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## Fimbriae-assisted bacterial surface display of heterologous peptides

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### Abstract

The display of peptide segments on the surface of bacteria offers many new and exciting applications in biotechnology and medical research. Fimbria-assisted display of heterologous sequences is a paradigm for chimeric organelle display on bacteria. Fimbriae are particularly attractive candidates for epitope display for several reasons: (1) they are present in extremely high numbers at the cell surface, (2) they are strong immunogens, (3) they possess inherent adhesive properties, and (4) they can be easily purified. The majority of work dealing with fimbria-assisted peptide display has been focused on the development of recombinant vaccines. A number of different fimbrial types have been used to display immune-relevant sectors of various foreign proteins. Chimeric fimbrial vaccines can be used in the context of purified proteins, however the potential also exists to exploit this technology for the development of live recombinant vaccines. Work has also been performed demonstrating the amenability of fimbriae towards the powerful technology of random peptide display. This review summarises the current state of research in this field.

**Key words:** surface display of foreign epitopes – chimeric proteins – type 1 fimbriae

### Introduction

Bacteria are able to display a wide range of proteins on the cell surface. Such proteins are key players in a number of natural processes like adhesion, colonization of target surfaces, motility, signal transduction, enzymatic degradations etc. However, many proteins of interest in medicine and biotechnology are not surface displayed. An attractive way to obtain surface display of an important protein or sectors of such a protein is to graft it to a naturally occurring bacterial surface protein and express the chimeric protein on the cell surface. The ability to display heterologous proteins on the surface of bacteria is leading to a number of applications, such as recombinant vaccines, reagents for

diagnostics, whole cell biocatalysts and bioadsorbants, and systems for scanning peptide libraries (Georgiou et al., 1997). Fimbriae are adhesive bacterial surface structures which enable bacteria to target and to colonize specific host tissues (for reviews see Klemm, 1994). 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 and Bunn, 1993). Such results have indicated that fimbriae normally are

very good immunogens both in the context of live vaccines and as purified proteins. A number of different fimbriae types have been used for the display of heterologous sequences (Table 1).

Fimbriae in Gram negative bacteria are in most cases assembled via the chaperone/usher pathway. The initial translocation of organelle components across the cytoplasmic membrane is dependent on the normal type II export system. However, further export from the periplasm to the cell exterior is mediated by a specific two-component system consisting of a periplasmic chaperone and an usher, an outer membrane-located pore, which serves as assembly platform (Hultgren et al., 1996; Klemm and Schembri, 2000). A highly choreographed series of specific molecular interactions ultimately leads to the formation of the fimbrial organelle, a polymeric structure in which hundreds of subunits are held together by non-covalent subunit-subunit interactions. Specific motifs present on the structural proteins are involved in interactions with the transport machinery and subunit-subunit interactions and are obviously non-permissible regions for heterologous insertion.

This review to a large degree focuses on type 1 fimbrial display systems but other examples are also included. Type 1 fimbriae are found on the majority of *Enterobacteriaceae* including *Escherichia coli*. 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 and Klemm, 1988). It has been shown that the minor components, FimF, FimG and FimH, are involved in fimbrial length regulation (Klemm and Christiansen, 1987; Russell and Orndorff, 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 system-specific export system consists of the FimC chaperone and the FimD usher proteins (Klemm, 1992; Klemm and Christiansen, 1990).

The biogenesis machinery of type 1 fimbriae has been shown to be quite tolerant in accepting similar

