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Bacterial Phage Receptors, Versatile Tools for Display of Polypeptides on the Cell Surface

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Four outer membrane proteins of *Escherichia coli* were examined for their capabilities and limitations in displaying heterologous peptide inserts on the bacterial cell surface. The T7 tag or multiple copies of the myc epitope were inserted into loops 4 and 5 of the ferrichrome and phage T5 receptor FhuA. Fluorescence-activated cell sorting analysis showed that peptides of up to 250 amino acids were efficiently displayed on the surface of *E. coli* as inserts within FhuA. Strains expressing FhuA fusion proteins behaved similarly to those expressing wild-type FhuA, as judged by phage infection and colicin sensitivity. The vitamin B_{12} and phage BF23 receptor BtuB could display peptide inserts of at least 86 amino acids containing the T7 tag. In contrast, the receptors of the phages K3 and λ, OmpA and LamB, accepted only insertions in their respective loop 4 of up to 40 amino acids containing the T7 tag. The insertion of larger fragments resulted in inefficient transport and/or assembly of OmpA and LamB fusion proteins into the outer membrane. Cells displaying a foreign peptide fused to any one of these outer membrane proteins were almost completely recovered by magnetic cell sorting from a large pool of cells expressing the relevant wild-type platform protein only. Thus, this approach offers a fast and simple screening procedure for cells displaying heterologous polypeptides. The combination of FhuA, along with with BtuB and LamB, should provide a comprehensive tool for displaying complex peptide libraries of various insert sizes on the surface of *E. coli* for diverse applications.

The display of peptides on the surface of bacteria has become very attractive for a variety of applications such as the development of recombinant bacterial vaccines (32, 33, 34) and the screening of polypeptide libraries for protein-protein interactions (5, 27, 36). In *Escherichia coli*, the outer membrane proteins OmpA, LamB, and PhoE and also the flagellar and fimbrial proteins flagellin, FimH, and PapA (for a review, see reference 18) have been used to display peptides or proteins on the cell surface. However, insertion of peptides longer than 60 amino acids was shown to perturb the conformation of LamB and PhoE (1, 11), resulting in interference with proper cell surface localization. Similarly, the subunits of cellular appendages were also reported not to be suitable for the display of larger polypeptides (for a review, see reference 18). Although the lambda receptor is restricted for the size of insertion, it had been shown that a diverse variety of peptides could be displayed on the surface when fused to LamB (11). Subsequently, the adhesin AIDA-I (39) and the TraT protein (10) from *Escherichia coli*, as well as the ice-nucleation protein of *Pseudomonas mobilis* (26), were used to display heterologous polypeptides on the surface of *E. coli*. Whereas only peptide sequences of up to 100 amino acids were examined for display using the TraT protein, the AIDA-I and the ice-nucleation protein were shown to be capable of displaying a full-length protein. For the latter two proteins, only a few individual examples were examined for surface display. In addition, the AIDA-I and the ice-nucleation fusion proteins were generated by C-terminal addition, while peptides were always inserted within loops of the bacteriophage receptors.

E. coli possesses numerous outer membrane proteins which are involved in different activities to acquire nutrients from the outside milieu. Hydrophilic substrates with molecular masses below 700 Da diffuse through channels formed by the porins OmpC and OmpF, sucrose enters the cell via the ScrY protein (46), and nucleosides through the Tsx pore (4). In contrast, receptor-mediated transport requires the binding of substrates to a receptor, and translocation across the outer membrane is energy and TonB dependent. FhuA facilitates the uptake of ferrichrome (13), FepA transports ferric enterobactin (37, 45), and BtuB mediates uptake of vitamin B_{12} (21). The elucidation of the three-dimensional structures of outer membrane proteins has shown that they in general consist of numerous antiparallel β-barrels connected by turns exposed to the periplasm and loops facing the exterior (29). While the β-barrel structure anchors the protein within the outer membrane, the flexible extracellular loops are well suited to accommodate and display foreign peptide inserts on the cell surface. Importantly, the function of outer membrane proteins as phage and colicin receptors demonstrates that the loops are accessible to extracellular ligands of considerably different sizes. In addition, it indicates that even large structures might be efficiently and stably linked to the bacterial surface via outer membrane proteins.

The ferrichrome and phage T5 receptor FhuA exposes 11 loops to the extracellular milieu and 10 turn regions to the periplasm (14, 35). Most of these structures have been predicted by mutagenic and subsequent functional analyses of mutant FhuA proteins (28). These studies have also shown that small peptide insertions in loops 4, 5, and 10 of the ferrichrome receptor did not interfere with the sensitivity for phage T5 and

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TABLE 1. *E. coli* K-12 strains used in this study

Strain	Genotype	Source or reference
DH10B	F′ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara leu*)*7697 araD139 galU galK nupG rpsL* λ$^-$	Life Technologies
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *gal phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Life Technologies
XL1-Blue MRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)]	Stratagene
Pop6510	*thr leu tonB thi lacY1 recA dex-5 metA supE*	M. Hofnung (2)
UH203	*lac supF ompA recA proAB rpsL* (F′ *lacI*^{q} *lacZ*ΔM15 *proAB*$^+$)	R. Freudl (16)
AM6	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *gal phoA supE44* λ$^-$ *thi-1 gyrA96 relA1 ompA* (P1 transduction of DH5α with lysate grown on UH203)	This study
UL4	*aroB tsx malT thi fhuA recA* Δ*srl*	V. Braun (47)
RK5016	*araD139* Δ(*argF-lac*)*169 flbB5301 ptsF25 relA1 rpsL150 rbsR22 deoC1 gyrA219 non-9 metE70 argH1 btuB461 recA56*	R. Kadner (21)

colicin M, which is an indication of the proper conformation and assembly of the fusion protein in the outer membrane. Although the three-dimensional structure has not been solved for the vitamin B_{12} receptor from *E. coli*, structure-function analyses similar to those of FhuA have also been performed with BtuB. Insertion of single restriction sites into the gene and experiments with deletion, as well as duplication, mutants allowed the prediction of extracellular loops and periplasmic turns (23, 30). The analysis of these mutant BtuB proteins with respect to their functionality as receptors for bacteriophage BF23 and family E colicins indicated that BtuB could be modified without disturbing the conformation of the protein or its proper insertion into the outer membrane.

This study sought to provide platform proteins for the display of randomly generated, genomic libraries of various insert sizes suitable for rapid screening with diverse ligands. We have therefore analyzed the ability of the two outer membrane proteins, FhuA and BtuB, to present large polypeptide inserts on the bacterial surface. In addition, we examined OmpA and LamB, which were previously shown to accept smaller peptide inserts (11, 16), with regard to their restrictions for efficient surface display and selection by magnetic cell sorting (MACS). Two loops of the FhuA protein were evaluated with multiple copies of the myc epitope and differently sized fragments of gene *10* from phage T7 encoding the T7 tag epitope in order to determine the size restriction for foreign polypeptides. The vitamin B_{12} and phage BF23 receptor BtuB, as well as OmpA and LamB, were assessed for their ability to accept fragments of variable sizes of gene *10* from phage T7 encoding the T7 tag epitope. While BtuB showed a moderately increased tolerance for the display of polypeptides in comparison with OmpA and LamB, FhuA was capable of presenting polypeptides of up to 249 amino acids in size. This would be sufficient to encompass complete structural and/or functional domains of proteins.

