Intimin-Mediated Export of Passenger Proteins Requires Maintenance of a Translocation-Competent Conformation

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Intimins from pathogenic bacteria promote intimate bacterial adhesion to epithelial cells. Several structurally similar domains form on the bacterial cell surface an extended rigid rod that exposes the carboxy-terminal domain, which interacts with the translocated intimin receptor. We constructed a series of intimin-derived fusion proteins consisting of carboxy-terminally truncated intimin and the immunoglobulin light-chain variable domain RELv, ubiquitin, calmodulin, β-lactamase inhibitor protein, or β-lactamase. By systematically investigating the intimin-mediated cell surface exposure of these passenger domains in the presence or absence of compounds that interfere with outer membrane stability or passenger domain folding, we acquired experimental evidence that intimin-mediated protein export across the outer membrane requires, prior to export, the maintenance of a translocation-competent conformation that may be distinct from the final protein structure. We propose that, during export, competition exists between productive translocation and folding of the passenger domain in the periplasm into a stable conformation that is not compatible with translocation through the bacterial outer membrane. These results may expand understanding of the mechanism by which intimins are inserted into the outer membrane and expose extracellular domains on the cell surface.

The dual membrane envelopes of gram-negative bacteria provide two barriers of unlike nature that pose formidable problems concerning the transport of molecules into and out of these organisms. Nutrients and essential cofactors must be actively transported into the cells, and end products of metabolism, toxic molecules, and proteins need to be extruded. While gram-positive bacteria, eukaryotes, and archaea exhibit just three known secretory systems for protein transport across the cytoplasmic and endoplasmic reticulum membranes (7, 37, 45), gram-negative bacteria have evolved multiple systems for protein transport across the whole-cell envelope; the proteins may remain attached to the surface or be released into the extracellular milieu (8, 25, 40, 50). They serve as, for example, substrate-degrading enzymes, adhesion anchors, or pathogenicity factors that interfere with host metabolism or immune defense.

Most machineries for translocating proteins across gram-negative bacterial membranes are composed of numerous proteins that form heterooligomeric structures, which mediate the simultaneous export of a passenger protein across both membranes (25). Two exceptions are known: the type V secretion pathway (19) and the autodisplay of intimins and invasins (34). In these cases, all of the necessary elements for translocation across the outer membrane are located within their own polypeptide sequences. Members of the family of type V secreted virulence factors comprise three functional domains in a single autoexport protein: an N-terminal targeting sequence, a C-terminal translocation domain, and the passenger domain in between. The C-terminal domain is supposed to form in the outer membrane a β-barrel structure that mediates the translocation of the fused passenger domain, which may eventually be released into the extracellular medium upon proteolytic cleavage (19). Members of this autotransporter family include virulence factors of human pathogens, such as the immunoglobulin A (IgA) β protease from Neisseria spp. (35), the AIDA-I adhesin from pathogenic Escherichia coli (5), and the cytotoxin VacA from Helicobacter pylori (10).

The second, unrelated family of outer membrane proteins that expose passenger domains on the bacterial outer surface are the intimins and invasins, nonfimbrial adhesins from pathogenic bacteria, which specifically interact with host cell surface receptors and mediate bacterial attachment or invasion. They are integrated into the bacterial outer membrane with the amino-terminal region, while the carboxy-terminal region of the polypeptide is surface exposed (4, 18). Invasins bind to high-affinity members of the β1 family of integrins and mediate bacterial entry into eukaryotic cells (21). Intimins are surface proteins of enteropathogenic E. coli and enterohemorrhagic E. coli (EHEC) that promote the intimate bacterial adhesion associated with attaching and effacing lesion formation (1). Both intimins and invasins expose on the bacterial cell surface structurally similar domains that form an extended rigid rod made up of domains resembling eukaryotic members of the immunoglobulin superfamily. The carboxy-terminal domain has a folding topology related to that of C-type lectin-like domains capable of binding to a eukaryotic cell surface receptor (4). The transmembrane regions of all outer membrane proteins whose structures are known are β barrels. In accordance with these data, Touze et al. recently showed by circular dichroism spectroscopy that the transmembrane region of intimin is also composed largely of β strands (44). The architecture of intimin, which has been shown to form...
dimers (44), may resemble the model shown in Fig. 1A, where the protein monomer is composed of a periplasmic region, a β-barrel membrane anchor, and a cell-binding region that projects away from the bacterial surface and is positioned to contact the translocated intimin receptor located on the host cell surface (30).