**Table 1.** Summary of fimbriae-displayed peptides/epitopes.

Fimbriae	Subunit	Displayed polypeptide/epitope	Insert size (aa)	Reference
Type 1	FimA	Hepatitis B surface antigen epitope	7–16	Hedegaard and Klemm, 1989
		Poliovirus VPI coat protein	11	Hedegaard and Klemm, 1989
		Foot-and-mouth disease virus epitope	19	Hedegaard and Klemm, 1989
		Cholera toxin B subunit	15–34	Stentebjerg-Olesen et al., 1997
	FimH	PreS2 segment of hepatitis B surface antigen	52	Pallesen et al., 1995
		Cholera toxin B subunit	15	Pallesen et al., 1995
		Heavy-metal-binding sequences	9–36	Schembri and Klemm, 1998
	Random peptide libraries	9–36	Schembri and Klemm, 1998; Schembri et al., 1999; Kjærsgaard et al., 2000	
K88	FaeG	Human influenza virus epitope	7–12	Thiry et al., 1989
		Human somatostatin epitope	14	Thiry et al., 1989
		<i>Neisseriae gonorrhoeae</i> piliin epitope	11	Bakker et al., 1990
		Foot-and-mouth disease virus epitope	11	Bakker et al., 1990
		Human immunodeficiency virus epitope	11	Bakker et al., 1990
		Hepatitis B surface antigen epitope	20	Pedersen and Andersen, 1991
P	FelA	Foot-and-mouth disease virus epitope	8–20	van Die et al., 1988; van Die et al., 1990
		Human immunodeficiency virus epitope	15–26	van Die et al., 1990
		<i>Mycobacterium leprae</i> 65 kDa protein epitope	9	van Die et al., 1990
		<i>Plasmodium falciparum</i> surface protein epitope	10	van Die et al., 1990
		Gonadotropin-releasing hormone		van der Zee et al., 1995
987P	FasA	Herpes simplex virus epitopes	8–9	Rani et al., 1999
		Transmissible gastroenteritis virus epitope	10	Rani et al., 1999
CS31A	ClpG	Transmissible gastroenteritis virus epitopes	18–51	Bousquet et al., 1994; Der Vartanian et al., 1994; Mechin et al., 1996; Der Vartanian et al., 1997
Type 4	Major	Foot-and-mouth disease virus epitope	16	Jennings et al., 1989

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. We have used two components of type 1 fimbriae for surface display of foreign peptides, viz. the major structural protein, FimA, and the fimbrial adhesin, FimH. In both cases a strategy employing in-frame fusion of the heterologous sequences into permissible sites has been used. If insert positions are chosen which do not interfere with the bioassembly of the organelles, normal fimbriae will result (Fig. 1).

### Heterologous antigen display in the major type 1 fimbrial protein, FimA

In the wild-type Fim system a single fimbriated bacterium carries several hundred thousand copies of FimA on the surface. This makes FimA an ideal candidate for high-valency display of heterologous peptide segments. Apart from the high number of foreign inserts that can be displayed in the FimA system there are several other advantages, notably in connection with vaccine development; type 1 fimbriae are excellent immunogens, which could boost the immune response against a passenger epitope, and the fimbriae are easily detached from the bacteria and amenable to purification.

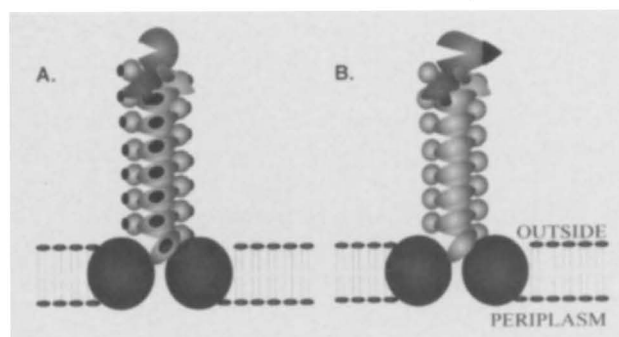
In a preliminary study (Hedegaard and Klemm, 1989) heterologous DNA segments, encoding consensus sequences for restriction enzymes or, in a few cases, mimicking foreign epitopes, were inserted into naturally available restriction sites in the *fimA* gene. However, it was not clear from this study whether the foreign sequences were authentically displayed in the context of chimeric FimA proteins, and could evoke an immune response directed against the parental protein. Recently a more systematic study was carried out (Stentebjerg-Olesen et al., 1997). Based on algorithms for prediction of such parameters as hydrophilicity and secondary structure, four positions were selected for insertion of a reporter epitope. As a reporter epitope a well characterized region of the cholera toxin B chain (CTB) was chosen. This consists of a 15-residue segment which comprises a conformational loop on CTB and was previously shown to elicit antibodies that bind to and neutralize cholera toxin (Jacob et al., 1983; Merrit et al., 1994; Shoham et al., 1995). A synthetic DNA segment encoding the cholera epitope was made

and inserted in both single copy and as tandem repeats in the selected sites in *fimA*. The inserted CTB epitope was authentically displayed in three out of four employed positions in FimA as evidenced by immunofluorescence microscopy and immunoelectron microscopy employing serum raised against CTB. Purified chimeric FimA-CTB fimbriae containing a single CTB segment in a permissive position were used to immunize rabbits. Immunoblot analysis demonstrated that serum from immunized animals was specifically able to recognize natural CTB.