Importantly, bacteria displaying polypeptides in the context of these outer membrane proteins could be quickly and efficiently recovered using MACS from a large pool of cells, especially with the proteins FhuA, LamB, and BtuB. We therefore suggest that complex and diversely sized libraries for bacterial surface display can be obtained by combining the use of several outer membrane proteins.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The bacterial strains used in this study are listed in Table 1. Strain UL4 is a *fhuA* mutant and was used for all FhuA sensitivity assays. The bacteriophages T5 and ϕ80 use the FhuA protein as a receptor and were propagated on *E. coli* XL1-Blue MRF′ (Stratagene). The *btuB*-deficient strain RK5016 was used for BtuB sensitivity assays with phage BF23 and colicins E1 and E3. Phage BF23 was propagated on *E. coli* XL1-Blue MRF′. The *ompA*-deficient strain AM6 was generated from *E. coli* DH5α (Life Technologies) by P1 transduction with phage grown on UH203. Colicin M was produced from the *E. coli* M57T containing plasmid pTO4 (43). Colicins E1 and E3 were produced from the *E. coli* K-12 W3110 strains 105640 and 105646 obtained from the Collection de l'Institut Pasteur.

The plasmids pBluescript-myc3 and pBluescript-myc9 harbor three and nine copies of the sequence encoding the myc epitope (EQKLISEEDLN) inserted into the *Xba*I and *Spe*I sites of plasmid pBluescript, respectively (C. Michaelis, unpublished results). All other plasmids are listed in Table 2. Plasmid pAJC264 contains a *Bam*HI site downstream of the sequence encoding S155 in loop 4 of the LamB protein which was used for insertion of fragments encoding the T7 tag epitope (MASMTGGQQMG). These fragments were generated by PCR amplification of gene *10* of phage T7 encompassing the T7 tag epitope at its N terminus by using plasmid pET17xb (Novagen) as a template. *Bam*HI sites were introduced at both ends by this PCR, which allowed in-frame cloning of the fragments resulting in the insertion of peptides of 27 (ICC.40 and ICC.38), 47 (ICC.40 and ICC.37), 67 (ICC.40 and ICC.39), and 87 (ICC.40 and ICC.41) amino acids, respectively. Plasmid pEV218 contains a polylinker downstream of the sequence encoding G154 of the OmpA protein (16) and was treated in a similar fashion. An insertion encoding 31 amino acids was created by ligation of the annealed oligonucleotides T7-UP and T7-DOWN into the *Sac*I/*Xba*I sites of pEV218 (9). Fragments generated by PCR from plasmid pET17xb with the listed oligonucleotides resulted in the insertion of peptides of 41 (T7L5 and PCR1-3), 51 (ICC.44 and ICC.42), 61 (T7L5 and PCR2-3), or 71 (ICC.44 and ICC.43) amino acids in length, each containing the T7 tag epitope. All fragments that were generated by PCR were cloned into the *Sac*I/*Xba*I sites of pEV218. pHIE3 is based on the vector pEH1 (20) that carries the gene for kanamycin resistance, the *lacI* repressor, and the *lacUV5* promoter. The *fhuA* gene was amplified by PCR from genomic DNA of *E. coli* DH5α using the oligonucleotides ICC.95 and ICC.96 and cloned into the *Nco*I/*Eco*RI sites of pEH1 via these newly introduced restriction sites. Plasmid pHIE6 was generated from pHIE3 by insertion of a *Not*I site downstream of the sequence encoding P405 in loop 5 of the FhuA protein by PCR mutagenesis by using the oligonucleotides ICC.72 and ICC.130. A linker consisting of *Fse*I, *Xba*I, and *Not*I sites was inserted downstream of the sequence encoding P405 in loop 5 of FhuA by PCR mutagenesis by using oligonucleotides ICC.72 and ICC.209 and plasmid pHIE3 as a template, resulting in plasmid pHIE11. pHIE7, pHIE8, pHIE9, pHIE12, and pHIE13 were created by insertion of various repeats of the sequence encoding the myc epitope into pHIE6, yielding inserts of 18, 46, 89, 126, and 166 amino acids, respectively. A single myc epitope encoding DNA fragment was inserted into pHIE6 with the annealed oligonucleotides ICC.113 and ICC.114 to generate pHIE7, pHIE8 was created by insertion of three myc epitopes into pHIE6 generated by PCR from pBluescript-myc3 as a template with oligonucleotides ICC.99 and ICC.100. The *Not*I fragment encoding three myc epitopes was excised from pHIE8, and two

TABLE 2. Plasmids used in this study

Platform protein	Type of insert	Length of insert (amino acids)	Plasmid name[a]
OmpA L4 (G154)	*Fse*I/*Xba*I/*Not*I	9	pMAL5
	CS2 linker	25	pEV218
	T7	31	pEV/T7-10
	T7	41	pEV/T7-20
	T7	51	pEV/T7-30
	T7	61	pEV/T7-40
	T7	71	pEV/T7-50
LamB L4 (S155)	*Fse*I/*Xba*I/*Not*I	10	pMAL9.1
	*Bam*HI	4	pAJC264
	T7	27	pMAL9/T7
	T7	27	pAJC/T7-20
	T7	47	pAJC/T7-40
	T7	67	pAJC/T7-60
	T7	87	pAJC/T7-80
FhuA wt[b]			pHIE3
			pHIE10
FhuA L4 (P321)	*Not*I	3	pICCS6
	1× myc	18	pICCS9
FhuA L4 (A324)	*Not*I	3	pICCS7
	1× myc	18	pICCS10
	3× myc	46	pICCS14
	9× myc	126	pICCS15
FhuA L4 (A333)	*Not*I	3	pICCS8
	1× myc	18	pICCS11
	3× myc	46	pICCS12
	9× myc	126	pICCS13
	18× myc	249	pICCS16
FhuA L5 (P405)	*Not*I	3	pHIE6
	*Fse*I-*Xba*I-*Not*I	9	pHIE11
	1× myc	18	pHIE7
	3× myc	46	pHIE8
	6× myc	89	pHIE9
	9× myc	126	pHIE12
	12× myc	166	pHIE13
	S. aureus peptide	95	pHIE11-Sa95
	T7	56	pHIE14
	T7	86	pHIE21
	T7	126	pHIE22
	T7	166	pHIE23
BtuB wt			pMAL10
BtuB L3 (G236)	*Fse*I-*Xba*I-*Not*I	9	pMAL10.1
	T7	56	pMAL10.2
	T7	86	pMAL10.3
	T7	126	pMAL10.4
	T7	166	pMAL10.5
	S. aureus peptide	95	pMAL10.1-Sa95

[a] All pMAL, pHIE, and pICCS plasmids are pEH1-based vectors (20) that carry the kanamycin resistance cassette; only pHIE10 is pEH3 based and confers resistance to chloramphenicol. All pEV plasmids are based on pEV218 (16); all pAJC plasmids are based on pAJC264 (3).
[b] wt, wild type.

