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Display of Passenger Proteins on the Surface of *Escherichia coli* K-12 by the Enterohemorrhagic *E. coli* Intimin EaeA

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Intimins are members of a family of bacterial adhesins from pathogenic *Escherichia coli* which specifically interact with diverse eukaryotic cell surface receptors. The EaeA intimin from enterohemorrhagic *E. coli* O157:H7 contains an N-terminal transporter domain, which resides in the bacterial outer membrane and promotes the translocation of four C-terminally attached passenger domains across the bacterial cell envelope. We investigated whether truncated EaeA intimin lacking two carboxy-terminal domains could be used as a translocator for heterologous passenger proteins. We found that a variant of the trypsin inhibitor *Ecballium elaterium* trypsin inhibitor II (EETI-II), interleukin 4, and the Bence-Jones protein REI_v were displayed on the surface of *E. coli* K-12 via fusion to truncated intimin. Fusion protein net accumulation in the outer membrane could be regulated over a broad range by varying the cellular amount of suppressor tRNA that is necessary for translational readthrough at an *amber* codon residing within the truncated *eaeA* gene. Intimin-mediated adhesion of the bacterial cells to eukaryotic target cells could be mimicked by surface display of a short fibrinogen receptor binding peptide containing an arginine-glycine-aspartic acid sequence motif, which promoted binding of *E. coli* K-12 to human platelets. Cells displaying a particular epitope sequence fused to truncated intimin could be enriched 200,000-fold by immunofluorescence staining and fluorescence-activated cell sorting in three sorting rounds. These results demonstrate that truncated intimin can be used as an anchor protein that mediates the translocation of various passenger proteins through the cytoplasmic and outer membranes of *E. coli* and their exposure on the cell surface. Intimin display may prove a useful tool for future protein translocation studies with interesting biological and biotechnological ramifications.

In recent years there has been considerable progress in the development of expression systems for the display of heterologous peptides and proteins on the surfaces of bacteria and yeasts (6, 16, 31). Cells displaying peptides and proteins such as receptors, antibodies, and enzymes are of considerable value for various biotechnological applications, such as bioseparations, vaccine development, and combinatorial library screening. Numerous anchor proteins that mediate the translocation of passenger proteins through the cytoplasmic and outer membranes of *Escherichia coli* and their exposure on the cell surface have been used. Short peptides (less than approximately 50 amino acids [aa]) were successfully displayed on the cell surface by insertion into surface-exposed loops of fimbrial proteins (24) or outer membrane proteins like LamB (7) or PhoE (2). Larger passenger domains could be presented on the *E. coli* cell surface by insertion into a surface-exposed domain of the *E. coli* flagellin FliC (50), by carboxy-terminal fusion to Lpp-OmpA (a hybrid protein consisting of parts of the *E. coli* lipoprotein Lpp and OmpA protein [9, 12]), by using the peptidoglycan-associated lipoprotein PAL as an outer membrane anchor (15), by amino-terminal fusion to the β-domain of immunoglobulin A (IgA) protease of *Neisseria gonorrhoeae* and other autotransporters (22, 30, 35), or by C-terminal fusion to InaZ, the ice nucleation protein of *Pseudomonas syringae* (18).

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Pathogenic gram-negative bacteria have developed several distinct secretion mechanisms for the efficient surface display of binding domains, which specifically interact with their complementary receptors on host cell surfaces (23). Among them, intimins and invasins are members of a family of bacterial adhesins which specifically interact with diverse eukaryotic cell surface receptors, thereby mediating bacterial adherence and invasion (48). Enteropathogenic *E. coli* and enterohemorrhagic *E. coli* (EHEC) produce attaching and effacing lesions in the intestinal mucosa. The intimate bacterial adhesion associated with attaching and effacing lesion formation is promoted by intimin, an EHEC surface protein. Intimin targets the translocated intimin receptor (Tir), which is exported by the bacteria and integrated into the host cell membrane (20). At least five different subtypes of intimin have been described (1, 37). They are integrated into the *E. coli* outer membrane with their amino-terminal region, while the carboxy-terminal 280 amino acids are surface exposed (13).

The EaeA intimin from EHEC O157:H7 is composed of 939 amino acids. The cell binding activity of EaeA intimin has been localized to its C-terminal 280 residues (13), and the structure of the carboxy-terminal domains has been determined recently by both X-ray crystallography and nuclear magnetic resonance (3, 19, 32). It is assumed that the amino-terminal 550 residues of intimin form a porin-like structure (43) and are folded into an antiparallel β-barrel (32). The entire extracellular segment of intimin is an elongated and relatively rigid rod made up of three immunoglobulin-like domains and a C-terminal lectin-like domain to interact with the receptors (Fig. 1A) (19, 32). This domain resides on a rigid extracellular arm, which is most

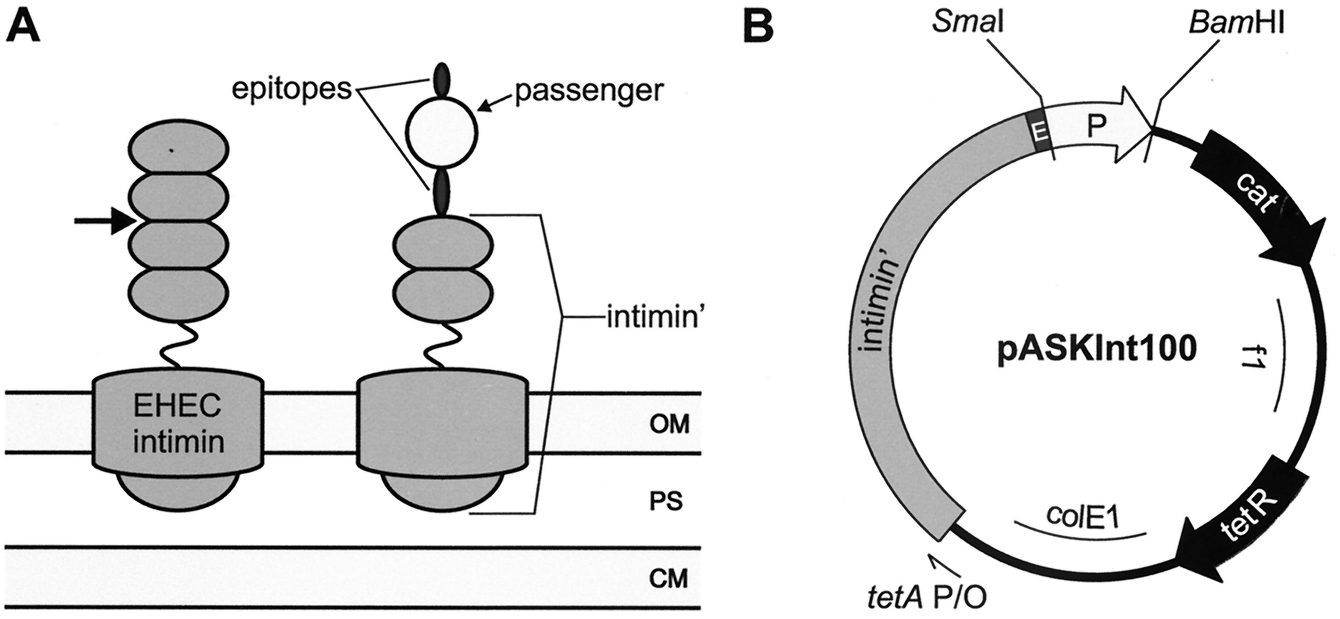

FIG. 1. (A) Schematic drawing of intimin and intimin′ used in this study for the display of heterologous passenger domains. The arrow indicates the site of truncation at residue 659 of intimin. The passenger domain is flanked at its amino-terminal and carboxy-terminal ends by short epitope sequences and fused to carboxy-terminally truncated intimin. OM, outer membrane; PS, periplasmic space; CM, cytoplasmic membrane. (B) Schematic representation of the display vector pASKInt100 harboring the structural gene of intimin′. f1, f1 replication origin; *cat*, chloramphenicol resistance marker; *tetR*, tetracycline repressor encoding gene; colE1, ColE1 replication origin; intimin′, truncated *eaeA* gene of EHEC O157:H7 from codon 1 to 659; E, E^{tag} epitope-encoding sequence. Unique *Sma*I and *Bam*HI restriction sites allow the in-frame fusion of genes encoding various passenger domains (P).

likely anchored to the amino-terminal transmembrane domain through a flexible hinge made by two glycine residues, allowing mechanical movement between the extracellular rod and the bacterial outer membrane (32).

Apparently, intimin provides a structural scaffold ideally suited to the cell surface display of receptor binding domains remote from the bacterial cell surface. This prompted us to investigate whether a truncated intimin that lacks the carboxy-terminal receptor binding domain could be used as a platform for the display of heterologous passenger domains on the surface of *E. coli* K-12 cells. We found that protein fusion to truncated intimin results in cell surface exposure of foreign passengers at high copy numbers. Furthermore, intimin′-based display of a foreign peptide ligand enables *E. coli* K-12 cells to adhere to eukaryotic target cells that present the corresponding receptor and also allows the isolation of peptide-presenting cells by fluorescence-activated cell sorting (FACS).