While the extracellular transport of passenger domains through the barriers of the inner and outer membranes via the...
For the growth and maintenance of Bacteria were grown at 37°C, except where otherwise noted. The medium used was M9 medium (17) supplemented with the following final concentrations of antibiotics: spectinomycin, 50 μg/ml; ampicillin, 50 μg/ml; and gentamicin, 25 μg/ml. Other drugs were used at the following final concentrations: tetracycline, 10 μg/ml; and chloramphenicol, 25 μg/ml.

**MATERIALS AND METHODS**

### E. coli strains

The *E. coli* strains used in this study are listed in Table 1. Bacteria were grown at 37°C, except where otherwise noted. The medium used for the growth and maintenance of *E. coli* strains was M9 medium (17) supplemented with the following final concentrations of antibiotics: spectinomycin, 50 μg/ml; ampicillin, 50 μg/ml; and gentamicin, 25 μg/ml. Other drugs were used at the following final concentrations: tetracycline, 10 μg/ml; and chloramphenicol, 25 μg/ml. **Reagents.** Restriction enzymes and DNA-modifying enzymes were purchased from MBI Fermentas (Vilnius, Lithuania) or New England Biolabs (Beverly, Mass.). Tj polymerase was obtained from Promega (Madison, Wis.), and biotinylated goat anti-mouse antibody was obtained from Sigma (St. Louis, Mo.). A monoclonal antibody (VII-E-7) against a 13-residue C-terminal epitope (Sendt) encoding gene sequence was amplified by PCR with oligonucleotide primers BLIP-containg vector (47), as a template and with primers BLIP-up and BLIP-low. The resulting PCR product was digested with SmaI and BglII and used as a template for the construction of a C-terminally truncated intimin–calmodulin fusion protein in the bacterial outer membrane and allows the translocation of four C-terminally attached passenger domains across the bacterial cell envelope. Here we describe the construction of fusion proteins consisting of carboxy-terminally truncated intimin and passenger polypeptide.

### Plasmids

- **pASKInt100**: Vector for expression of Eae A’ fusion proteins under tetA promoter–kan-1 operator control; contains a chloramphenicol resistance marker, tetR gene
- **pASKInt100-REI**: Vector for expression of REL; derivative of pASKInt100
- **pASKInt100-BLIP**: Vector for expression of β-lactamase inhibitor protein from Streptomyces clavuligerus; derivative of pASKInt100
- **pASKInt100-Cal**: Vector for expression of human calmodulin; derivative of pASKInt100
- **pASKInt100-Ubi**: Vector for expression of ubiquitin; derivative of pASKInt100
- **pASKInt100-Bla**: Vector for expression of TEM-1 β-lactamase; derivative of pASKInt100
- **pBRR22β-Bla**: Vector for expression of soluble wild-type TEM-1 β-lactamase with Sendt epitope and His tag
- **pBRR22β-C89Y**: Vector for expression of soluble C89Y TEM-1 β-lactamase with Sendt epitope and His tag

**DNA procedures.** Standard DNA procedures, such as plasmid isolation, ligation, and restriction analysis and isolation of DNA fragments, were carried out as described previously (39). PCR with Tj polymerase was carried out 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.