copies were inserted into pHIE6, yielding pHIE9. pHIE8 was digested with *Xba*I and ligated to two copies of the PCR fragment amplified from pBluescript-myc3 with oligonucleotides M13-forward and M13-reverse and digested with *Xba*I, resulting in plasmid pHIE12. pHIE13 was constructed by the insertion of a PCR fragment, which was amplified from pBluescript-myc9 with oligonucleotides M13-forward and M13-reverse and digested with *Spe*I, into pHIE8 digested with *Xba*I. The pICCS series was constructed by PCR mutagenesis of pHIE3, inserting a *Not*I site downstream of the sequence encoding P321 (pICCS6) by using oligonucleotides ICC.72, ICC.95, ICC.203, and ICC.204; A324 (pICCS7) was constructed by using oligonucleotides ICC.72, ICC.95, ICC.205, and ICC.206; and A333 (pICCS8) was constructed by using oligonucleotides ICC.72, ICC.95, ICC.207, and ICC.208. Various repeats of the sequence encoding the myc epitope were cloned into these vectors utilizing the pHIE series as described for the P405 insertion site (see Table 2). A single myc epitope-encoding DNA fragment was generated by insertion of the annealed oligonucleotides ICC.113 and ICC.114. The DNA fragments encoding repeats of three and nine myc epitopes were excised from pHIE8 and pHIE12, respectively. Plasmid pMAL10 is derived from pEH1 and contains the *btuB* gene amplified from genomic *E. coli* DH5α DNA with the oligonucleotides ICC.265 and ICC.268 and cloned into the *Nco*I/*Sac*I sites of pEH1. pMAL10.1 contains an *Fse*I/*Xba*I/*Not*I linker inserted downstream of the sequence encoding G236 of BtuB in plasmid pMAL10 by PCR mutagenesis by using the oligonucleotides ICC.265, ICC.269, ICC.266, and ICC.268. Fragments of gene *10* of phage T7 of 56, 86, 126, and 166 amino acids in length containing the T7 tag epitope were amplified with plasmid pETx17b as a template and the oligonucleotides ICC.283 and ICC.284, ICC.283 and MOL.797, ICC.283 and MOL.798, and ICC.283 and MOL.799, respectively. All four fragments were cloned via the *Fse*I/*Not*I sites into plasmid pMAL10.1 or pHIE11, resulting in plasmids pMAL10.2, pMAL10.3, pMAL10.4, and pMAL10.5 or plasmids pHIE14, pHIE21, pHIE22, and pHIE23, respectively. The antigenic determinant from *S. aureus* of 95 amino acids in length (unpublished data) was transferred from plasmid pHIE11-Sa95 into the *Fse*I/*Not*I sites of pMAL10.1, resulting in plasmid pMAL10.1-Sa95. All oligonucleotide sequences are available upon request.

Antibodies. T7 tag monoclonal antibody (MAb; Novagen) was used for Western blot analysis at a dilution of 1:10,000 and for fluorescence-activated cell sorting (FACS) analysis at a dilution of 1:500. The α-myc MAb 9E10 was immunoglobulin G (IgG) purified (3.2 mg/ml) and used for Western blots at a dilution of 1:5,000 and for FACS at a dilution of 1:500. The α-LamB MAb LBS-1 (17) was used for Western blot analysis at a dilution of 1:1,000, and the α-OmpA polyclonal antiserum (22) was used at a dilution of 1:5,000. FhuA polyclonal antiserum was generated by injecting rabbits with peptides corresponding to amino acids 407 to 428 (loop 5), amino acids 454 to 474 (loop 6), or amino acids 544 to 565 (loop 8) of the FhuA protein and coupled to keyhole limpet hemocyanin according to standard procedures. IgG antibodies (10 mg/ml) specific for loop 5 of FhuA were purified by affinity chromatography by using the corresponding peptide and used for Western blot analysis at a dilution of 1:100,000.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Bacterial cultures were induced for protein expression in mid-exponential phase with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 30 to 120 min (see figure legends for details). Proteins from crude bacterial lysates (5×10^6 bacteria/sample) were separated on SDS–10% polyacrylamide minigels and subsequently transferred onto Hybond C membrane (Amersham Pharmacia Biotech) by semidry transfer. α-LamB and α-myc antibodies were detected with rabbit anti-mouse horseradish peroxidase-labeled immunoglobulin (Ig-HRP; Dako), and α-OmpA and α-FhuA were detected with donkey anti-rabbit Ig-HRP (Amersham Pharmacia Biotech), all at a dilution of 1:5,000. Detection was performed by using the ECL detection kit (Amersham Pharmacia Biotech).

FACS analysis. Surface exposure of the epitopes inserted into *E. coli* outer membrane proteins was confirmed by FACS analysis. Freshly inoculated cultures were induced with 1 mM IPTG at an optical density at 600 nm (OD_{600}) of 0.5 for 30 min (FhuA and BtuB) or 90 min (OmpA and LamB) and subsequently harvested. About 10^6 bacteria were washed once with 1 ml of ice-cold phosphate-buffered saline (PBS) and 0.5% bovine serum albumin (BSA) and incubated with α-myc MAb 9E10 or T7 tag MAb in PBS–0.5% BSA blocking solution for 30 min on ice. Unbound antibodies were removed by washing with PBS–0.5% BSA, and the cells were subsequently exposed to fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins (Dako) at a dilution of 1:500. After being washed, the bacteria were fixed in PBS–1% paraformaldehyde. The fluorescence intensity was analyzed by using a FACSCalibur flow cytometer (Becton Dickinson). A total of 10,000 bacteria were counted and analyzed with the WinMDI software.

Sensitivity of the FhuA and BtuB fusion proteins to bacteriophages and colicins. The response to the bacteriophages T5 and φ80, as well as to the bacterial toxin colicin M, was tested with *E. coli* strain UL4 transformed with the *fhuA* constructs as listed in Table 2. About 10^7 cells of an overnight culture were suspended in 3 ml of molten soft agar and poured onto an agar plate containing the selective antibiotic. About 10^7 PFU of each bacteriophage in 1 μl or 1 μl of a culture supernatant of the colicin M-producing strain *E. coli* M57T pTO4 was spotted onto the top agar. The plates were inspected after overnight incubation at 37°C. Similarily, phage BF23 and colicin E1 and E3 sensitivities were determined with *E. coli* strain RK5016 transformed with the *btuB* constructs as listed in Table 2.

MACS screening. About 5,000 cells expressing the FhuA or BtuB fusion protein and carrying the resistance marker for kanamycin were mixed with approximately 10^7 bacteria harboring the respective wild-type gene on a plasmid encoding chloramphenicol resistance. The same ratio was used for cells containing plasmids encoding the OmpA and LamB platform fusion proteins and am-

picillin resistance and for those encoding the wild-type protein and kanamycin resistance. After induction of protein expression with 1 mM IPTG for 30 to 90 min, the cell mixture was washed twice with Luria-Bertani (LB) medium and incubated with 10 to 100 ng of α-myc MAb 9E10, 10 to 100 ng of α-T7 tag MAb, or 0.05 μl of mouse serum in 50 μl of LB medium overnight at 4°C. The mouse serum was preadsorbed against *E. coli* cells expressing the relevant wild-type platform protein. The cells were then washed and incubated with biotinylated goat anti-mouse IgG antibody (Southern Biotechnology) at 0.2 μg/sample in LB medium for 30 min at 4°C. After another wash with LB medium, 10 μl of MACS microbeads coupled to streptavidin (Miltenyi Biotech) and 40 μl of LB medium were added, and the incubation was continued for 20 min at 4°C. Thereafter, 950 μl of LB medium was added, and the MACS microbead cell suspension was loaded onto the equilibrated MS column (Miltenyi Biotech) which was attached to the magnet. The column was washed twice with 3 ml of LB medium. The elution was performed by removing the magnet and washing with 2 ml of LB medium. After the column was washed with 3 ml of LB medium, the eluate was loaded a second time on the same column, and the washing and elution process was repeated. The loading, washing, and elution process was performed a third time, resulting in a final eluate of 1 ml. Aliquots were plated onto LB plates containing chloramphenicol or kanamycin to select for the clones expressing the wild-type platform protein or the corresponding platform fusion protein, respectively.