### Heterologous antigen display in the type 1 fimbrial adhesin, FimH

The mannose-specific adhesin FimH is a minor component of type 1 fimbriae. FimH is produced as a 300-amino-acid precursor that is processed into a mature form of 279 amino acids, i.e. roughly twice the size of the other structural elements of the fimbrial organelle. FimH has been shown to be located in a short tip-fibrillum (Jones et al., 1995) and several reports have suggested that it is additionally intercalated along the fimbrial shaft (Abraham et al., 1987; Krogfelt et al., 1990). Recently the 3D structure of a FimH-FimC, adhesin-chaperone, complex was elucidated (Choudhury et al., 1999). According to this, FimH is a two-domain protein, an N-terminal adhesive domain (residues 1–156) linked by a tetrapeptide loop to a C-terminal organelle integration domain (residues 160–279).

The FimH protein was probed by linker insertion mutagenesis in order to identify permissive sites into which heterologous sequences could be inserted without significant interference with the function/structure of the protein (Schembri et al., 1996). Two potential positions were identified by this approach, viz. 225



**Fig. 1.** Model for differential-valency display of a heterologous peptide displayed by the major type 1 fimbrial protein, FimA (panel A), and the minor fimbrial component, FimH (panel B). Black knobs represent the passenger peptide. See main text for further details.

and 258, both located in the C-terminal domain. Subsequently, two different heterologous reporter peptides were inserted into both of the permissive positions. These were: a 15-amino-acid sector of the CTB subunit (see previous section for details) and a 52-amino-acid peptide mimicking the preS2 region of the hepatitis B surface antigen known to contain protective antigenic determinants (Itoh et al., 1986). In all cases the insert positions proved to be compatible with integration of the heterologous sequences with regard to surface display and at least partial conservation of the mannose-binding function of the chimeric FimH proteins (Palleesen et al., 1995). Furthermore, both the CTB segment and the preS2 hepatitis segment were displayed on the surface of the chimeric FimH proteins in conformations which were immunologically similar to the conformations in the parental proteins, as evidenced by immunofluorescence microscopy and immunoelectron microscopy (Palleesen et al., 1995).

### Random library display in FimH

The fact that FimH could accommodate and display diverse heterologous sequences evidenced from the work on the display of immuno-relevant sequences led us to believe that this bacterial surface organelle component would be an ideal candidate for bacterial surface display of random peptide libraries. The rationale behind random library display is to create a high number of randomly permuted nucleotide sequences in a permissive site on the gene encoding the carrier protein. From the huge population of displayed peptides specific peptide sequences can be isolated on the basis of their biological activity, e. g. binding affinity to a target molecule (Georgiou et al., 1997).

Random libraries were constructed by inserting synthetic double-stranded oligonucleotides into a permissive position, i.e. codon 225, in the *fimH* gene. The oligonucleotides consisted of nine random codons flanked by identical restriction sites. This structure permitted insertion of single or polymeric oligonucleotides, a feature which enhanced the complexity of the libraries considerably. Individual libraries were calculated to contain  $10^7$ – $10^8$  individual clones (Schembri and Klemm, 1998). Serial selection and enrichment of the random libraries was performed with either ionic metal-NTA resins or with selected metal oxides. After 5–10 rounds of selection and enrichment specific binders to a given target substrate could be isolated and characterized. Clones expressing chimeric FimH with peptide inserts that recognized a number of heavy metals or heavy metal oxides were identified, viz.  $\text{Ni}^{2+}$ , NiO, CuO, PbO<sub>2</sub>, CdO (Schembri and Klemm, 1998; Schembri et al., 1999). Even inserts that were able to

distinguish between a metal oxide and the corresponding ionic form, e.g. ZnO and  $\text{Zn}^{2+}$ , were identified (Kjærsgaard et al., 2000). Such findings strongly suggest the huge potential of the system. We are currently developing this technique to identify immuno-relevant peptides suitable for vaccine development.