**Construction of plasmids.** pASKInt100-AP was constructed by cleavage of pASKInt100-IL-4 (47) with Smal and BglII, followed by religation of the vector after filling in of DNA ends with T4 DNA polymerase. To obtain vector pASKInt100-Bla, the gene for RTEM-1 was amplified with PCR with oligonucleotides BlaSmaUp and Bla-Bgl12dwn and with pHK5-20 as the template DNA. The PCR product was digested with SmaI and BglII and ligated to similarly digested pASKInt100-EETI-CKSend (47). For the construction of a C-terminally truncated intimin–calmodulin fusion vector (pASKInt100-Cal), pASKInt100-TmDegP was digested with Smal and BglII and ligated to similarly digested PCR product encompassing the calmodulin gene, which was obtained from PCR amplification with a human heart cDNA library as a template and oligonucleotide primers Calmodulin-Smal and Calmodulin-BglII-low. Finally, to obtain plasmid pASKInt100-Ubi, the ubiquitin-encoding gene sequence was amplified with PCR with a BLIP-containing vector (kindly provided by N. C. J. Strynadka) as a template and with primers BLIP-up and BLIP-low. The resulting PCR product was digested with Smal and BamHI and ligated to similarly digested pASKInt100-EETI-CKmod (47). For the construction of a C-terminally truncated intimin–calmodulin fusion vector (pASKInt100-Cal), pASKInt100-TmDegP was digested with Smal and BglII and ligated to similarly digested PCR product encompassing the calmodulin gene, which was obtained from PCR amplification with a human heart cDNA library as a template and oligonucleotide primers Calmodulin-Smal and Calmodulin-BglII-low. Finally, to obtain plasmid pASKInt100-Ubi, the ubiquitin-encoding gene sequence was amplified with PCR with oligonucleotides UbiSmal and UbiBglII and with human heart cDNA as a template. The resulting product was digested with Smal and BglII and ligated to similarly digested pASKInt100-EETI-CKmod (47). **Preparation of an E. coli membrane fraction.** Cultures of *E. coli* strains containing the respective expression plasmids were grown overnight and subcultured 1:50 until they reached an optical density at 600 nm (OD600) of 0.8. After induction with hydrotetracycline (0.2 μg/ml) for 10 min, the cells were pelleted by centrifugation and resuspended in 100 mM Tris-Cl (pH 8.0). The membrane fraction was prepared as described previously (16), with minor modifications. Cells were lysed by sonication with a Branson (Danbury, Conn.) Sonifier. Remaining large bacterial fragments were sedimented by centrifugation at 5,000 × g for 10 min. After incubation of the lysate on ice for 30 min in 100 mM Tris-HCl (pH 8.0)–10 mM EDTA–1% (wt/vol) Triton X-100, the membrane fraction was isolated by centrifugation of the cleared solution at 100,000 × g for 120 min at 15°C. 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-poly-
acrylamide gel electrophoresis (PAGE) (12.5% acrylamide-bisacrylamide [30:0.8]) followed by immunoblotting.

Trypsin treatment of intact cells and preparation of periplasmic proteins. Cells were grown in dYT and treated with trypsin as previously described (47). After being washed, cells were resuspended in 200 mM Tris-Cl (pH 9.0)–100 mM EDTA–20% (wt/vol) sucrose and incubated on ice for 1 h. Cells were pelleted by centrifugation and subjected to osmotic shock by resuspension in 10 mM Tris-Cl (pH 9.0). Bacterial fragments were removed by centrifugation at 13,000 × g for 30 min. The cleared osmotic shock fluid was subjected to SDS-PAGE (12.5% acrylamide-bisacrylamide [30:0.8]) followed by immunoblotting.

Flow cytometric analysis. For flow cytometric analysis, cultures of E. coli strains containing the respective expression plasmids were grown overnight and subcultured 1:50 until they reached an OD600 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 antibodies (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 (1:1 diluted). After 5 min of incubation at room temperature and the addition of 500 μl of PBS, cells were pelleted as described above and resuspended in 100 μl of PBS for flow cytometric analysis. A total of 300,000 events were collected with a Cytometer MoFlo cell sorter. Parameters were set as follows: forward scatter and side scatter—730 (LIN mode, amplification factor 6); FL1 (fluorescein isothiocyanate)—600 (LOG mode); FL2 (PE—LOI mode); and trigger parameter—side scatter. The sample flow rate was adjusted to an event rate of approximately 30,000 s−1.

Binding of MykBlaSend to cell surface-exposed calmodulin. Cells harboring pASK100-Cal were grown overnight and subcultured 1:50 in dYT containing 20 mM EGTA until they reached an OD600 of 0.2. For the induction of gene expression 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, resuspended in 50 μl of Tris-Cl (pH 7.5)–1 mM EDTA–15 mM 2-ME, and incubated on ice for 15 min.

