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MINI-REVIEW

Fimbrial surface display systems in bacteria: from vaccines to random libraries

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Overview

The double membrane system of Gram-negative bacteria is an efficient barrier instrumental in maintaining a steady environment in the cytoplasm. Only a few low-molecular-mass substances are able to cross this barrier unassisted from the cell exterior. On the other hand, the Gram-negative cell envelope also constitutes a formidable obstacle for proteins destined for the cell exterior. Gram-negative bacteria have developed various systems for protein export to the cell exterior; four major systems can be distinguished (Lory, 1998). By these systems a wide range of proteins can be routed to the surface, and many of these remain physically attached to and displayed on the cell surface. Such proteins are key players in a number of natural processes, such as adhesion, colonization of target surfaces, biofilm formation, motility, signal transduction, enzymic degradations, etc. However, many proteins of interest in medicine and biotechnology are not surface-displayed and/or not of bacterial origin. An attractive way to obtain surface display of such proteins or sectors thereof is to graft them into permissible positions on a naturally occurring bacterial surface protein and express the chimeric protein on the cell surface. Surface display of heterologous proteins on bacteria has resulted in 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 organelles which enable bacteria to target and to colonize specific host tissues (for reviews, see Klemm, 1994). They are long, thread-like surface structures, found in up to about 500 copies per cell. A large diversity of fimbriae, mostly of Gram-negative origin, are known. In Gram-negative bacteria, fimbriae are in most cases assembled via the chaperone/usher pathway. The initial translocation of organelle components across the cytoplasmic membrane is dependant 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 & 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; these are obviously non-permissible regions for heterologous insertion.

This review primarily deals with display systems based on type 1 fimbriae. These can be considered as paradigms for fimbrial display, firstly because type 1 fimbriae are the best structurally characterized fimbrial system and are very representative of this type of organelle; secondly, because most display studies have been carried out with this system. 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. The bulk of the organelle is made up of about 1000 subunits of the major building element, the FimA protein, stacked in a helical cylinder (Brinton, 1965). Additionally, small amounts of minor components are present as integral, primarily tip-located constituents (Krogfelt & Klemm, 1988; Jones *et al*., 1995). The minor components, viz. FimF, FimG and FimH, are involved in initiation of organelle synthesis and consequently in length regulation (Klemm & Christiansen, 1987; Russell & 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

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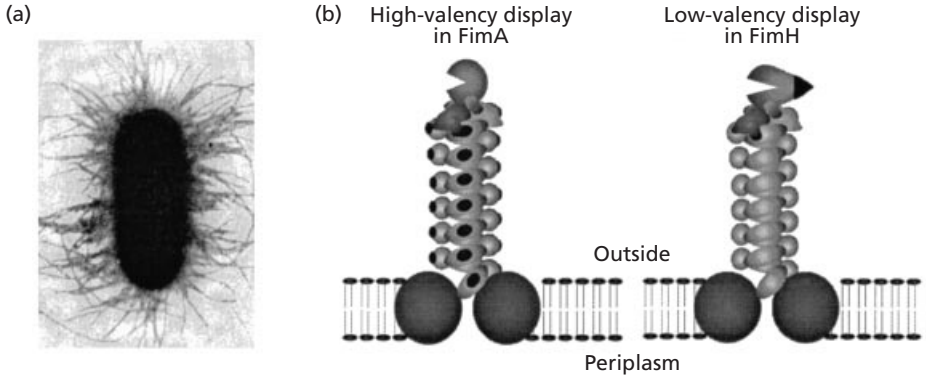

Fig. 1. (a) Fimbriated *E. coli*. (b) Model for high- and low-valency display of heterologous peptides by type 1 fimbriae. Black knobs represent the passenger epitopes.