### Bacterial surface display by P fimbriae

Chimeric P fimbriae of *E. coli* have been constructed by insertion of foreign epitopes within the hypervariable regions of the F11 serotype subunit protein Fela (van Die et al., 1988, 1990). Antigenic determinants from the VPI coat protein of foot-and-mouth disease virus (FMDV), two different epitopes from the gp 120 coat protein of human immunodeficiency virus (HIV), one epitope from a *Mycobacterium leprae* protein, and one epitope from *Plasmodium falciparum* have all been inserted into hypervariable regions 1 or 4, or both. Recognition by specific monoclonal antibodies revealed that epitopes consisting of up to 15 amino acids could be correctly displayed on the fimbrial surface. Furthermore, mice injected with purified F11 fimbriae carrying an FMDV epitope inserted into region 1 elicited specific anti-FMDV antibodies. More recently, recombinant F11 fimbriae were constructed to display the brain peptide gonadotropin-releasing hormone (GnRH) as a means to develop a contraceptive vaccine for fertility control of domestic animals (van der Zee et al., 1995). Hybrid fimbriae containing authentically displayed GnRP peptide sequences were expressed efficiently on the *E. coli* cell surface. Most significantly, the vaccination of female rats and young bull calves with purified GnRH-containing fimbriae induced both a serological and a pharmacological effect that altered the reproductive characteristics of both animals.

### K88 and CS31A fimbriae

Several studies have investigated the use of K88 fimbriae from porcine enterotoxigenic *E. coli* as carriers of foreign epitopes (Thiry et al., 1989; Bakker et al., 1990; Pedersen and Andersen, 1991). Hypervariable regions of the K88 subunit protein were used as fusion sites to insert epitopes from human influenza virus, HIV-1, the FMDV VP1 coat protein, the *N. gonorrhoea* pilin subunit protein and the hormone somatostatin. The position of insertion and type of insert affected both the number of fimbriae produced on the bacterial cell surface and the immunogenicity of the presented epitope. Hybrid K88 fimbriae expressing epitopes from influenza virus, FMDV or HIV-1 elicited



ed specific antibodies in either rats or mice when used as purified preparations.

CS31A fimbriae are K88-related surface organelles produced by *E. coli* and *Klebsiella pneumoniae* (Girardeau et al., 1988). The CS31A major subunit protein ClpG has been used to successfully display various antigenic peptides of the transmissible gastroenteritis virus (Bousquet et al., 1994; Der Vartanian et al., 1994; Mechin et al., 1996; Der Vartanian et al., 1997). In addition to the insertion of single epitopes, tandem repeats of different epitopes have also been displayed on the *E. coli* surface. The ClpG subunit appears to be highly flexible towards epitope display as exemplified by the different nature of the inserted epitopes with regards to amino acid composition, charge and hydrophobicity. Using either live recombinant bacteria or purified chimeric CS31A fimbriae, intraperitoneally immunized outbred mice elicited serum peptide antibodies with titres that were capable of recognizing native virus particles. However, only immunization with purified chimeric fimbriae could produce virus-neutralizing titres (Der Vartanian et al., 1997).

## Bacterial surface display by other fimbriae

Immunogenic epitopes from herpes simplex virus type 1 (HSV-1) and transmissible gastroenteritis virus surface proteins have been authentically displayed on the enteroadhesive 987P fimbriae of *E. coli* (Rani et al., 1999). Random linker mutagenesis was used to identify permissive insertion sites in the 987P major subunit protein FasA that did not effect fimbriation levels. Optimal expression of the chimeric subunits was observed when the epitopes were inserted near the N-terminus of FasA. Rabbits immunized with purified chimeric fimbriae elicited high levels of antibodies against both epitopes.

The major subunit of *Bacteroides nodosus* type 4 fimbriae has also been used to display an epitope from the VPI coat protein of FMDV (Jennings et al., 1989). Four different chimeric constructs could be recognized to varying degrees by anti-FMDV antibodies when expressed in *E. coli*. One of the chimeric constructs was also expressed in *Pseudomonas aeruginosa*, however it was not determined whether the hybrid fimbriae could be detected by specific anti-FMDV serum.

## Conclusions

Over the last decade a diverse range of heterologous peptides have been successfully displayed on bacteria by a wide spectrum of fimbriae. In spite of the highly

diverse character of the displayed peptides the issue of insert size limitation has not been systematically addressed. However, judging from the various reports, it seems that such a size limitation may indeed exist and it is noteworthy that the largest displayed peptides are in the 50–60 amino acid range. Furthermore, the presence of cysteines in the passenger sequences is not readily accepted by fimbrial display systems. Notwithstanding these limitations the technology has achieved a number of successes: workable vaccines based on fimbrial display are now in the last stages of testing or have already been commercialized, and the ability to display random libraries by fimbriae seems promising for a wide range of applications.

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