RESULTS

Selection of platform proteins for the display of foreign peptide inserts on the cell surface of *E. coli*. In order to develop an approach facilitating the display of comprehensive and variably sized peptide libraries derived from genomic DNA on the cell surface, we sought to test and apply multiple platform proteins for surface presentation, since it had been shown for most proteins that biological constraints exist that exclude certain sequences and/or sizes of peptides and proteins from display. The family of outer membrane proteins of *E. coli* provides a large repertoire of candidates, each of which has multiple potential insertion sites due to the presence of multiple extracellular loops. Since all proteins serve as receptors for various phages and toxins to enter the bacterial cell, the proper conformation of the phage receptor within the outer membrane can be assessed by a biological assay for phage or toxin sensitivity. The porins OmpA and LamB were shown previously to be capable of presenting diverse, small peptide inserts on the bacterial surface and were therefore chosen for a detailed analysis. In order to enable the presentation of protein domains which require correct folding for the interaction with an exogenous compound or protein, it was a prerequisite for the platform protein to be able to present polypeptides exceeding 60 amino acids in size. For this purpose, the TonB-dependent receptors BtuB and FhuA were examined for the display of larger polypeptides. Subsequently, all platform proteins were assessed for their potential to allow selection of *E. coli* cells via the displayed foreign polypeptide by MACS.

Surface display of the T7 tag via OmpA fusion proteins. OmpA was shown to accept insertions in loop 4, when a polylinker encoding 25 amino acids was cloned into its gene downstream of the sequence encoding G154 (16) and in loop 2, where hexapeptides were inserted to facilitate binding of cadmium to *E. coli* cells (40). In order to systematically determine the size of the insert that would be tolerated by OmpA in loop 4 without negatively affecting the folding and insertion of the fusion protein into the outer membrane, PCR primers were designed to amplify fragments of gene *10* of phage T7 with lengths of between 31 and 71 amino acids and including the T7 tag. The insertion of gene *10* fragments into *ompA* was facilitated by plasmid pEV218 containing the linker CS2 (16) (Table 2). Western blot analysis with polyclonal antibodies directed against OmpA revealed that all fusion proteins with insertions in loop 4 of OmpA were expressed, but expression was reduced when more than 41 amino acids were inserted (Fig. 1A). While the reduction in expression was moderate for OmpA fusion proteins with 51 and 61 amino acids inserted, it was strongly decreased for the protein with an insertion of 71 amino acids. Efficient surface presentation as assayed by FACS analysis with T7 tag MAb was detected with OmpA fusion proteins containing inserts of up to 41 amino acids. The display of 51 and 71 amino acids was completely abolished (Fig. 1B), although cytoplasmic expression of the OmpA fusion with 51 amino acids was only slightly reduced in comparison to expression of those with smaller insertions. Surprisingly, OmpA with an insert of 61 amino acids within loop 4 was presented on the cell surface. However, the growth of these cells was greatly impaired, which is also reflected in the presence of weakly or unstained cells in the FACS analysis. These results indicate that the size of the insert in loop 4 of OmpA should not exceed ca. 40 amino acids to ensure efficient surface display of the foreign peptide. All experiments were performed in the *E. coli* strain AM6 (*ompA*), since the expression of OmpA fusion proteins on the surface was not detectable in *E. coli* strains

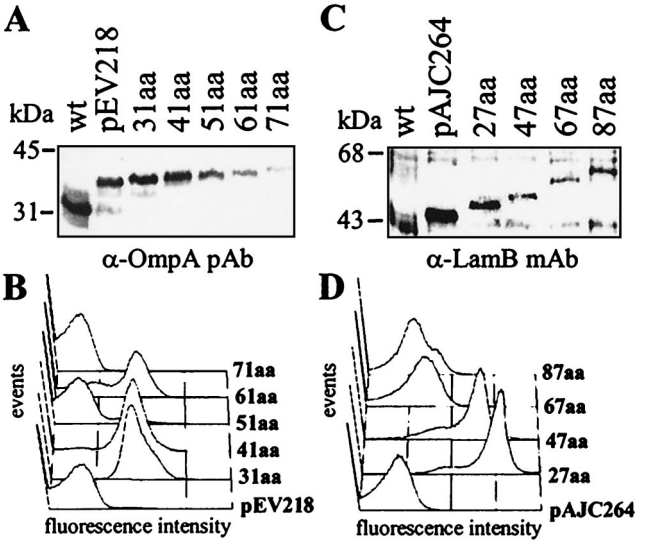

FIG. 1. Detection of LamB and OmpA T7 tag fusion proteins by Western blot and FACS analysis (for detailed information on the fusion proteins, see Table 2). Expression of the proteins in *E. coli* strains AM6 for OmpA and Pop6510 for LamB was induced for 90 min with 1 mM IPTG. (A and C) Proteins in lysates from approximately 5×10^6 bacteria were separated by SDS-PAGE and blotted onto HybondC membrane. Detection of the OmpA (A) and LamB (C) proteins was performed with the polyclonal α-OmpA antibody (pAb) or with the α-LamB MAb LBS-1. Molecular masses are indicated in kilodaltons on the left. The size of the insert within the respective protein or the relevant construct is indicated for each lane (in amino acids [aa]). DH5α cell lysates were loaded as wild-type control for both proteins (wt). (B and D) FACS analysis was carried out with α-T7 tag antibody and cells expressing OmpA (B) and LamB proteins (D) as described in Table 2 and in Materials and Methods. The fluorescence intensity reflects the amount of fusion protein displayed on the bacterial surface.

expressing wild-type OmpA protein, such as DH5α or DH10B (unpublished data).

Presentation of peptides on the cell surface via LamB. Various polypeptides have been displayed on the bacterial surface by using the lambda receptor (loop 4) as a platform protein (12, 42, 49). Although four protein A IgG binding domains with a size of 232 amino acids were tolerated by LamB (50), previous experiments have shown that only inserts of up to 60 amino acids are efficiently displayed at the cell surface (11). In order to assess the possibility of using LamB for MACS selection, gene *10* fragments including the T7 tag epitope were inserted into the *Bam*HI site present in plasmid pAJC264 (3), yielding LamB fusion proteins with 27 to 87 amino acids inserted in loop 4. Expression of all lambda receptor fusion proteins with insertions distal to amino acid S155 was analyzed in the *lamB* strain Pop6510 to avoid detection of the LamB protein expressed from the chromosome. Western blot analysis with α-LamB MAb LBS-1 (17) showed that insertions of 47 amino acids or more reduced the expression level of the fusion protein significantly (Fig. 1C). Accordingly, the shift in fluorescence intensity as determined by FACS analysis with the T7 tag MAb showed a continuous decrease with increasing insert size (Fig. 1D). In order to generate a plasmid encoding LamB and a different resistance marker for MACS experiments, we subsequently constructed plasmid pMAL9.1. We also transferred the *lamB* gene encoding the T7 tag with 27 amino acids into plasmid pEH1 which showed a tighter control of protein expression than pAJC264-based plasmids (pMAL9/T7) and therefore improved the growth of cells harboring the pEH1-based plasmid. *E. coli* DH5α cells containing pMAL9/T7 displayed the T7 tag efficiently on the surface, as measured by FACS analysis (unpublished results), indicating that peptides can be displayed on the bacterial surface in cells that express wild type LamB from the chromosome. Whereas the expression of larger inserts within OmpA basically abolished surface presentation, the display on the surface of larger inserts when fused to LamB is clearly detectable, albeit to a strongly reduced level. These data agree well with previously published data (11).