MATERIALS AND METHODS

Bacterial strains. All *E. coli* strains used in this study are listed in Table 1. Bacteria were grown at 37°C. The medium used for growth and maintenance of *E. coli* strains was dYT (1% yeast extract, 1.6% Bacto tryptone, 0.5% NaCl). Anhydrotetracycline (Acros, Morris Plains, N.J.) was added to liquid media at a final concentration of 0.2 μg/ml. Antibiotics were used at the following final concentrations if required: chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 50 μg/ml; tetracycline, 12.5 μg/ml.

Reagents. Restriction enzymes and DNA-modifying enzymes were purchased from MBI Fermentas and New England BioLabs. *Tfl* polymerase was obtained from Promega. Biotinylated goat anti-mouse antibody was obtained from Sigma. The monoclonal antibody (VII-E-7) to a 13-residue C-terminal epitope of Sendai virus L protein (DGSLGDIEPYDSS) (11) was a gift from H. Einberger and H. P. Hofschneider (Max-Planck-Institut für Biochemie, Martinsried bei München, Germany). Anti-E monoclonal antibody was obtained from Pharmacia Biotech. A streptavidin–R-phycoerythrin conjugate was purchased from Molecular Probes. All other chemicals were of analytical grade and obtained from Sigma.

Oligonucleotides. Synthetic oligonucleotides were purchased from Metabion (Martinsried, Germany), SigmaARK (Darmstadt, Germany), and MWG Biotech (Ebersberg, Germany). The oligonucleotides were as follows: IL-4-up, 5′-GCG CCCCGGGCACAAGTGCGATATCACC-3′; IL-4send-lo, 5′-GCGCGGATCC TTATGATGAATCGTATGGTTCGATATCACCTAATGATCCATCGCTCG AACACTTTGAATATT-3′; intimin-amber-lo, 5′-GCGCGAATTCTAATTAA CATAAAAAAACAATCC-3′; intilo1, 5′-GCGCCAATTGCGCTGGCCTTGG TTTGATC-3′; intiminup, 5′-GCGCTCTAGATAACGAGGGCAAAAAATGA TTACTCATGGTTGTTATAC-3′; pASK-cat5′-seqlo, 5′-TATCAACAGGGAC ACCAGG-3′; REI-lo-BglII, 5′-GCGCAGATCTCCTAGTGATTTGAAGCTT AG-3′; RGD-up, 5′-GCGCCCCGGGTGCATCCCTCGAGGGGACTACCGT TGCWAACAGGACTCCGACTG-3′; RSPX, 5′-GTGAATTTCGACCTCTA G-3′; SupE2-Eco-up, 5′-GCGCGAATTCACCAGAAAGCGTTGTACGG-3′; SupE2-Mlu-lo, 5′-GCGCACGCGTAAGACGCGGCAGCGTCGC-3′; EheI-up, 5′-CAGCTGTTGCCCGTCTCG-3′; and AWcatlo, 5′-CGCGTCGACAAG CTTGAAAACGTTTCAGTTTGC-3′.

DNA procedures. Standard DNA procedures, such as plasmid isolation, ligation, restriction analysis of plasmids, and isolation of DNA fragments, were as described elsewhere (42). PCR using *Tfl* polymerase was as follows: 30 s of denaturation at 94°C, 30 s of annealing at 53°C, and 30 s of elongation at 72°C for 30 cycles. Plasmids constructed and used in this study are listed in Table 1.

Construction of plasmid pASKInt100. For construction of plasmid pASKInt100, the following cloning strategy was applied. Codons 1 to 659 of the *eaeA* gene were PCR amplified directly from heat-inactivated cells of EHEC O157:H7 strain 933 (a gift from J. Hacker, University of Würzburg) using the oligonucleotides intiminup and intilo1. The resulting PCR product was digested with *Xba*I and *Mun*I and ligated to *Xba*I/*Eco*RI-digested vector pASKC21-EETI to give pASKInt1. pASKC21-EETI is a derivative of plasmid pASK21-EETI (9), where the coding sequence for an E epitope was placed 5′ to the *Ecballium elaterium* trypsin inhibitor II (EETI-II) coding sequence. It contains a chloramphenicol resistance marker instead of the ampicillin resistance gene which has codons 8 to 281 deleted. To place an *amber* codon at codon position 35 of the *eaeA* gene, part of the *eaeA* coding sequence was PCR amplified with the primers intiminup and intimin-amber-lo. The resulting PCR product was digested with

TABLE 1. *E. coli* strains and plasmids used in this work

Strain or plasmid	Genotype or phenotype	Reference
DH5αZ1	F$^-$ (ϕ80d*lacZ*ΔM15) Δ(*lacZYA-argF*)*U169 deoR recA1 hsdR17*(r_k^- m_k^-) *endA1 supE44* λ$^-$ *thi-1 gyrA*(Nal^r) *relA1 tetR*$^+$ *lacR*$^+$ Sp^r	33
71-18	F′ *lacI*^{q} (*lacZ*ΔM15) *proA*$^+$*B*$^+$ Δ(*lac-proAB*) *supE thi-1*	51
71-18mutS	F′ *lacI*^{q} (*lacZ*ΔM15) *proA*$^+$*B*$^+$ Δ(*lac-proAB*) *supE thi-1 mutS*::Tn*10*	27
WK6	F′ *lacI*^{q} (*lacZ*ΔM15) *proA*$^+$*B*$^+$ Δ(*lac-proAB*) *galE strA* Nal^r	28
pASK21-EETI-CK^{Send}	EETI-CK^{Send} coding sequence fused to display module *lpp′-ompA′*, derivative of pASK75 (47)	9
pASK75cat	Derivative of pASK75 (47), contains an additional chloramphenicol resistance marker	This work
pASKInt1	Cell surface display vector containing an E-epitope-encoding sequence fused to a truncated *eaeA* gene under *tetA* promoter/operator control, chloramphenicol resistance marker, *tetR* gene	This work
pASKInt100	Derivative of pASKInt1, contains amber codon 35 in the intimin′ gene	This work
pASKInt100-EETI-CK^{Send}	Vector for display of EETI-CK^{Send}, derivative of pASKInt100	This work
pASKInt100-IL4	Vector for display of IL-4, derivative of pASKInt100	This work
pASKInt100-REI	Vector for display of REI, derivative of pASKInt100	This work
pASKInt100-REIΔSend	Same as pASKInt100-REI but lacking the Sendai epitope-encoding sequence	This work
pASKInt100-RGD	Vector for display of RGD peptide, derivative of pASKInt100	This work
pASKInt100-EETI-CK^{RGD}	Vector for display of EETI-CK^{RGD}, derivative of pASKInt100	This work
pMX-RGD	Vector for periplasmic production of MalE-RGD, derivative of pHKREI (26)	This work
pMX-EETI-CK^{RGD}	Vector for periplasmic production of MalE–EETI-CK^{RGD}, derivative of pHKREI (9)	This work
pREP4supE	Contains *amber* suppressor tRNA under P_{Llac} promoter control, derivative of pREP4 (Qiagen)	This work

*Xba*I and *Eco*RI and ligated to similarly digested vector pASKInt1, yielding pASKInt100. The complete nucleotide sequence of this plasmid is available on our website (http://www.gwdg.de/~hkolmar).

Construction of plasmids pASKInt100-EETI-CK^{Send}, pASKInt100-IL-4, and pASKInt100-REI. The EETI-CK^{Send} gene was obtained from pASK21-EETI-CK^{Send} (9) by digestion with *Sma*I and *Bam*HI and ligated to similarly digested pASKInt100 to give vector pASKInt100-EETI-CK^{Send}. The interleukin 4 (IL-4)-encoding gene was amplified by PCR using the vector pRPR9IL-4FD (29) as a template and the primer pair IL-4-up and IL-4send-lo. IL-4send-lo hybridizes at the 3′ end of the IL-4 gene and introduces the nucleotide sequence encoding the 13-residue epitope from the Sendai virus L protein (9). The resulting PCR product was digested with *Sma*I and *Bam*HI and ligated to similarly digested pASKInt100. For the construction of an *eaeA′-rei* gene fusion, the vector pASKInt100-TmDegP, a derivative of pASKInt100 which contains a *Bgl*II restriction site preceding the coding sequence for the Sendai virus L-protein epitope, was digested with *Sma*I and *Bgl*II and ligated to the *Eco*RV- and *Bgl*II-digested PCR product containing the *rei* gene, which was obtained by PCR amplification with vector pHKREI (26) as the template and the oligonucleotide primer pair EheI-up and REI-lo-BglII to give pASKInt100-REI. To obtain pASKInt100-REIΔSend, the Sendai virus epitope-encoding sequence, which is flanked in pASKInt100-REI by a *Bgl*II and a *Bam*HI restriction site, was removed by digestion of pASKInt100-REI with *Bgl*II and *Bam*HI followed by ligation of the vector fragment.