Table 1. Summary of fimbriae-displayed epitopes

Fimbriae	Subunit	Displayed polypeptide/epitope	References
Type 1	FimA	Hepatitis B surface antigen epitope	Hedegaard & Klemm (1989)
		Poliovirus VPI coat protein	Hedegaard & Klemm (1989)
		Foot and mouth disease virus epitope	Hedegaard & Klemm (1989)
		Cholera toxin B subunit	Stenteberg-Olesen *et al*. (1997)
	FimH	PreS2 segment of Hepatitis B surface antigen	Pallesen *et al*. (1995)
		Cholera toxin B subunit	Pallesen *et al*. (1995)
		Heavy-metal-binding sequences	Schembri & Klemm (1998)
		Random peptide libraries	Schembri & Klemm (1998); Schembri *et al*. (1999); Kjærgaard *et al*. (2000)
P	FelA	Foot and mouth disease virus epitope	van Die *et al*. (1988, 1990)
		Human immunodeficiency virus epitope	van Die *et al*. (1990)
		Mycobacterium leprae 65 kDa protein epitope	van Die *et al*. (1990)
		Plasmodium falsiparum surface protein epitope	van Die *et al*. (1990)
		Gonadotropin releasing hormone	van der Zee *et al*. (1995)
K88	FaeG	Human influenza virus epitope	Thiry *et al*. (1989)
		Human somatostatin epitope	Thiry *et al*. (1989)
		Neisseriae gonorrhoeae pilin epitope	Bakker *et al*. (1990)
		Foot and mouth disease virus epitope	Bakker *et al*. (1990)
		Human immunodeficiency virus epitope	Bakker *et al*. (1990)
		Hepatitis B surface antigen epitope	Pedersen & Andersen (1991)
987P	FasA	Herpes simplex virus epitopes	Rani *et al*. (1999)
		Transmissible gastroenteritis virus epitope	Rani *et al*. (1999)
CS31A	ClpG	Transmissible gastroenteritis virus epitopes	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	Jennings *et al*. (1989)

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fimbriae. The specific export system consists of the FimC chaperone and the FimD usher proteins (Klemm, 1992; Klemm & Christiansen, 1990).

The biogenesis of type 1 fimbriae has been shown to be quite adaptable with regard to incorporation of homologous structural components and their assembly into fimbriae. Thus, components from F1C fimbriae exhibiting as little as 34% identity were readily incorporated into type 1 fimbriae, resulting in hybrid organelles (Klemm *et al*., 1994). This and other structure–function information suggested that type 1 fimbriae could be used as carriers of heterologous sequences. Two structural components of type 1 fimbriae have been used to display foreign peptides, viz. FimA and 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).

Fimbrial display of foreign epitopes

A large variety of fimbriae are known, and have, where this aspect has been studied, turned out to be completely non-toxic proteins. Indeed, vaccines based on wild-type fimbriae have been highly successful, for example in protecting particular domestic animals against various diarrhoea-causing enterotoxigenic *E. 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. This aspect, as well as their high-valency display, initially spurred the interest in using fimbriae as tools for display of foreign epitopes. The fact that fimbrial proteins exhibited a fair amount of sequence variability (probably due to immunological pressure) indicated that variable sectors would be amenable for grafting of foreign sequences. This was indeed found to be the case and a number of different fimbriae types have over the years been used for the display of heterologous sequences (Table 1).

Heterologous antigen display in the major structural protein and the adhesin of type 1 fimbriae

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. Type 1 fimbriae are highly immunogenic, which might boost the immune response against a passenger epitope. Also, the fimbriae are easily detached from the bacteria and amenable to purification. In a preliminary conceptual study (Hedegaard & Klemm, 1989), heterologous segments, 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 in which a cholera toxin B chain (CTB) epitope was inserted into a number of positions in FimA (Stentebjerg-Olesen *et al*., 1997). Inserted in single- and as tandem repeats, the CTB epitope was authentically displayed in three different positions in FimA as evidenced by immunofluorescence microscopy (Fig. 2). Furthermore, serum from animals immunized with purified FimA-CTB fimbriae was specifically able to recognize natural CTB.

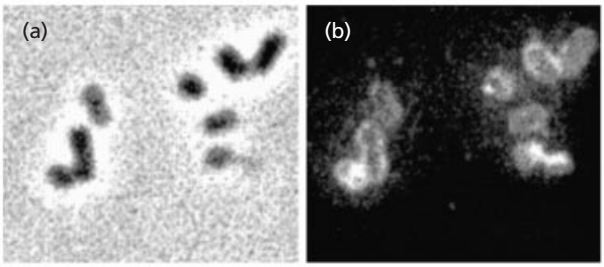

Fig. 2. Surface display of *E. coli* cells presenting an epitope of CTB within the FimA major subunit of type 1 fimbriae. Cells are shown as visualized by phase-contrast microscopy (a) and by immunofluorescence microscopy after reaction with anti-CTB serum (b).