BtuB, a novel platform for display of foreign and large peptide inserts. The two porins OmpA and LamB are well suited to display small peptides on the cell surface, but their size restriction for surface display prompted us to study the capacity of two other outer membrane proteins from the family of TonB-dependent receptors, BtuB and FhuA. The vitamin B_{12} receptor has not been employed previously to present peptide inserts on the cell surface. Nevertheless, structure-function analyses including small peptide insertions, gene fragment duplications, and gene fragment deletions have suggested the existence of extracellullar loop structures within BtuB (23, 30). Sequence comparison and published data indicate the localization of the first 160 amino acids of BtuB, including a TonB box within the cytoplasm similar to FepA and FhuA (8), but there is poor sequence conservation of BtuB with the sequence constituting their barrel structure. However, by using the GenTHREADER algorithm to predict relationships of BtuB to proteins of known structure (25), FepA and FhuA were identified as the closest relatives. This analysis suggests that BtuB has an organization similar to that of FepA (7) and FhuA (14, 35). In agreement with the tolerance for

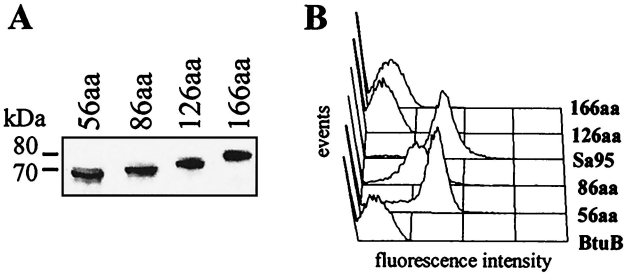

FIG. 2. Western blot and FACS analysis of BtuB with fragments of different lengths inserted in an extracellular loop of the protein (for more detailed information, see Table 2). Expression of the proteins in *E. coli* DH5α was induced for 40 to 120 min with 1 mM IPTG. BtuB fusion proteins were separated as described in Fig. 1. (A) BtuB fusion proteins were detected with α-T7 tag antibody. (B) Surface display of BtuB fusion proteins as determined by FACS analysis with α-T7 tag antibody (inserts 56, 86, 126, and 166) or mouse serum (insert Sa95) as described in Materials and Methods. The number indicates the insert size within BtuB. Sa95, BtuB with an insertion of 95 amino acids encoding an antigenic determinant from *S. aureus*. The fluorescence intensity reflects the amount of fusion protein displayed on the bacterial surface.

mutational changes at this position in regard to bacteriophage BF23 and family E colicin sensitivities, amino acid G236 would be predicted to be located in an extracellular loop 3 of BtuB. This led us to choose putative loop 3 for the insertion of foreign peptides. A linker consisting of the three restriction sites *Fse*I, *Xba*I, and *Not*I was inserted downstream of the sequence encoding G236 in order to allow directional cloning of DNA fragments into this site. Subsequently, fragments of gene *10* encoding 56, 86, 126, and 166 amino acids, including the T7 tag, were cloned into the *Fse*I/*Not*I sites of the *btuB* gene. All fusion proteins were expressed as determined by Western blot analysis with the T7 tag MAb (Fig. 2A). It was not possible to compare the expression level with plasmid encoded, wild-type BtuB protein, since no antibody directed against BtuB was available. When surface presentation was assessed by FACS analysis with the T7 tag MAb, BtuB was able to display peptide inserts of 56 and 86 amino acids in length within loop 3, but no or only very low presentation on the surface was detectable for the polypeptide of 126 and 166 amino acids in length, respectively (Fig. 2B). In accordance with the data obtained by FACS analysis, the sensitivities toward phage BF23 and colicins E1 and E3 were decreased only slightly for the insertion of 56 and 86 amino acids, but especially the sensitivity toward colicin E1 was strongly decreased with larger inserts (Table 3). The BtuB protein thus shows a greater potential to present polypeptides on the cell surface than OmpA and LamB, offering the display of at least 86 amino acids in size.

The ferrichrome and T5 receptor FhuA is a superior and versatile display platform. In contrast to BtuB, the three-dimensional structure of FhuA has been resolved recently (14, 35). The analysis of the structure revealed that the two extracellular loops 4 and 5 reach farthest out from the surface, making them good candidates to present foreign peptides to exogenously added reagents (Fig. 3A). Furthermore, experiments with MAbs directed against the FhuA protein and insertional mutagenesis with DNA fragments encoding short

TABLE 3. Properties of FhuA and BtuB fusion proteins

Fusion protein	Length of insert (amino acids)	Relative sensitivities[a] to:			Surface presentation (α-myc or α-T7 tag MAb)[b]
		T5 (BF23)	ϕ80 (ColE1)	Colicin M (ColE3)	
FhuA wt[c]	−	+++	+++	+++	−
P321:					
*Not*I	3	+	−	++	−
1× myc	18	+	−	++	+++
A324:					
*Not*I	3	+++	+++	+++	−
1× myc	18	++	++	++	+++
3× myc	46	+	−	++	+++
9× myc	126	+	−	+	++
A333:					
*Not*I	3	+++	+++	+++	−
1× myc	18	+++	+++	+++	+++
3× myc	46	++	++	+++	+++
9× myc	126	++	++	++	+++
18× myc	249	+	+	+	++
P405:					
*Not*I	3	+++	+++	+++	−
1× myc	18	+++	+++	+++	+++
3× myc	46	+++	+++	+++	+++
6× myc	89	+++	+++	+++	+++
9× myc	126	+++	++	++	+++
12× myc	166	++	+	++	++
T7	56	+++	+++	+++	+++
T7	86	+++	+++	+++	+++
T7	126	++	++	++	+
T7	166	++	++	++	++
BtuB wt	−	(+++)	(+++)	(+++)	−
T7	56	(+++)	(++)	(++)	+++
T7	86	(++)	(++)	(++)	++
T7	126	(++)	(−)	(+)	−
T7	166	(++)	(+)	(++)	+

[a] Sensitivities are given in relative values: +++, wild-type sensitivity; ++ and +, intermediate sensitivities; −, complete resistance. The sensitivities to BF23, ColE1, and ColE3 (i.e., for BtuB) are enclosed in parentheses.

[b] Surface presentation measured by flow cytometry using α-myc MAb 9E10 or α-T7 tag antibody. The data from Fig. 2 and 4 are summarized here in order to facilitate the comparison with the phage and colicin sensitivities. +++, Maximal shift (10^2); ++, slightly reduced surface presentation; +, strongly reduced surface presentation; −, no surface presentation.

[c] wt, wild type.

peptides of up to 16 amino acids in length indicated the surface location of 12 loops prior to the determination of the three-dimensional structure (28, 41) (Fig. 3B). The latter study also showed that phage T5 infection and colicin M sensitivity were largely unaffected by insertions at positions P321 and A333 in loop 4 and P405 in loop 5, suggesting that the conformation of these FhuA fusion proteins was not drastically changed compared to wild-type FhuA. Since amino acid A324 was determined by X-ray crystallography to be located at the very tip of loop 4, most distal to the outer membrane (14, 35), this site was also chosen for the insertion of peptides. PCR mutagenesis was performed in order to insert a *Not*I restriction site immediately downstream of the sequences encoding amino acids P321, A324, A333, and P405, allowing the construction of genes encoding multiple copies of the myc epitope or the T7 tag fused to FhuA at the respective position.