Construction of plasmids pASKInt100-RGD and pASKInt100-EETI-CK^{RGD}. pASKInt100-EETI-CK^{Send} was used as template DNA for PCR with the oligonucleotide pair RGD-up and AWcatlo. RGD-up introduces the coding sequence for CIP**RGD**YRC, which replaces the EETI-II inhibitor loop. Due to its degeneration, it contains either a TAA stop codon or an AAA (Lys) codon after the nonameric peptide coding sequence. The resulting PCR product was cloned as described above for the construction of pASKInt100-EETI-CK^{Send} to give pASKInt100-RGD and pASKInt100-EETI-CK^{RGD}. These coding sequences were introduced in a similar manner into pMX-EETI (9) to give plasmids pMX-RGD and pMX-EETI-CK^{RGD}. The nucleotide sequences of all cloned genes were confirmed by nucleotide sequence analysis.

Construction of plasmid pREP4supE. To place the *supE* tRNA coding sequence under P_{Llac} promoter/operator control, a DNA segment containing the coding sequence for tRNA$^{Gln(CAA)}$, tRNA$^{Gln(CCT)}$, tRNA^{Met}, *supE* tRNA, and tRNA$^{Gln(AAT)}$ was amplified by PCR using chromosomal DNA of 71-18 as a template and the oligonucleotide pair SupE2-Eco-up and SupE2-Mlu-lo. The resulting PCR product was digested with *Eco*RI and *Mlu*I and ligated to the similarly digested vector pZA22-MCS1 (33). The resulting vector was digested with *Xho*I and *Xba*I to give a DNA fragment with the tRNA gene cluster under P_{Llac} promoter/operator control followed by a fill-in reaction using T4 DNA polymerase. The fragment was ligated to *Sma*I-digested vector pREP4 (Qiagen) to give pREP4supE.

Flow-cytometric analysis and FACS. For flow-cytometric analysis, cultures of *E. coli* strains containing the appropriate expression plasmid were grown overnight at 37°C and subcultured 1:50 at 37°C until they reached an optical density at 600 nm (OD_{600}) of 0.2. After induction with anhydrotetracycline (0.2 μg/ml) for 60 min, cells (200 to 500 μl) were pelleted by centrifugation in a tabletop centrifuge for 1 min and resuspended in 10 μl of phosphate-buffered saline (PBS). After the addition of 1 μl of the respective antibody (1 mg/ml), cells were incubated for 5 min at room temperature. After the addition of 500 μl of PBS, cells were centrifuged for 1 min and resuspended in 10 μl of PBS containing biotinylated goat anti-mouse immunoglobulin (1:10 dilution). After 5 min of incubation at room temperature and addition of 500 μl of PBS, cells were pelleted again and resuspended in 10 μl of PBS containing streptavidin–R-phycoerythrin conjugate (1:10 dilution) followed by 5 min of incubation at room temperature. Finally, after the addition of 500 μl of PBS, cells were pelleted and resuspended in 100 μl of PBS for flow cytometry analysis. A total of 300,000 events were collected on a Cytomation MoFlo cell sorter. Parameters were set as follows: forward scatter, side scatter, 730 (LIN mode, amplification factor 6); FL1 (fluorescein isothiocyanate), 600 (LOG mode); FL2 (phycoerythrin), 600 (LOG mode); trigger parameter, side scatter. The sample flow rate was adjusted to an event rate of approximately 30,000 s^{-1}.

Titration of *E. coli* cell surface-exposed antibody binding sites. A 100-μl aliquot of induced cells from DH5αZ1 containing pASKInt100-EETI-CK^{Send} was diluted with PBS to 3 ml. The total number of cells was determined by flow cytometry and found to be 4.5×10^7. In parallel, 100 μl of the induced culture were centrifuged, resuspended in 10 μl of PBS, and incubated with serial dilutions of anti-E antibody ranging from 1 ng (6.7 fmol) to 2 μg (13.3 pmol). Cells were incubated for 5 min at room temperature. After addition of 500 μl of PBS, cells were centrifuged for 1 min and successively incubated with an excess over the anti-E antibody of biotinylated goat anti-mouse immunoglobulin and with streptavidin–R-phycoerythrin conjugate, as described above. Cells were analyzed by flow cytometry analysis. A total of 300,000 events were collected on a Cytomation MoFlo cell sorter.

Trypsin treatment of intact cells and membrane preparation. Cultures of *E. coli* strains containing the respective expression plasmid were grown overnight at 37°C and subcultured 1:50 at 37°C until they reached an OD_{600} of 0.8. After induction with anhydrotetracycline (0.2 μg/ml) for 180 min, cells (50 ml) were pelleted by centrifugation and resuspended in PBS. The bacterial suspension was adjusted to an OD_{600} of 10 and incubated for 10 min at 37°C with trypsin at a final concentration of 50 μg/ml. To remove trypsin after the reaction, cells were centrifuged and washed with PBS. The membrane fraction was prepared as described previously (17), with minor modifications. The cells were lysed by passage through a French pressure cell at 1,000 lb/in^2. Remaining large bacterial fragments were sedimented by centrifugation at 5,000 × *g* for 10 min. After incubation of the lysate on ice in 100 mM Tris-HCl (pH 8.0)–10 mM EDTA–1% (wt/vol) Triton X-100 for 30 min, the membrane fraction was obtained by cen-

trifugation of the cleared solution for 120 min at 100,000 × *g* and 15°C. The membranes were solubilized in sample buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% [wt/vol] sodium dodecyl sulfate [SDS], 0.1% (wt/vol) bromophenol blue, 10% [vol/vol] glycerol) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide-bisacrylamide, 30:0.8) followed by immunoblotting.

Protein purification. The MalE-RGD fusion protein was affinity purified (34) using an amylose column (Pharmacia). MalE–EETI-CK^{RGD}, which contains six carboxy-terminal histidines, was purified by metal chelate affinity chromatography as described elsewhere (9). The protein concentration was determined from the A_{280} (38).

Determination of IC_{50}s for inhibition of platelet aggregation. Human platelets were obtained from the Göttingen University hospital. The platelet suspension was diluted to a concentration of approximately 250,000 U/μl and preincubated with various concentrations of MalE-RGD and MalE–EETI-CK^{RGD} proteins. Aggregation was stimulated by adding 25 μM ADP, and the change in OD_{650} was monitored by photometry. Values were expressed as percent aggregation, which represents the percentage of light transmission standardized to fully and not aggregated samples (8). The IC_{50} is the concentration of the protein at which the platelet aggregation is inhibited by 50%.

Enrichment of RGD peptide-displaying *E. coli* cells via binding to human platelets. SilanePrep slides (Sigma) were coated with glutaraldehyde (6.25% [vol/vol]) for 30 min. After extensive washing with water, they were immediately used for platelet capture. Platelets were resuspended in Tris-buffered saline (TBS; 100 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$ and sequestered to the glutaraldehyde-treated slides for 30 min. Remaining aldehyde groups were blocked with TBS containing 2% (wt/vol) bovine serum albumin. Slides were preincubated with untransformed 71-18 to saturate unspecific binding sites. Induced cells from a 50-ml culture of 71-18 containing pASKInt100-RGD or pASKInt100-EETI-CK^{RGD} were harvested by centrifugation and resuspended in 50 ml of TBS containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$.The bacterial cell suspension was transferred into a 50-ml plastic tube, and the slide was immersed in the cell suspension and agitated for 30 min at room temperature. The slides were carefully but thoroughly washed with TBS containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The bacteria were propagated by placing the slides into flasks containing rich medium with the appropriate antibiotic and overnight incubation at 37°C.

RESULTS

Targeting of intimin′ to the surfaces of *E. coli* K-12 cells. To determine whether the amino-terminal fragment of intimin was sufficient for outer membrane translocation of a heterologous passenger domain using *E. coli* K-12 as the expression host, a derivative of EETI-II was used as a reporter. This protein is a member of the cystine knot family of protease inhibitors and provides a stable framework for the display of conformationally constrained peptides of various length and sequence (9, 49). The variant used here (EETI-CK^{Send}) contains a 13-residue epitope sequence from Sendai virus L protein in place of the original inhibitor loop and can easily be detected using a monoclonal anti-Sendai virus antibody (11).

The coding sequence for intimin lacking the two carboxy-terminal domains (intimin′) which spans codons 1 to 659 of the *eaeA* gene from EHEC O157:H7 (*eaeA′*) was amplified by PCR and placed under the control of the tightly regulated *tetA* promoter/operator in the vector pASK21-EETI-CK^{Send} (9) to give pASKInt1-EETI-CK^{Send}. Finally, codon 35 (CAG) of the intimin′-encoding gene was replaced by an *amber* stop codon. The resulting expression vector, pASKInt100-EETI-CK^{Send}, carries a tripartite gene fusion which encodes (i) the truncated intimin, (ii) an epitope (E^{tag}) from human bone Gla protein which is specifically recognized by a monoclonal anti-E antibody (40), and (iii) the EETI-CK^{Send} cystine knot protein. Tight regulation of gene expression is achieved by the presence of the structural gene for the tetracycline repressor *tetR* on the same plasmid (Fig. 1B).