The FimH adhesin is a minor component of type 1 fimbriae. FimH is produced as a 300 aa precursor that is processed into a mature form of 279 aa, i.e. roughly twice the size of the other structural elements of the fimbrial organelle. FimH has been shown to be located at the organelle tip in a short fibrillum (Jones *et al*., 1995) and perhaps additionally intercalated along the fimbrial shaft (Krogfelt *et al*., 1990). The three-dimensional structure of FimH was recently elucidated (Choudhury *et al*., 1999), revealing a two-domain architecture consisting of 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 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 insert positions were identified, both located in the C-terminal domain. Subsequently, a 52 aa peptide mimicking the preS2 region of the hepatitis B surface antigen and the previously mentioned CTB epitope were inserted into both positions. In all cases, the insert positions proved to be compatible with integration of the heterologous sequences with regard to surface display and full or at least partial conservation of the mannose-binding function of the chimeric FimH proteins (Pallesen *et al*., 1995). Furthermore, both the CTB- and the preS2

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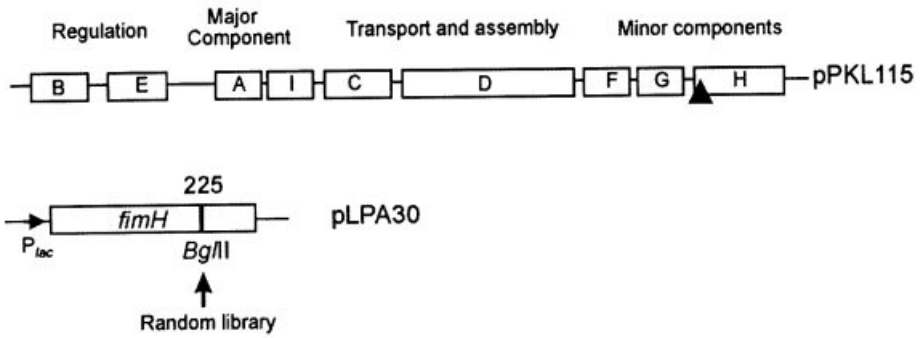
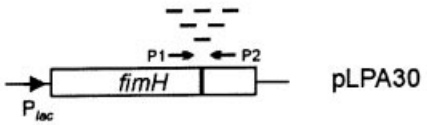
(a) Plasmids used in fimbrial display system

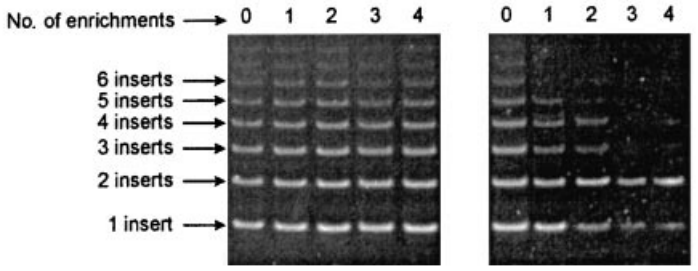


(b) Genetic structure of random library

(c) Model demonstrating monitoring and complexity of library



(d) Monitoring of enrichment steps in selection procedure



(e) Example of wild-type (i) and enriched (ii) metal-oxide-binding clones

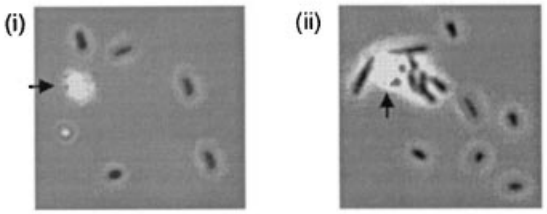


Fig. 3. Overview of random peptide display in type 1 fimbriae. (a) The two plasmids used in heterologous display by FimH. Plasmid pPKL115 contains the entire *fim* gene cluster with a translational stop linker inserted into the *fimH* gene (indicated by a triangle). The *fimH* expression vector is shown along with the *Bgl*II site at position 225. (b) Genetic structure of the random library inserted into *fimH*. The two oligonucleotides were annealed, extended with Klenow fragment of DNA polymerase I, and the product was purified after digestion with *Bgl*II. N indicates an equimolar mixture of all nucleotides and V indicates an equimolar mixture of A, C and G. The use of a VNN coding system prevents the introduction of functional stop codons in an amber-suppressing host. (c) Model demonstrating the complexity of the random library as indicated by the ability to introduce multiple copies of the random insert sequence. Also shown are the

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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 (Pallesen *et al*., 1995). Recent research has established that the FimH adhesin is a key player in *E. coli*-mediated urinary tract infections (UTI) (Connell *et al*., 1996; Sokurenko *et al*., 1998). This knowledge subsequently spurred significant efforts in the development of a FimH-based anti-UTI vaccine. Indeed, a number of promising results support the feasibility of this line of approach (Langermann *et al*., 1997, 2000). In this respect, it is interesting to note that the ability of FimH to display heterologous epitopes might pave the way for FimH-based multivalent, single-protein vaccines.