FhuA with large insertions maintains its phage and colicin receptor functions. As a first evaluation of the ability of the chosen sites in loops 4 and 5 to tolerate insertions of foreign peptides, one copy of the myc epitope (18 amino acids) was fused with FhuA at each position (Table 2). The proper conformation and insertion of the fusion protein in the outer membrane was assessed by means of phages T5 and ϕ80, as well as via colicin M sensitivity assays. While the insertions at positions A324, A333, and P405 showed little or no effect on phage and colicin sensitivity, the fusion with the myc epitope between amino acids P321 and A322 conferred strongly reduced sensitivities toward these toxic agents (Table 3). Therefore, only the three positions A324, A333, and P405 were tested for larger peptide insertions. DNA fragments encoding three copies of the myc epitope were inserted at the respective positions under conditions allowing insertion of multiple repeats of this fragment. A maximum of 9, 12, and 18 myc epitopes were cloned by this strategy downstream of the sequence encoding A324, P405, and A333, respectively. Cells expressing FhuA with insertions larger than 18 amino acids at position A324 were clearly affected by the fusion with the foreign peptide. Phage ϕ80 infectivity was completely abol-

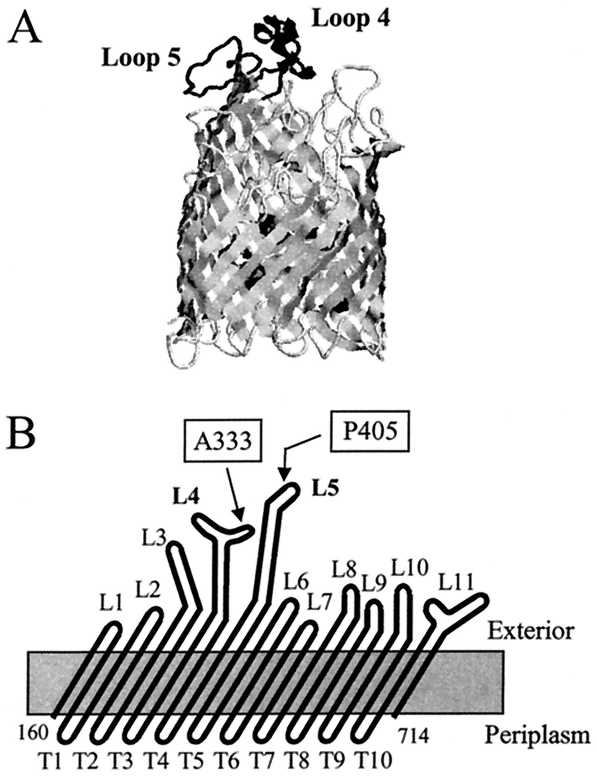

FIG. 3. Structure of the outer membrane protein FhuA. (A) Three-dimensional structure of the FhuA protein with the loops 4 and 5 (highlighted in dark gray) directed toward the extracellular milieu (35). (B) Schematic model of the topology of the FhuA protein. L and T refer to extracellular loops and periplasmic turns, respectively. The gray box represents the lipid bilayer. Residues A333 and P405, at which positions fragments of different lengths were inserted, are indicated. The numbers of the first and last amino acids which are shown are indicated.

ished and phage T5 and colicin M sensitivity reduced substantially (Table 3). Positions A333 and P405, on the other hand, proved to be tolerant for the insertion of large polypeptides. Only FhuA with the largest insertion at either position showed a significant decrease in phage and colicin M sensitivities, while polypeptides of up to 126 amino acids had little effect (Table 3). These data strongly suggest that FhuA can accept and display polypeptides as large as 249 and 166 amino acids in loops 4 and 5, respectively.

FhuA presents large peptides efficiently on the cell surface. In order to evaluate the potential of FhuA for bacterial surface display, the expression and surface presentation of the protein with insertions distal to A324 and A333 in loop 4, as well as P405 in loop 5, were analyzed. The level of FhuA expression was first examined in total cellular lysates by Western blot analysis with polyclonal α-FhuA antibodies directed against a peptide derived from loop 5 as well as with the α-myc MAb 9E10. Expression of all myc-FhuA fusion proteins was detectable, although larger inserts resulted in a reduced expression level compared to the wild-type plasmid-encoded FhuA (Fig. 4A and B). In addition, degradation products were detectable with the FhuA fusion proteins containing larger insertions.

The α-myc MAb 9E10 was used to analyze surface presentation of FhuA fusion proteins by FACS. All fusion proteins were displayed on the cell surface; only FhuA with 126 amino acids inserted between A324 and P325 showed a small decrease in fluorescence shift, indicating a lower level of surface presentation of the fusion protein (Fig. 4C and Table 3). Insertions of up to 126 amino acids in positions A333 and P405 did not reduce presentation of the myc epitope at the bacterial surface, and even myc epitopes consisting of 166 and 249 amino acids inserted distal to P405 and A333, respectively, were displayed via FhuA, although with slightly reduced efficiency.

In order to allow a direct comparison of the results obtained with FhuA with those of the other three outer membrane proteins, plasmids were constructed expressing FhuA fusion proteins with the T7 tag inserted into position P405 of FhuA. All T7 tag-FhuA fusion proteins were expressed to similar levels, and less degradation was observed compared to the myc-FhuA fusion proteins (Fig. 4A and B). T7 tag-FhuA fusions with inserts of 56, 86, and 166 amino acids in loop 5 were efficiently displayed on the surface, as determined by FACS analysis (Fig. 4C), and inserts had little effect on phage and colicin M sensitivities (Table 3). As had been observed for BtuB, the insert of 126 amino acids containing the T7 tag caused a significant reduction in surface expression and sensitivity toward phage and colicin M.

Based on these experiments, the FhuA protein provides the greatest potential to display large polypeptides on the surface of *E. coli*.

Selection of *E. coli* cells displaying peptide inserts via outer membrane proteins by MACS. We sought to establish an approach to select bacterial cells by MACS utilizing antibodies directed against the displayed peptide. Such a selection process can only be successful when the level of surface presentation is suitable to separate cells expressing the foreign peptide of choice from cells expressing unrelated peptides. At the same time the expression level has to be suitable to sustain viability of cells during the selection process. In order to establish the selection procedure for bacterial cells, approximately 10^3 *E. coli* cells presenting the foreign epitope were mixed with up to 10^8 cells expressing the wild-type platform protein. The selection by MACS was performed with antibodies directed against the inserted peptide and biotinylated secondary antibodies. The antibody-cell complex was then immobilized via streptavidin coupled to magnetic beads. To distinguish cells displaying a relevant epitope from cells expressing the wild-type platform protein, plasmids were constructed expressing the wild-type protein in combination with a different antibiotic marker than those expressing the fusion protein (Table 2).

Relevant fusions of all four platform proteins were analyzed by MACS with the respective MAb as the capture reagent. As anticipated from the FACS data, cells displaying the T7 tag within OmpA and LamB were efficiently recovered when the insert size did not exceed 41 and 27 amino acids, respectively (Table 4). Larger inserts drastically reduced the specific recovery with T7 tag MAb. The BtuB protein supported efficient selection of epitope displaying cells with 56 and 86 amino acids inserted into loop 3, but the insertion of 126 and 166 amino acids was ineffective (Table 4). In contrast to OmpA, LamB, and BtuB, the FhuA protein was capable of presenting peptides ranging from 18 to 249 amino acids on the cell surface