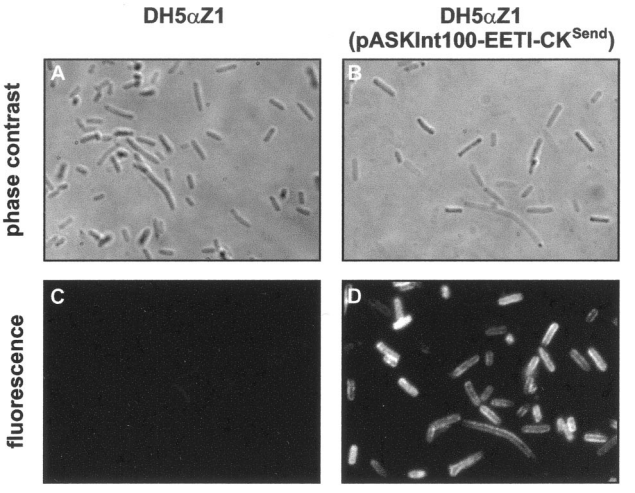

FIG. 2. Phase-contrast and fluorescence micrographs of the same field of DH5αZ1 and DH5αZ1(pASKInt100-EETI-CK^{Send}) after consecutive incubation of induced cells with anti-E antibody, biotinylated anti-mouse antibody, and streptavidin–R-phycoerythrin conjugate.

The *amber* suppressor host DH5αZ1 was used for expression of the intimin′–EETI-CK^{Send} fusion protein. The presence of the intimin′ fragment in the outer membrane of *E. coli* DH5αZ1 cells containing pASKInt100-EETI-CK^{Send} was confirmed by indirect immunofluorescence labeling of intact cells with anti-E antibody which recognizes an epitope sequence immediately adjacent to the intimin′ carboxy terminus. To this end, *E. coli* DH5αZ1 cells were grown at 37°C and induced at an OD_{600} of 0.2 with 0.2 μg of anhydrotetracycline per ml. After 60 min of induction, cells were washed and incubated with anti-E antibody. After washing, they were incubated with biotinylated anti-mouse antibody followed by labeling with streptavidin–R-phycoerythrin conjugate. As judged by fluorescence microscopy, all cells containing pASKInt100-EETI-CK-^{Send} were fluorescently labeled by this procedure (Fig. 2). No immunofluorescence staining was detected with untransformed DH5αZ1 control cells or with control cells containing a pASKInt100-EETI-CK^{Send} derivative lacking the E-epitope coding sequence (data not shown).

Influence of intimin′ expression on cell growth and survival. Induction of gene expression by addition of the inducer anhydrotetracycline to the growth medium of DH5αZ1(pASKInt1-EETI-CK^{Send}) resulted in an arrest of cell growth and a drastic reduction of cell viability (data not shown). Overproduction of outer membrane-linked proteins often results in changes in the structure of the outer membrane and, as a consequence, in periplasmic enzyme leakage and cell death (9, 16). To restore cell viability, net accumulation of fusion protein in the outer membrane was reduced to a tolerable level by introducing an *amber* codon at position 35 of the *eaeA′* sequence and utilizing the *amber* suppressor strain DH5αZ1 as the expression host. DH5αZ1 contains a glutaminyl *amber* suppressor tRNA, which by recognition of the TAG codon gives rise to translational readthrough. Due to the reduced efficiency of in vivo nonsense suppression compared to the translation of a CAG codon at the same position (36), yields of the encoded protein are diminished.

To assess the influence of reduced *eaeA′* expression on cell viability, the survival rate of induced DH5αZ1 cells harboring plasmid pASKInt100-EETI-CK^{Send} was compared to those of untransformed DH5αZ1. Cells were grown in rich medium to an OD_{600} of 0.2. Aliquots were withdrawn 1, 2, and 4 h after addition of the inducer anhydrotetracycline. Single cells were spotted with the single-cell deposition unit of the MoFlo cell sorter in 20-by-20 arrays on agar plates using a sorting gate that covered all fluorescence channels. Plates were incubated overnight at 37°C, and the number of colonies was counted in order to determine the percent surviving cells of DH5αZ1 (pASKInt100-EETI-CK^{Send}) compared to DH5αZ1 (Fig. 3A). The survival rate of *E. coli* cells producing the intimin′–EETI-CK^{Send} fusion protein was only slightly reduced after 1 h of induction compared to that of DH5αZ1. Even after 4 h of induction, the survival rate was found to be over 60% of that of untransformed DH5αZ1.

To determine whether intimin′-presenting *E. coli* cells show a significant growth disadvantage in liquid culture under conditions of continuous induction of intimin′ expression compared to control cells, a competitive growth experiment was performed. *E. coli* strain 71-18mutS was used as an expression host, since it contains a chromosomally located tetracycline resistance gene. This allowed the cells to grow in the presence of high concentrations of tetracycline, which ensured that sufficient amounts of the inducer were available during overnight growth to fully induce intimin′–EETI-CK^{Send} expression from the *tetA* promoter/operator. Overnight cultures of 71-18mutS harboring either pASK75cat as a control or pASKInt100-EETI-CK^{Send} grown in the presence of chloramphenicol were mixed at a ratio of 1:1. A 2-μl portion of the mixture was used to inoculate 50 ml of rich medium containing chloramphenicol and tetracycline. The resulting overnight culture was used as a starter culture to inoculate with 2 μl each of three flasks containing 50 ml of fresh medium supplemented with chloramphenicol and tetracycline. After overnight growth, 2 μl of each culture was used to inoculate 50 ml of fresh medium. This procedure was repeated eight times. Finally, aliquots were withdrawn from the overnight cultures and cells were labeled with anti-E antibody followed by flow cytometry analysis (Fig. 3B). After cultivation of the mixture for over 100 generations, the fraction of intimin′–EETI-CK^{Send}-presenting cells dropped from 40% to approximately 5%, which indicates that control cells display only a slight growth advantage over intimin′-producing cells.

Regulation of cellular net accumulation of the intimin′–EETI-CK^{Send} fusion protein. In order to obtain an estimate of the net accumulation of the intimin′–EETI-CK^{Send} fusion protein in the outer membrane of a single bacterial cell, the number of anti-E antibody molecules required to obtain saturation of the immunofluorescent staining of 4.5×10^7 induced bacterial cells was determined. Saturation of antibody binding was reached at approximately 0.4 μg (2.67 pmol) of anti-E antibody, which corresponds to approximately 36,000 molecules per bacterial cell (Fig. 4).

Previous attempts to regulate the net accumulation of an Lpp-OmpA fusion protein in the *E. coli* outer membrane which is expressed under *tetA* promoter control by varying the inducer concentration failed (10; A. Wentzel, unpublished observations). Cells were found to be either uninduced or fully induced, and only the fraction of fully induced cells increased with increasing concentrations of the inducer anhydrotetracycline. Since transcriptional regulation of gene expression of outer membrane fusions failed, we investigated whether the net accumulation can be adjusted on the basis of translational regulation, i.e., by varying the amount of suppressor tRNA that is necessary for translational readthrough at the *amber* codon at position 35 of the *eaeA* gene (Fig. 5A). pREP4supE contains

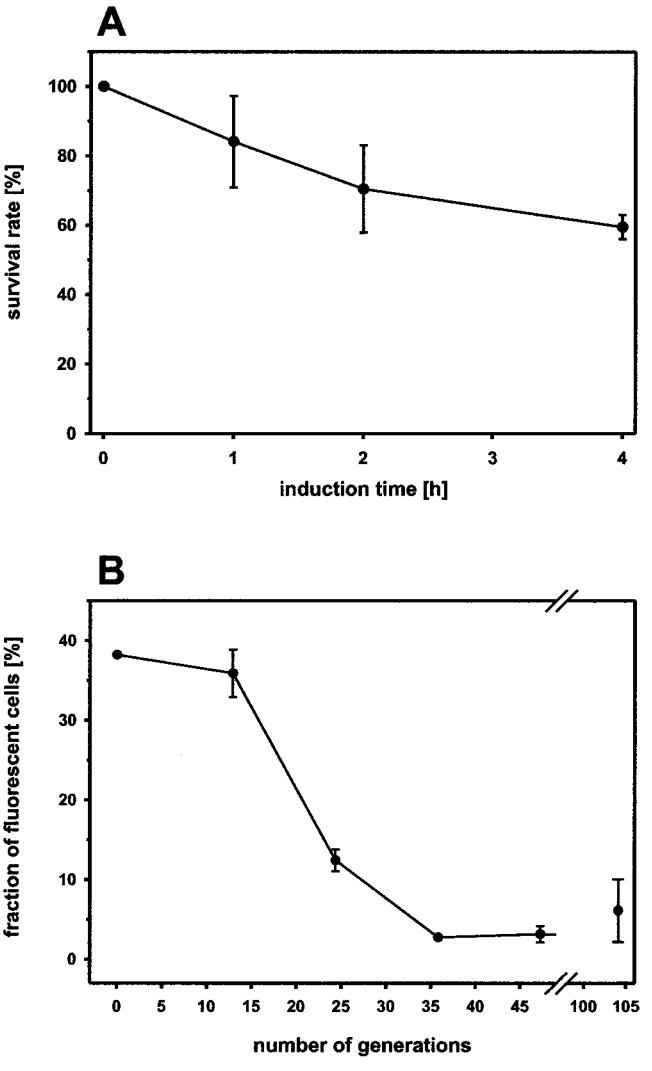

FIG. 3. (A) Survival of DH5αZ1 harboring pASKInt100-EETI-CK^{Send} after induction of intimin′–EETI-CK^{Send} production by addition of anhydrotetracyline (time zero). At each data point, single cells were spotted with a MoFlo cell sorter onto agar plates. The number of colonies after overnight incubation at 37°C was compared with the number of untransformed DH5αZ1 colonies, which was set at 100%. Values are means and standard deviations from three independent experiments. (B) Growth competition of induced 71-18mutS harboring pASKInt100-EETI-CK^{Send} compared to 71-18mutS harboring pASK75cat. A 1:1 mixture of both plasmid-containing cells was propagated in the presence of tetracycline for the number of generations indicated. The proportion of intimin′-displaying *E. coli* cells was determined by fluorescent labeling of culture aliquots with anti-E antibody. Values are means and standard deviations from three independent experiments.