Antigen display by other fimbriae

P fimbriae of *E. coli* are structurally closely related to type 1 fimbriae. A number of foreign epitopes mimicked by up to 15 aa inserts have been displayed in hypervariable regions of the major structural component, FelA, of F11 fimbriae, a type of P fimbriae (Table 1) (van Die *et al*., 1988, 1990). As a demonstration of this technology, recombinant F11 fimbriae were constructed to display the brain peptide gonadotropin releasing hormone (GnRH) for the development of a contraceptive vaccine for fertility control in 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. Importantly, the vaccination of female rats and young bull calves with purified GnRH-containing fimbriae induced both serological and pharmacological effects that altered the reproductive characteristics of both animals.

K88 fimbriae from porcine enterotoxigenic *E. coli* are excellent immunogens and in fact the first recombinant vaccine (against porcine diarrhoea) was based on wild-type K88 fimbriae. Several studies have addressed the potential of K88 fimbriae as display organelles for foreign epitopes (Thiry *et al*., 1989; Bakker *et al*., 1990; Pedersen & Andersen, 1991). Hypervariable regions of the K88 subunit protein were used as fusion sites to insert epitopes from human influenza virus, human immunodeficiency virus 1 (HIV-1), the foot and mouth disease virus (FMDV) VP1 coat protein, the *Neisseria gonorrhoea* pilin subunit protein and the hormone somatostatin. Specific antibodies were elicited in animal models against the passenger epitopes when the chimeric fimbriae were used as purified preparations.

CS31A fimbriae are K88-related organelles produced by *E. coli* and *Klebsiella pneumoniae* (Girardeau *et al*., 1988). A number of peptides representing antigenic determinants from the transmissible gastroenteritis virus have been displayed in ClpG, the major subunit protein of CS31A fimbriae (Bousquet *et al*., 1994; Der Vartanian *et al*., 1994, 1997; Mechin *et al*., 1996). These studies indicate that the ClpG subunit is highly flexible with regard to epitope display. Using either live recombinant bacteria or purified chimeric CS31A fimbriae, intraperitoneally immunized mice elicited serum peptide antibodies that were capable of recognizing native virus particles. However, only immunization with purified chimeric fimbriae could produce titres capable of virus neutralization (Der Vartanian *et al*., 1997).

The major component of type 4 fimbriae of *Bacteroides nodosus* has been used for presentation of a 16 aa segment mimicking the principal antigenic determinant of the VP1 coat protein of FMDV (Jennings *et al*., 1989). When expressed in *E. coli*, four different chimeras could be recognized by FMDV-specific antibodies. In addition, one of the constructs was expressed in *Pseudomonas aeruginosa*; however, the antigenic properties were not tested.

Random library display

Expression systems have been used to display both defined and random peptide sequences at exposed regions of surface proteins of filamentous bacteriophage virions, bacteria and yeasts (Boder & Wittrup, 1997; Georgiou *et al*., 1997). Phage display is a powerful technology that has been used successfully for applications such as epitope mapping and antibody engineering (Hill & Stockley, 1996). Bacterial surface display systems have been shown to be a powerful complement to this technology. Random peptide libraries inserted into the *E. coli* outer-membrane protein LamB and the major flagellar protein FliC have been successfully screened for specific target recognition sequences by panning techniques involving sequential binding and elution (Brown, 1992; Lu *et al*., 1995). In recent years, research in our laboratory has been strongly focused towards using type 1 fimbriae as display organelles for random peptide sequences. One of the attractive features of bacterial surface display technologies lies in the ability to use fluorescence-activated cell sorting (FACS) for high throughput screening. The use of FACS technology for phage display systems is limited because of the small size of bacteriophages. Indeed, enrichment factors as high as 10000:1 per round have been reported using FACS for *E. coli* displaying protein fusions in outer membrane proteins (Georgiou *et al*., 1997).

Random library display in FimH

The fact that FimH could accommodate and display diverse heterologous sequences as evidenced from the work on the display of immune-relevant sequences led

positions of the primers used to monitor the distribution of the insert population. (d) PCR analysis of a mock enrichment demonstrating the stability of the insert population in the absence of selection (left) and the insert population after enrichment for sequences that bind to ZnO_2 (right). (e) Phase-contrast microscopy of a control (i) and an enriched (ii) clone demonstrating specific binding to ZnO_2. Metal oxide particles are indicated by arrows.

