edited by P. Klemm. Boca Raton, FL: CRC Press.

- McMichael, J. C. & Ou, J. T. (1979). Structure of common pili from *Escherichia coli*. *J Bacteriol* **138**, 969–975.
- Merrit, E. A., Sarfaty, S., van den Akker, F., L'Hoir, C. E., Martial, J. A. & Hol, W. G. J. (1994). Crystal structure of cholera toxin B-pentamer bound to receptor G_{MI} pentasaccharide. *Protein Sci* **3**, 166–175.
- Moon, H. W. & Bunn, T. O. (1993). Vaccines for preventing enterotoxigenic *Escherichia coli* infections in farm animals. *Vaccine* **11**, 213–219.
- Newton, S. M. C., Jacob, C. O. & Stocker, B. A. D. (1989). Immune response to cholera toxin epitope inserted into *Salmonella* flagellin. *Science* **244**, 70–72.
- Pallesen, L. & Klemm, P. (1994). Chimeric fimbrial vaccines. In *Fimbriae, Adhesion, Genetics, Biogenesis and Vaccines*, pp. 289–294. Edited by P. Klemm. Boca Raton, FL: CRC Press.
- Pallesen, L., Poulsen, L. K., Christiansen, G. & Klemm, P. (1995). Chimeric FimH adhesin of type 1 fimbriae: a bacterial display system for heterologous sequences. *Microbiology* **141**, 2839–2848.
- Pugsley, A. P. (1993). The complete secretory pathway in Gram-negative bacteria. *Microbiol Rev* **57**, 50–108.
- Russell, P. V. & Orndorff, P. E. (1992). Lesions in two *Escherichia coli* type 1 pilus genes alter pilus number and length without affecting receptor binding. *J Bacteriol* **174**, 5923–5935.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Schembri, M., Pallesen, L., Connell, H. & Klemm, P. (1996). Linker insertion mutagenesis of the FimH adhesin of type 1 fimbriae in an *Escherichia coli* *fimH*-null host. *FEMS Microbiol Lett* **137**, 257–263.
- Shoham, M., Scherf, T., Anglister, J., Levitt, M., Merrit, E. A. & Hol, W. G. J. (1995). Structural diversity in a conserved cholera toxin epitope involved in ganglioside binding. *Protein Sci* **4**, 841–848.
- Wu, J. Y., Newton, S., Judd, A., Stocker, B. & Robinson, W. S. (1989). Expression of immunogenic epitopes of hepatitis B surface antigen with hybrid flagellin proteins by a vaccine strain of *Salmonella*. *Proc Natl Acad Sci USA* **86**, 4726–4730.

Received 26 November 1996; revised 30 January 1997; accepted 3 February 1997.