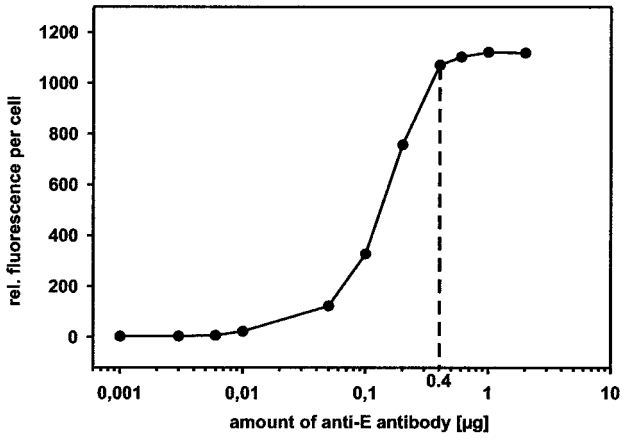

FIG. 4. Titration of anti-E antibody binding to induced cells. Induced cells of DH5αZ1 containing pASKInt100-EETI-CK^{Send} were incubated with increasing amounts of anti-E antibody and immunofluorescently labeled with excess biotinylated anti-mouse antibody and streptavidin–R-phycoerythrin conjugate. Saturation of mean cellular fluorescence is reached at approximately 0.4 μg of anti-E antibody.

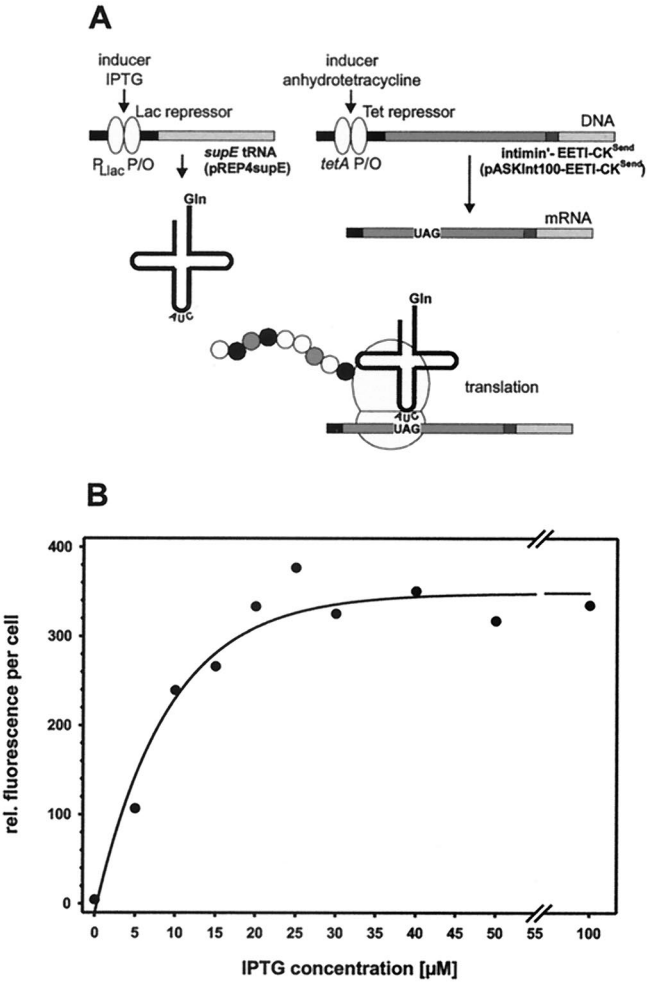

FIG. 5. Regulation of intimin′ outer membrane accumulation. (A) Schematic outline of IPTG-induced synthesis of *supE* tRNA that acts as an *amber* suppressor tRNA at codon 35 (UAG) of the *eaeA′* transcript. The *eaeA′* mRNA is synthesized upon induction of transcription of the *eaeA′* gene, which resides in vector pASKInt100 under *tetA* promoter/operator control. (B) Dose-response curve depicting the mean relative cellular fluorescence of intimin′–E^{tag}–EETI-CK^{Send}-displaying cells labeled with anti-E antibody as a function of IPTG concentration.

the coding sequence for glutaminyl *amber* suppressor tRNA under P_{Llac} promoter/operator control. This plasmid contains a p15A replication origin and a kanamycin resistance marker and is therefore compatible with derivatives of pASKInt100. Cells of the nonsuppressor strain WK6 containing plasmids pREP4supE and pASKInt100-EETI-CK^{Send} were grown overnight in selective media at 37°C in the presence of various concentrations of IPTG (isopropyl-β-D-galactopyranoside) to induce the transcription of the *amber* suppressor tRNA. Cells from these cultures were subcultured at 37°C in rich media containing the appropriate IPTG concentration. At an OD_{600} of 0.2, transcription of the intimin′ fusion gene from the *tetA* promoter was fully induced by addition of anhydrotetracycline at a final concentration of 0.2 μg/ml. After 60 min of induction, cells were immunofluorescently labeled with anti-E antibody and analyzed by flow cytometry (Fig. 5B). The average cellular fluorescence per cell was found to increase with the IPTG concentration and reached a maximum value at about 20 μM IPTG. Similar dose-response curves have been observed for the IPTG modulation of transcriptional regulation of gene expression under P_{Llac} promoter/operator control (33). The maximum of mean cellular fluorescence, approximately 350 relative fluorescence units, was approximately twofold lower than the one obtained from constitutive *supE* transcription in an *amber* suppressor strain. Most likely, the reduced net accumulation of *supE* tRNA which results from the lower rate of transcription initiation from the P_{Llac} promoter compared to the very strong rRNA promoter accounts for that difference.

Probing the surface display of various passenger domains. Besides the 35-aa cystine knot protein EETI-CK^{Send}, two other protein domains were chosen as passengers to probe surface display via intimin′ fusion, namely, human IL-4, a 128-aa four-helix-bundle protein, and REI_v, a 108-aa immunoglobulin variable light chain domain. The corresponding genes were introduced into pASKInt100-EETI-CK^{Send} by replacement of the EETI-CK^{Send} coding sequence. The 13-residue Sendai virus epitope tag was placed at the carboxy terminus of both domains. Intact cells containing pASKInt100-EETI-CK^{Send}, pASKInt100-IL-4, or pASKInt100-REI were labeled by indirect immunofluorescence with anti-E antibody or anti-Sendai virus antibody. The fluorescently labeled cells were analyzed with a MoFlo flow cytometer (Fig. 6A to C). All three constructs with different passenger domains could be fluorescently labeled with both antibodies, corroborating the cell surface exposure of the epitope sequences flanking the various passenger domains.