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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 permutated 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.

The strategy employed to generate random libraries in the type 1 fimbrial display system is outlined in Fig. 3. Random libraries were constructed by inserting various numbers of synthetic double-stranded oligonucleotides into a permissive position in the *fimH* gene. The oligonucleotides consisted of nine random codons flanked by identical restriction sites. This genetic structure permitted the insertion of single or polymeric oligonucleotides, a feature that considerably enhanced the complexity of the libraries. Individual libraries were calculated to contain 10^7–10^8 individual clones (Schembri & Klemm, 1998). Clones expressing chimeric FimH with peptide inserts that recognized a number of heavy metals or heavy metal oxides were selected (Schembri & Klemm, 1998; Schembri *et al*., 1999). A number of common structural characteristics were observed in the amino acid sequences selected in line with previous studies on metal–protein interactions (Barbas *et al*., 1993). Even inserts that were able to distinguish between a metal oxide and the corresponding ionic form were identified (Kjærgaard *et al*., 2000).

Taking into account the natural binding ability of FimH to saccharides, one of the potential uses of this technology may be the construction of heterobifunctional adhesins that can adhere simultaneously to both metal and saccharides. We have shown that peptide library display can be combined with alterations in the natural receptor-binding region to independently modulate the binding of FimH to two ligands simultaneously (Schembri & Klemm, 1998; Schembri *et al*., 2000). An obvious advantage inherent in this binary system could be that the immobilization of bacteria using one adhesive domain may facilitate the use of the cells in detection systems for metals, or perhaps directly as biosorption agents for the removal of toxic or precious metals from the environment.

Conclusions

Over the last decade a diverse range of heterologous peptides has been successfully displayed on bacteria by a wide spectrum of fimbriae. The display of peptide segments on the surface of bacteria offers many new and exciting applications in biotechnology, medical research and vaccinology. 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.

Although many different and highly varied heterologous sequences have been displayed in fimbriae, some constraints with regard to composition and size seem to exist. It is noteworthy that the displayed peptides are relatively hydrophilic and essentially devoid of cysteine residues. Arguably, the presence of highly hydrophobic segments is not compatible with surface display in an aqueous environment, and added cysteines might interfere with the passage of the protein to the cell exterior, due to unfavourable S–S bridge formation. 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 a limitation in insert size may indeed exist. It is noteworthy that major structural proteins of various fimbriae can only accommodate relatively modest-sized inserts (in the 10–30 aa range) without detrimental effects on organelle structure and surface display. However, the minor component, FimH, seems to be more generous and is capable of displaying peptides of >100 aa (unpublished results). In this regard, it is tempting to draw parallels to filamentous phage display, where insert size in the major structural protein, pVIII, is severely restricted whereas the minor component (tip protein pIII) can accommodate large inserts (Hill & Stockley, 1996). Indeed the architecture of fimbriae and filamentous phages is in many ways similar, and for display purposes fimbriae might in fact be visualized as surface-attached phages.

More than a decade has elapsed since the first reports on bacterial surface display systems appeared. Since then, an impressive number of different display systems have been developed and tested as antigen presentation vehicles, cellular adsorbents, biocatalysts and library-selection tools (Georgiou *et al*., 1997; Ståhl & Uhlén, 1997). In Gram-negative bacteria, the main impetus has been in using outer-membrane proteins and highly polymeric surface organelles (fimbriae and flagellae) as scaffolds for surface presentation of peptides (Georgiou *et al*., 1997; Sandkvist & Bagdasarian, 1996; Ståhl & Uhlén, 1997). Recently, very promising results have also been obtained with systems based on autotransporter proteins, which are characterized by the fact that all information required to reach the bacterial surface resides in one single peptide chain (Maurer *et al*., 1997; Suzuki *et al*., 1995). Many of these systems have proven versatile with respect to the size and composition of the passenger sequences that can be displayed. Arguably, fimbriae- and flagellae-based systems, and

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perhaps also autotransporter systems, would seem to have an obvious advantage because, like their outer-membrane-protein-based counterparts, they can be developed and used in the context of whole cells but, additionally, and contrary to the outer membrane protein systems, they are easy to purify in large amounts and can therefore also be used as simple protein preparations. Furthermore, when it comes to high-valency display none of the other systems come even close to the format of fimbriae- and flagellae-systems in which close to half a million copies of the major structural proteins are present on each bacterium.

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