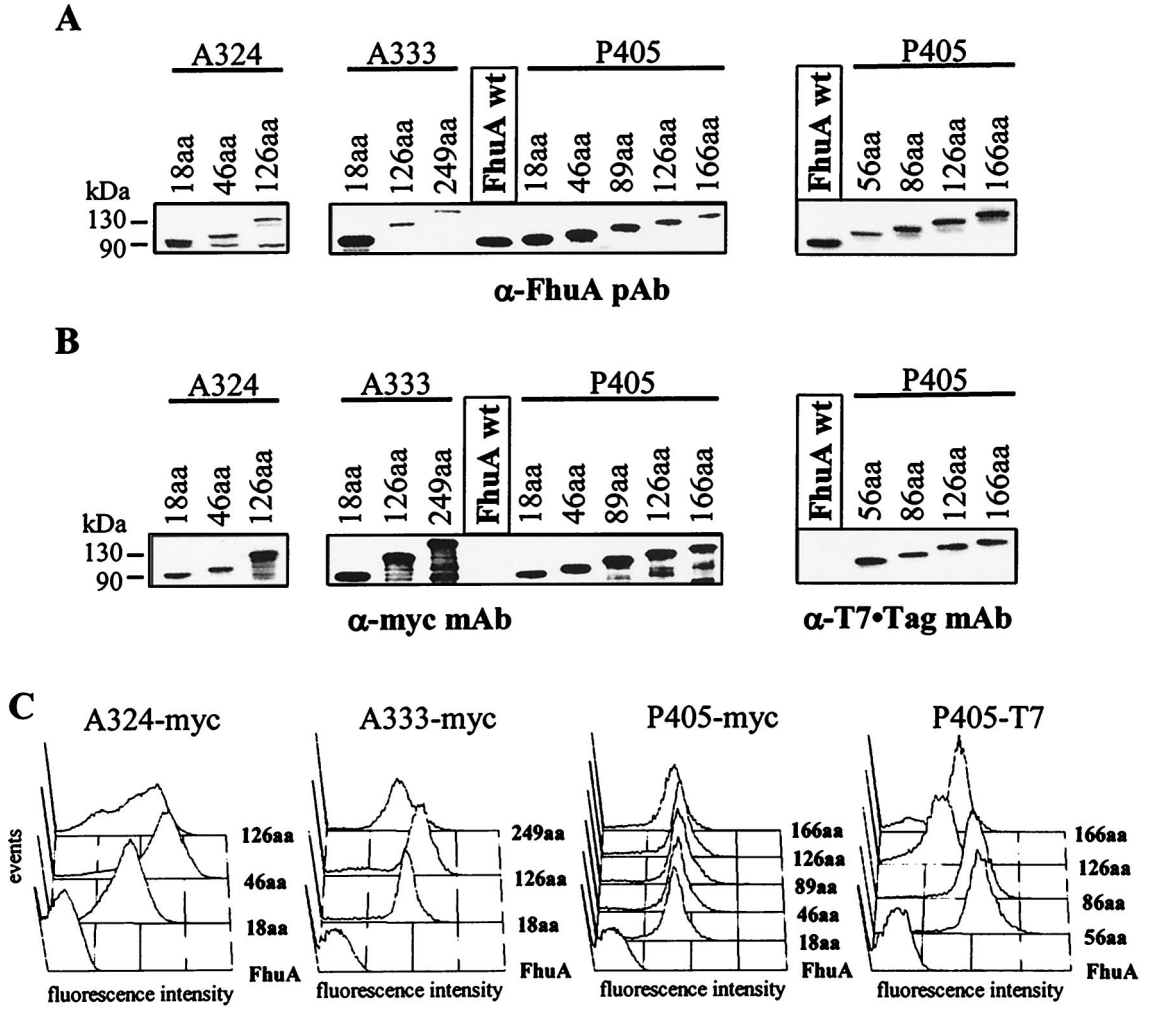

FIG. 4. Western blot and FACS analysis of FhuA with fragments of different lengths inserted in extracellular loops of the protein (for detailed information, see Table 2). Expression of the proteins in *E. coli* DH5α was induced for 30 min with 1 mM IPTG. FhuA and FhuA fusion proteins were separated as described in Fig. 1. (A and B) Detection of FhuA proteins was performed with polyclonal α-FhuA antibody (pAb), α-T7 tag MAb, or with α-myc MAb 9E10. The size of the insert and the insertion site is indicated for each lane (in amino acids [aa]). (C) FACS analysis of cells displaying FhuA or FhuA fusion proteins was carried out with α-myc antibody or α-T7 tag antibody as described in Materials and Methods.

(Fig. 4C), and cells expressing these fusion proteins were recovered with high efficiency (Table 4). Most interestingly, cells expressing the large insert in loops 4 and 5 showed no reduction in recovery, a finding consistent with the FACS analysis that showed only a minor reduction of surface presentation. Importantly, it was possible to obtain recovery rates for all four platform proteins exceeding 75% with a single round of selection, while the recovery of cells not displaying an epitope was <0.1%. In addition, it was possible to recover as few as 100 cells from a background of more than 10^7 cells. These results show that cells displaying a specific peptide can be quickly and efficiently selected by this method. Having shown that recovery of cells was very efficient with a model epitope and its cognate MAb, we wanted to compare this with the recovery of cells expressing an antigenic determinant identified from *Staphylococcus aureus* and crude mouse serum obtained after immunization of mice with *E. coli* cells expressing this antigen. The antigenic determinant was selected from a genomic *S. aureus* library screened with human IgG (H. Etz et al., unpublished data) and encodes a polypeptide of 95 amino acids in size derived from a novel protein encoded by Sa0723 (more information is available online [http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gsa]). The fusion of FhuA and BtuB with the antigen from *S. aureus* allowed us to examine surface presentation and selection with crude mouse serum. In both cases, more than 50% of the cells were recovered from a background of approximately 10^7 cells expressing the respective wild-type platform protein. This result shows that ligands do not need to be extensively purified for selection, and it confirms the capability of FhuA and BtuB to efficiently present large polypeptides on the cell surface.

DISCUSSION

In this study we describe a systematic analysis of four bacteriophage receptors to present polypeptides on the surface of *E. coli* in order to establish an approach allowing the comprehensive display of genome-derived peptide libraries. We have

TABLE 4. MACS recovery rates

Platform protein	Insert		Antibody used	% Recovery of positive cells	% Background of negative cells
	Type	Length (amino acids)			
OmpA L4 (G154)	T7	31	α-T7 tag MAb	88	1.4×10^{-3}
	T7	41	α-T7 tag MAb	66	2.3×10^{-3}
	T7	51	α-T7 tag MAb	0	1.0×10^{-3}
	T7	61	α-T7 tag MAb	0.5	0.6×10^{-3}
	T7	71	α-T7 tag MAb	0	2.0×10^{-3}
LamB L4 (S155)	T7	27	α-T7 tag MAb	75	3.6×10^{-2}
	T7	47	α-T7 tag MAb	0.3	2.2×10^{-2}
	T7	67	α-T7 tag MAb	0	9.0×10^{-2}
	T7	87	α-T7 tag MAb	0	1.0×10^{-1}
FhuA L4 (A324)	1× myc	18	α-myc MAb	88	1.2×10^{-6}
	3× myc	46	α-myc MAb	100	8.7×10^{-6}
	9× myc	126	α-myc MAb	31	3.0×10^{-6}
FhuA L4 (A333)	1× myc	18	α-myc MAb	95	1.4×10^{-5}
	3× myc	46	α-myc MAb	96	1.2×10^{-5}
	9× myc	126	α-myc MAb	67	1.2×10^{-6}
	18× myc	249	α-myc MAb	100	1.7×10^{-6}
FhuA L5 (P405)	1× myc	18	α-myc MAb	78	6.1×10^{-7}
	3× myc	46	α-myc MAb	75	2.5×10^{-6}
	6× myc	89	α-myc MAb	75	3.0×10^{-6}
	9× myc	126	α-myc MAb	87	6.4×10^{-7}
	12× myc	166	α-myc MAb	98	3.1×10^{-6}
	T7	56	α-T7 tag MAb	86	7.9×10^{-6}
	T7	86	α-T7 tag MAb	74	3.5×10^{-6}
	T7	126	α-T7 tag MAb	18	2.8×10^{-6}
	T7	166	α-T7 tag MAb	77	3.2×10^{-6}
	S. aureus peptide	95	Mouse serum	55	7.7×10^{-2}
BtuB L3 (G236)	T7	56	α-T7 tag MAb	95	1.0×10^{-1}
	T7	86	α-T7 tag MAb	40	6.1×10^{-2}
	T7	126	α-T7 tag MAb	0	4.2×10^{-2}
	T7	166	α-T7 tag MAb	0.5	4.7×10^{-2}
	S. aureus peptide	95	Mouse serum	72	7.9×10^{-2}

chosen the family of outer membrane proteins as platforms, since they have been shown to be capable of presenting small, synthetic peptide libraries (5) and because they provide a large variety of different candidates. In addition, the presence of multiple loops will allow the simultaneous insertion of two different peptides (51) and, furthermore, peptides will be fused with the platform protein at both ends, which may support their stability and surface presentation. The display of peptides on the surface of *E. coli* should then facilitate the recovery of cells by MACS, providing a fast and easy procedure for selection.