Protease treatment of intact cells is a suitable tool for probing the cell surface exposure of a heterologous passenger domain (12, 22). *E. coli* DH5αZ1 cells containing pASKInt100-EETI-CK^{Send}, pASKInt100-IL-4, or pASKInt100-REI were grown at 37°C and induced at an OD_{600} of 0.8 with 0.2 μg of anhydrotetracycline per ml. After 180 min of induction the

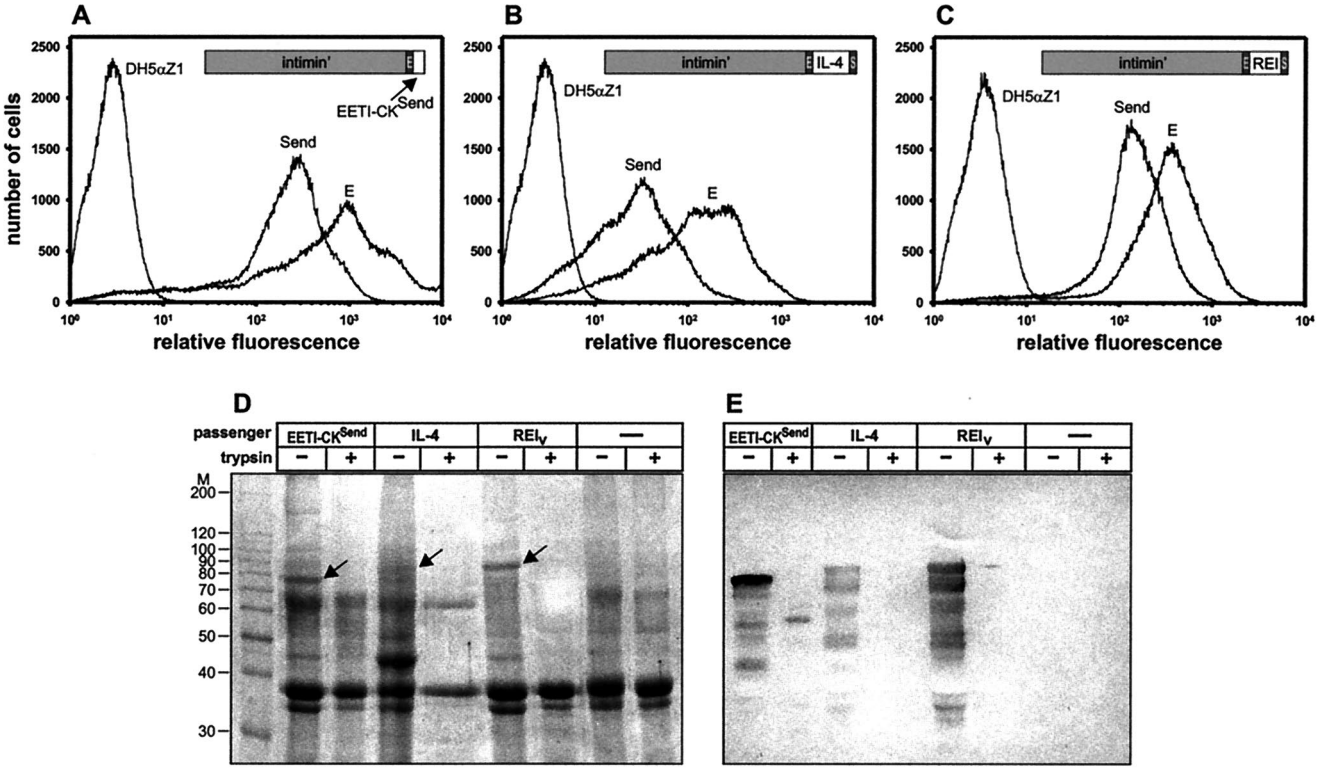

FIG. 6. Intimin′-mediated cell surface display of various passenger domains. (A to C) FACS histograms of recombinant DH5αZ1 harboring pASKInt100-EETI-CK^{Send} (A), pASKInt100-IL-4 (B), or pASKInt100-REI (C). Cells were successively incubated with anti-Sendai virus antibody (Send) or anti-E antibody (E), biotinylated anti-mouse antibody, and streptavidin–R-phycoerythrin conjugate. Untransformed DH5αZ1 labeled with anti-E antibody served as a control. (D and E) SDS-PAGE (D) and Western blot (E) analysis of surface targeting and protease accessibility of intimin′–EETI-CK^{Send}, intimin′–IL-4, and intimin′-REI$_v$ fusion proteins. Membrane preparations of *E. coli* DH5αZ1 expressing the respective passenger domains fused to intimin′ and of control cells without a plasmid were analyzed by SDS-PAGE (10% gel, Coomassie brilliant blue stain) and Western blots probed with anti-Sendai virus antibody. To verify the surface location of the various fusion proteins, bacteria were either subjected to trypsin treatment or left untreated. The intimin′ fusion proteins are indicated by arrows. M, marker proteins (sizes are in thousands).

outer membrane fraction was prepared from trypsin-treated and untreated cells and analyzed by SDS-PAGE (Fig. 6D) and Western blotting (Fig. 6E). A band migrating at the expected apparent molecular weight for the fusion protein was detected in the outer membrane fraction of induced plasmid-containing DH5αZ1 cells but was absent after trypsin treatment of intact cells, confirming the presence of the various intimin′ fusion proteins in the *E. coli* outer membrane with the respective passenger domains exposed at the cell surface. No unspecific staining was observed with untransformed DH5αZ1 cells. In addition to the full-length fusion proteins, numerous shortened fragments that were also susceptible to trypsin cleavage of intact cells were detected. IL-4 accumulated on the *E. coli* cell surfaces in smaller amounts than EETI-CK^{Send} and REI$_v$, which corresponds to the FACS analysis of IL-4-displaying cells labeled with C-terminal specific anti-Sendai virus antibody, where the maximum of cellular fluorescence was about 1 order of magnitude lower than those of intimin′–EETI-CK^{Send} and intimin′-REI$_v$.

Mimicking adhesin-host interaction by *E. coli* surface display of intimin′-RGD peptide fusions. The extracellular portion of intimin comprises a rod of immunoglobulin domains extending from the bacterial surface where the carboxy-terminal domain mediates the intimate attachment of enteropathogenic and enterohemorrhagic *E. coli* to the translocated intimin receptor on the mammalian host cells. For a number of applications, particularly for the study of receptor-ligand interactions, it is interesting to see whether a heterologous ligand fused to the amino-terminal intimin fragment is able to promote bacterial adhesion via binding to a corresponding receptor on the surface of mammalian cells.

To address this question, a simple model experiment was designed relying on the binding of fibrinogen to the $\alpha_{IIb}\beta_{III}$ integrin on the surfaces of human platelets. Many integrins, which are heterodimeric proteins with two membrane-spanning subunits, recognize short arginine-glycine-aspartic acid (RGD)-containing amino acid sequences (41). An RGD sequence resides in the cell attachment site of fibrinogen (44), and integrin binding peptides containing an RGD sequence have been identified from random peptide libraries displayed on phage (25) and from chemically synthesized libraries (8) by panning on purified fibrinogen receptors. To investigate whether an RGD-containing peptide displayed on the *E. coli* cell surface via fusion to intimin′ can promote binding of the bacteria to the fibrinogen receptor residing on platelets, two versions of intimin′-RGD peptide fusions were constructed (Fig. 7A). A synthetic gene fragment coding for the peptide sequence CIP**RGD**YRC (8) was fused in vector pASKInt100 to

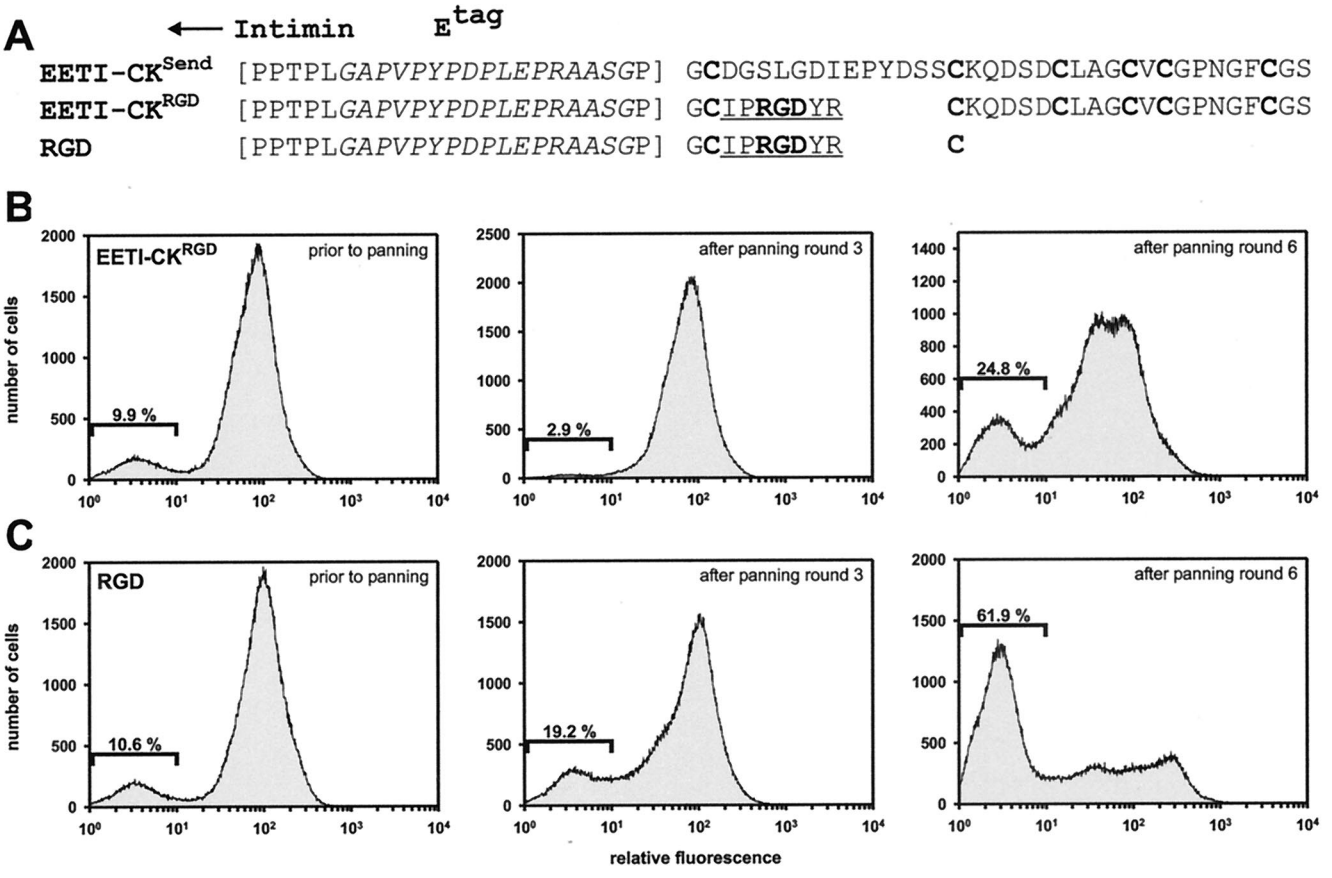

FIG. 7. (A) Amino acid sequences of EETI-CK^{Send}- and RGD-containing peptides. The sequence in brackets corresponds to a short linker region (PPTPL) and the E^{tag} epitope (italic) fused to intimin′. Cysteine residues that are involved in disulfide bond formation are in bold. The amino acid sequence of the RGD-containing peptide is underlined. (B) FACS histograms from enrichment of 71-18(pASKInt100-EETI-CK^{RGD}) from a 1:1,000 mixture with 71-18(pASKInt100-EETI-CK^{Send}). The mixture was labeled with anti-Sendai virus antibody and analyzed by FACS before and after three and six rounds of enrichment of 71-18(pASKInt100-EETI-CK^{RGD}) by panning on immobilized platelets. The bar in each graph represents the sorting gate defined as a negative event, i.e., bacteria which do not display the EETI-CK^{Send} epitope on their surfaces. The percentage of cells counted as negative events is indicated. (C) FACS histograms from enrichment of 71-18(pASKInt100-RGD) from a 1:1,000 mixture with 71-18(pASKInt100-EETI-CK^{Send}).

the *eaeA′* gene fragment. The same peptide sequence was introduced into the intimin′–EETI-II fusion at the position of the trypsin inhibitor loop of the EETI-II microprotein. Both peptides were also produced as soluble proteins via fusion to maltose binding protein (9), purified, and examined for their ability to specifically bind the fibrinogen receptor. RGD-containing peptides bind to the fibrinogen binding site of the receptor (39), thereby inhibiting platelet aggregation. The soluble proteins were used to inhibit platelet aggregation. IC_{50}s were 0.6 ± 0.2 and 2.1 ± 0.4 μM for MalE-RGD and MalE–EETI-CK^{RGD}, respectively, which are close to the IC_{50} of 0.33 μM obtained for the cyclic CIP**RGD**YRC (8). No inhibition of platelet aggregation was observed with MalE–EETI-II (data not shown).

To evaluate the feasibility of enrichment of RGD-presenting *E. coli* cells against a background of control cells, we devised a model experiment by mixing cells presenting the RGD peptide CIP**RGD**YRC or EETI-CK^{RGD} with EETI-CK^{Send}-presenting control cells at a ratio of 1:1,000. The mixture was used for panning on platelets that were immobilized on glass slides. The slides were immersed into the cell suspension, unbound cells were removed by careful and thorough washing, and the bound cells were propagated by placing the slide into a flask containing rich medium. The procedure was repeated over five additional rounds. After each round the fraction of the EETI-CK^{Send}-presenting control cells in the mixture was determined by labeling an aliquot with the monoclonal anti-Sendai virus antibody followed by flow cytometry analysis. After six rounds of panning, the fraction of unlabeled cells reached 24.8% for the mixture displaying the EETI-CK^{RGD} protein and 61.9% for the mixture displaying the CIP**RGD**YRC peptide (Fig. 7B and C). Four individual cultures derived from single cells of the last panning round from each experiment that were unable to bind the anti-Sendai virus antibody were probed for the presence of the RGD peptide coding sequence by PCR amplification and restriction fragment analysis. Of these, three of four clones in the CIP**RGD**YRC display experiment and all four in the EETI-CK^{RGD} display experiment contained the respective RGD peptide coding sequence, corroborating the enrichment of RGD peptide-displaying cells (data not shown).

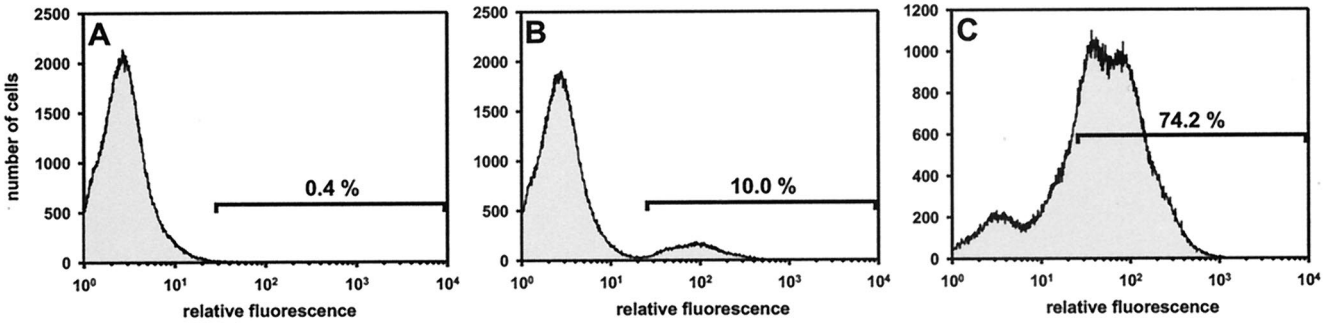

FIG. 8. Histogram data from enrichment of 71-18 containing pASKInt100-REI. The bar in each graph represents the sorting gate defined as a positive event. The percentage of cells counted as positive events is indicated. (A) A 1:200,000 mixture of 71-18(pASKInt100-REI) and 71-18(pASKInt100′-REIΔSend) prior to the first sorting round; (B) mixture after two consecutive runs of FACS and overnight cell growth; (C) mixture from round B after re-sorting and overnight cell growth.

Enrichment of cells displaying a Sendai virus epitope. To explore whether cells presenting a particular passenger protein can be enriched by FACS from a background of negative cells, the Sendai virus epitope fused to the carboxy terminus of the intimin′-REI_v fusion protein was used as an immunotag for fusion protein detection and FACS. 71-18 cells containing pASKInt100-REI were mixed at a ratio of 1:200,000 with cells containing pASKInt100-REIΔSend, which lack the Sendai virus epitope. Cells were subjected to labeling with anti-Sendai virus antibody, followed by biotinylated anti-mouse antibody and streptavidin–R-phycoerythrin conjugate. The cell population was run through the MoFlo cell sorter at an event rate of 30,000 s^{-1} and sorted on the basis of fluorescence intensity (Fig. 8). A sorting gate was chosen such that less than 0.5% of the control cells fell within the positive window. After immediate re-sorting, the collected cells were plated onto agar plates, grown overnight, labeled again, and re-sorted. After two consecutive sorting rounds, 10% of the total cell population were found in the positive fraction (Fig. 8B), and after an additional re-sorting, 74% of the cell population were stained with anti-Sendai virus antibody.

DISCUSSION

In this report we describe the export of passenger peptides and proteins to the surfaces of *E. coli* cells by fusion to intimin′, a truncated *E. coli* adhesin. To address the question of whether surface display via fusion to intimin′ provides a means of exposing various passenger domains in high copy numbers on the outer surfaces of *E. coli* cells, three different passenger proteins were probed for cell surface display. A derivative of the *Ecballium elaterium* trypsin inhibitor, the Bence-Jones protein REI_v, and human IL-4 were efficiently targeted to the surface of *E. coli* 71-18(pASKInt100) cells. This was demonstrated by successful immunofluorescence labeling using a monoclonal antibody that recognizes a carboxy-terminal epitope and by the accessibility of the fusion proteins residing on intact cells to exogenously added trypsin.