Based on the work of many laboratories, numerous proteins embedded or attached to the outer membrane have been established as platforms for the display of foreign peptides and proteins on the cell surface of gram-negative bacteria. Nevertheless, for most of these proteins it has been shown that the presentation of certain polypeptides is not possible or that the size for display is restricted (for a review, see reference 18). A similar observation has been made for the technique of phage display, wherein foreign peptides are fused with the bacteriophage adsorption protein gIIIp or the coat protein gVIIIp from filamentous phage (24, 48). While phage display is widely used for the library-based identification of protein-protein interactions, it has been reported that biological constraints also apply to this technique, restricting some peptides from expression and efficient surface display (38, 44). The more recently described platform protein AIDA-I was shown to anchor β-lactamase on the cell surface (31), but otherwise only shorter polypeptides were reported for surface display. The ice-nucleation protein of *Pseudomonas syringae* was fused to a number of different polypeptides and enzymes for recombinant bacterial vaccines and recombinant whole-cell catalysts, respectively (26, 33), but neither of these two platform proteins had yet been examined for the display of large peptide libraries. Georgiou and coworkers engineered a fusion protein consisting of the Lpp leader peptide and its first 9 amino acids and residues 46 to 154 of mature OmpA. This fusion protein was capable of presenting proteins such as β-lactamase and scFv on the surface of *E. coli* when fused to its C terminus (15, 19). However, it has been shown that the expression of Lpp-OmpA-Bla tripartite fusion proteins leads to major alterations in the outer membrane (19). Accordingly, we observed that the expression of the myc epitope and the T7 tag as fusion with Lpp-OmpA greatly impaired the survival of the respective *E. coli* strains (B.

Richter and A. Meinke, unpublished data). The heterologous proteins were fused with AIDA-I, the ice-nucleation protein, and Lpp-OmpA at their C terminus, leaving the C terminus of the displayed protein freely accessible. Since we were aiming to present randomly generated peptides on the surface, it seemed advantageous to provide a scaffold for the foreign peptide by anchoring it at both ends. The family of outer membrane proteins was therefore very well suited to present polypeptides on the bacterial surface, because they possess a robust β-barrel structure which anchors them in the outer membrane (29). The extracellular loops, which serve as receptors for bacteriophages, as well as toxins, such as colicins or microcins, are amenable to considerable modifications without interfering with the conformation of the protein. In addition, the outer membrane can accommodate a large number of individual outer membrane proteins, enabling the efficient presentation of multiple copies of a peptide on a single cell.

In order to facilitate the efficient display of a comprehensive genomic peptide library, it was important to identify outer membrane proteins and to determine the conditions for presentation of foreign peptide inserts on the surface which do not impair the growth of *E. coli*. OmpA is one of the most abundant proteins of the cell, and it stabilizes the outer membrane. Unfortunately, the level of surface display of peptides by OmpA was rather low in *E. coli* strains expressing the wild-type OmpA protein from the chromosome. This may be explained by the abundance of wild-type OmpA in the cell, which may lead to competition with the fusion protein for transport and incorporation into the outer membrane. While surface presentation was restored in OmpA-deficient *E. coli* strains, the use of OmpA as a platform for surface display of peptide libraries is hampered, because these strains show a severely reduced transformation rate by plasmid DNA than their parent OmpA-expressing strains (9). LamB, FhuA, and BtuB, in contrast, are present in *E. coli* cells only at small amounts, and their expression has to be induced by the appropriate environmental condition. Expression of the recombinant platform proteins carrying the foreign peptides was very well tolerated in *E. coli* strains such as DH5α, and a high level of surface presentation was possible in strains encoding the respective wild-type protein. Although we determined that only foreign peptides of approximately 30 amino acids in size are suitable for our approach, it has been shown that a wide variety of structures and hydrophobicities are tolerated by LamB (11). In addition, a random peptide library was displayed on the surface of *E. coli* by LamB and metal-binding polypeptides were successfully isolated (6).

In contrast to LamB, very limited data were available on surface display of peptides by the vitamin B_{12} receptor BtuB and the ferrichrome and T5 receptor FhuA. One distinct site for BtuB and three sites for FhuA were analyzed for the tolerance to accept large inserts. The data revealed that larger inserts interfere with the efficient display on the cell surface, as determined by FACS, and that different insertion sites and proteins behave differently. While both proteins facilitate the display of larger polypeptides on the cell surface, reducing the size restrictions imposed on the lambda receptor, the sites tested for FhuA showed a higher tolerance for insertions than the one analyzed for BtuB. The reduced display of the T7 tag encompassing 126 amino acids compared to the one containing 166 amino acids for both BtuB and FhuA indicates that not only the size of the insertion but also the insert as such will determine the efficiency of display. The presented data also show that overall protein expression does not strictly correlate with surface display but that the size and nature of the insert will influence the extent of proper incorporation into the outer membrane. We therefore argue that the use of multiple platform proteins will decrease the bias of surface presentation imposed on a single outer membrane protein.

FhuA not only showed the highest tolerance for peptide insertions but, of several analyzed insertion sites, two were identified to accept large polypeptides. Thus, it is possible to express libraries of peptides of sufficient length to encode domains able to fold independently as a fusion with FhuA. Importantly, this feature will facilitate the presentation of potential binding sites which require conformational information. The possibility to insert peptides in two different loops could also be used to display two different peptides simultaneously. Such a strategy was recently employed in order to express two B-cell epitopes in loops 5 and 9 of the LamB protein in an attenuated strain of *Salmonella enterica* serovar Typhimurium (51). On the other hand, an affinity tag could be added in one loop of the protein. This would facilitate detection of an FhuA fusion protein expressing a peptide for which no specific antibody is available and which is inserted in the second loop of FhuA. This feature might be especially useful for the construction of diverse peptide libraries, since the affinity tag would provide a tool to immediately perform experiments with a selected FhuA-peptide fusion protein.

E. coli cells displaying foreign peptide inserts fused to any one of the four examined phage receptors were very efficiently recovered from a large background of negative cells by MACS. The high recovery rates are especially important since they should allow the recovery of cells from a peptide library without extensive amplification of the library. Since we anticipated that our approach would be used to select peptides from libraries binding to distinct antibodies in crude serum preparations, it was important to test whether unspecific antibodies would interfere with a screen. The MACS selection experiment performed with FhuA and BtuB fused to an *S. aureus*-derived peptide and crude mouse serum has shown that high recovery rates can be obtained without extensively purifying the extracellular ligand applied for selection.

The outer membrane proteins FhuA, LamB, and BtuB thus provide a well-suited panel of platform proteins for bacterial surface display, which should facilitate the presentation of single polypeptides for applications, such as recombinant vaccines or whole-cell adsorbents. More interestingly, it is possible to generate comprehensive libraries of peptide sequence of up to 200 amino acids in size by combining the use of two or three platform proteins. This strategy would ensure that conformation-dependent binding sites are included, and it would also reduce or eliminate the possibility that peptides are excluded from the combined library by biological constraints imposed on one platform. We have already applied this strategy successfully to create genomic peptide libraries from the bacterial pathogens *S. aureus* and *S. epidermidis* in order to identify immunogenic B-cell epitopes from these pathogenic bacteria by MACS selection (H. Etz et al., unpublished results). These experiments have also shown that a large number of relevant

polypeptides can be displayed on the cell surface when two different platform proteins are employed. It seems therefore reasonable to propose that the outer membrane proteins and the approach described in this work will be valuable tools for the identification of protein-ligand interactions and for other bacterial surface display applications.

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