The *Ecballium elaterium* trypsin inhibitor was chosen as a model passenger domain, since it was shown to be successfully translocated through the outer membrane when fused to Lpp-OmpA or the C-terminal domain of the *N. gonorrhoeae* IgA protease precursor protein IgAβ (9, 49). EETI-II is a 28-residue peptide which is stabilized by three intramolecular disulfide bonds. The immunoglobulin variable light chain domain REI_v consists of 102 residues and is also mainly stabilized by a single intramolecular disulfide bond (14). It was shown previously that cell surface exposure of CtxB, a subunit of cholera toxin which contains a single intramolecular disulfide bond, was influenced by the conformational state of the polypeptide chain. Transport of CtxB via fusion to the IgAβ autotransporter across the outer membrane was blocked by intramolecular disulfide bonds and appeared to proceed only under reducing conditions (22). Protein translocation through IgAβ is thought to occur via passage of the unfolded linear polypeptide chain of the passenger through a hydrophilic pore in the center of the β-barrel formed by the outer membrane-anchored IgAβ domain (21). The mechanism by which the carboxy-terminal extracellular domains of the bacterial adhesin intimin reach the external surface is currently unknown. The finding that EETI-CK^{Send} (35 aa), containing three disulfide bonds, IL-4 (128 aa), containing two disulfide bonds, and REI_v (108 aa), containing one disulfide bond, are all efficiently translocated to the *E. coli* cell surface under oxidizing conditions does not necessarily imply that they are able to pass the outer membrane in a partially or completely folded state. Initial experiments to address this question indicate that passenger proteins are able to fold into their native structure on the surfaces of *E. coli* cells and that at least for some passengers periplasmic protein folding prevents efficient bacterial surface display (Wentzel, unpublished). Hence, it may be interesting to study whether the outer membrane translocation of passenger proteins depends on the velocity of disulfide bond formation and overall protein folding.

Surface display of recombinant proteins on bacteria and yeast is a promising tool for the analysis of macromolecular interactions. One major advantage over phage display lies in the ability to use FACS for high-throughput screening of polypeptide libraries. FACS screening of a library of single-chain F_v antibody fragments displayed on *Saccharomyces cerevisiae* allowed the isolation of variants with femtomolar antigen binding affinity, the highest monovalent ligand binding affinity yet reported for a monovalent protein (4). Besides yeast, *E. coli* is the preferred organism for the display of large populations of variant polypeptides, and numerous procedures have been developed to direct peptides or proteins to and

anchor them on the *E. coli* cell surface (for a review, see reference 16). Several requirements have to be fulfilled by a favorable display system. First, a maximum number of polypeptide molecules per cell should ideally be presented. It can be estimated that at least 10,000 fluorescent ligand molecules bound to the surface of the cell are required to achieve a sufficient fluorescence signal for FACS (5). By indirect staining using a biotinylated second antibody and streptavidin–R-phycoerythrin conjugate, which contains approximately 30 chromophores per molecule, substantial signal enhancement can be achieved, allowing detection of fewer than 1,000 antigenic sites per cell (Fig. 4). However, since the fluorescence signal-to-noise ratio of cells expressing high-affinity polypeptides to nonexpressing cells is a critical parameter for a successful FACS selection, it is desirable to reach a considerably higher level of expression. Second, expression of surface-exposed polypeptides should occur without detrimental effects on the outer membrane and without compromising cell viability. Third, the surface-displayed polypeptides should be remote from the outer membrane and the lipopolysaccharide layer in order to be freely accessible to the respective interaction partner.

These requirements are for the most part met by the intimin′-based surface display of passenger domains. We found by immunofluorescence titration with various amounts of the anti-E antibody, which recognizes an extracellular epitope at the junction of the intimin′ fragment and the carboxy-terminal domain, that approximately 35,000 intimin′ molecules are displayed per bacterial cell. This number is a minimal approximation, since it was calculated under the assumption of a 1:1 binding of antibody and intimin′ molecule. Since one antibody molecule contains two antigen binding sites and may therefore be capable of binding two intimin′ molecules, the actual number may even be higher. However, as can be seen from Western blot analysis of membrane preparations (Fig. 6), only small amounts of full-length intimin′-passenger protein fusions are found together with numerous shortened products, which are distributed over a broad size range. Since the epitope tag which is recognized by the anti-Sendai virus antibody is located at the carboxy-terminal end of the intimin′-passenger protein fusion, the observed shortened versions are likely the result of a proteolytic attack of the amino-terminal periplasmatic part and/or of the transmembrane regions connecting loops of intimin′. Despite their amino-terminal truncation, these shortened versions of the fusion protein remain anchored in the bacterial outer membrane and are capable of displaying the foreign passenger domain. Significant differences in the level of net accumulation are seen with the three passenger domains tested, where IL-4 surface accumulation is greatly reduced compared to that of the EETI-CK^{Send} and the REI_v passenger domain. Whether these differences are the consequences of reduced gene expression, of enhanced proteolysis, or of hampered passenger protein translocation through the cytoplasmic or outer membrane is currently under investigation.

For maintaining library diversity of passenger domain variants exposed on the cell surface by intimin′ fusion, it is necessary to prevent growth competition between different library members. Moreover, in cases where synthesis of the surface-exposed protein results in reduced growth rates and cell viability, randomly occurring clones, where the synthesis of the fusion protein is impeded, can overgrow the culture in only a few generations (10). To avoid these problems, the outer membrane net accumulation of intimin′-passenger fusion proteins was adjusted to a tolerable level by reducing the efficiency of intimin′ translation by introducing an *amber* codon in the *eaeA′* gene and using an *amber* suppressor strain as the expression host. Under conditions of fully induced intimin′ passenger gene transcription for more than 100 generations, the proportion of fusion protein-producing cells in a 1:1 mixture of producing and nonproducing cells decreased approximately 10-fold, which indicates that cell viability and growth rate were only slightly compromised by permanent overexpression of the fusion protein. This finding contrasts with surface display based on Lpp-OmpA, where display of approximately 30,000 molecules per cell resulted in growth inhibition and strongly reduced culture viability (10; Wentzel, unpublished).

Daugherty and coworkers have used the *tetA* and *araBAD* promoter to control the expression of an Lpp-OmpA passenger protein (10). In an attempt to regulate gene expression by addition of submaximal amounts of inducer to the growth medium, they found heterogeneous distribution of cellular expression levels. Variation of inducer concentration yielded mixed populations of uninduced and fully induced cells. Homogenous expression levels could be obtained only under conditions where the length of induction period was varied using saturating concentrations of inducer. With the *araBAD* promoter, induction times of several hours are required to reach intermediate levels of expression. Expression of Lpp-OmpA fusion proteins and intimin′ fusion proteins under *tetA* promoter control reached a maximum level after only approximately 45 min of induction (A. Christmann, unpublished results). In our hands, adjustment of gene expression levels by varying the induction time was hard to control, since minor differences in growth conditions resulted in larger experimental variations of fusion protein net accumulation. The problem could be overcome by controlling the expression of passenger proteins fused to truncated intimin at the level of translation instead of transcription. To achieve this, a glutaminyl suppressor tRNA gene was placed under P_{Llac} promoter/operator control on a helper plasmid, which also contained *lacI*, the gene encoding the Lac repressor. By varying the concentration of the inducer IPTG at saturating concentrations of tetracycline and keeping the induction duration constant, homogenous expression levels in uninduced and fully induced cells were reached by adjustment of suppressor tRNA net accumulation and efficiency of translational readthrough at the *amber* codon residing in the *eaeA′* gene. At least for regulation of gene expression by induction of transcription from the *ara* or *tetA* promoter, growth at various concentrations of inducer is not well suited to modulation of gene expression as long as expression levels reflect the proportion of cells that are fully induced rather than intermediate expression in any individual cell (10, 45, 46). Translational regulation by induction of suppressor tRNA synthesis allows us to overcome this problem and ensures a very tight repression of gene expression by dual control of target protein synthesis at the levels of both transcription and translation.

In this study we replaced the two carboxy-terminal domains of intimin which mediate the adhesion of enteropathogenic and enterohemorrhagic *E. coli* to target epithelia by various passenger proteins. By constructing a fusion protein of trun-

cated intimin with a conformationally constrained integrin binding RGD peptide, the bacterial adhesin could be newly functionalized towards an adhesin that mediates binding of *E. coli* cells to human platelets. This was confirmed by biopanning a 1:1,000 mixture of RGD peptide-presenting cells and control cells on immobilized platelets, which resulted in enrichment of RGD peptide-displaying bacteria. However, substantial unspecific binding of control cells to the coated platelets hampered the efficient enrichment of RGD peptide-presenting cells, and six rounds of panning were required for 1,000-fold enrichment. Nevertheless, this finding confirms that the heterologous passenger domain which is fused to the shortened intimin fragment is sufficiently remote from the bacterial cell surface and lipopolysaccharide layer to allow interaction with a receptor protein residing on eukaryotic cells, which are not natural targets for *E. coli* cell adhesion. Only three rounds of sorting were required to achieve an enrichment of cells displaying the Sendai epitope fused to the intimin′-REI_v fusion protein from a 1:200,000 to an approximately 1:1 ratio. With modern cell sorters, event rates of up to 100,000 s^{-1} can be applied, which opens up the possibility of screening large molecular repertoires with over 10^9 initial bacterial cells displaying different peptide or protein variants fused to intimin′ for binders to a particular target molecule in a short time.

In conclusion, carboxy-terminal truncated intimin expressed from the newly constructed pASKInt100 display vector directs fused polypeptides to the extracellular surfaces of *E. coli* cells in high copy numbers without detrimental effects on the integrity of the cell envelope. It may be useful for various applications, including combinatorial library screening, study of ligand-receptor interaction, and the production of live vaccines and cellular adsorbents.

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