Cells were pelleted and resuspended in 10 μl of 100 mM horeate buffer (pH 7.0) containing MykBlaSend fusion protein (350 μg/ml). MykBlaSend is a tripartite protein consisting of a calmodulin-binding segment (MKRRWKKNFIA VSAANRFKKISSSGAL) of human light-chain myosin kinase, RTEM β-lactamase, and a Send epitope. After 30 min of incubation on ice, cells were successively incubated with anti-Send epitope antibody, biotinylated goat anti-mouse antibody, and Streptavidin R-PE conjugate as described above. Finally, cells were resuspended in 20 μl of PBS for fluorescence microscopy by using a Zeiss (Göttingen, Federal Republic of Germany) Axioseecope with Zeiss filter set 487715.

Generation of Bla mutants. E. coli strain 71-18mutS (26) was successively transformed with plasmids pASK100-Blundp5 (H. Kolmar, unpublished results), a derivative of low-copy-number plasmid pZA22-MCS1 (31) containing the tetA gene under P1 promoter control. mutDS encodes a dominant-negative variant of the DNA polymerase III ε subunit lacking proof-reading activity (49). Cells were grown overnight in 50 ml of dYT supplemented with the appropriate antibiotics, 0.6% (wt/vol) SDS, 1 mM EDTA, and 15 mM 2-mercaptoethanol (2-ME). At an OD600 of 0.2, the expression of the C-terminally truncated intimin–Blundp5 fusion was induced by the addition of tetracycline to a 10-ml portion of the culture. Cells were grown for 2 h, and then 5 ml of this culture was used to inoculate 50 ml of fresh dYT supplemented with the appropriate antibiotics, 0.6% (wt/vol) SDS, 1 mM EDTA, and 15 mM 2-ME. Cells were grown at 30°C overnight and subcultured 1:50 in fresh dYT supplemented with the appropriate antibiotics, 0.6% (wt/vol) SDS, 1 mM EDTA, and 15 mM 2-ME until they reached an OD600 of 0.2. The expression of the C-terminally truncated intimin–Blundp5 fusion was induced by the addition of anhydrotetracycline. Cells were grown for 1 h, successively labeled with anti-Send epitope antibody, biotinylated anti-mouse antibody, and Streptavidin R-PE conjugate, and subjected to fluorescence-activated cell sorting (FACS).

To examine whether these fusion proteins accumulate in the outer membrane as full-length proteins and to assess susceptibilities to proteolysis, membrane fractions of E. coli 71-18 cells carrying the respective expression plasmids were analyzed by SDS-PAGE and immunoblotting (Fig. 2). Each fusion protein could be detected in the membrane fraction by Western blot analysis with a monoclonal antibody that recognizes the C-terminal Send epitope, albeit with various yields of net accumulation. This experiment also revealed significant proteolysis of the hybrid proteins, which was not altered in the presence of protease inhibitors. This result suggests that the observed proteolysis does not affect the integrity of the constructs.
ence of 2-ME or EGTA. The same results were obtained with a monoclonal antibody to the internal E epitope (data not shown). Since the antibody used in the Western blot analysis recognizes the carboxy-terminal epitope of the fusion protein, the proteolysis products correspond to fusion proteins with a truncated amino-terminal periplasmic domain of intimin.

Translocation of passenger domains to the E. coli cell surface and localization studies. To investigate whether the C-terminally truncated intimin domain mediated the translocation of the passenger domains through the outer membrane, mid-exponential-growth-phase cultures of E. coli 71-18 cells expressing the respective fusion proteins were probed for display of the passenger domains by immunofluorescence staining with anti-E epitope and anti-Send epitope monoclonal antibodies, which recognize epitopes flanking the respective passenger domains. Both antibodies are specific for their respective tags and do not recognize C-terminally truncated intimin. As a control, the cell surface display of a C-terminally truncated intimin fusion protein lacking a passenger domain was investigated with 71-18(pASKInt100-ΔP) cells, in which the E epitope is directly fused to the Send epitope. Labeled cells were analyzed by FACS.

As shown in Fig. 3, all fusion protein-producing cells were immunofluorescently stained with the anti-E epitope antibody, while the extent of immunofluorescence obtained with the anti-Send epitope antibody, recognizing the C-terminal epitope of the fusion protein, varied greatly. Both epitope tags flanking ubiquitin and—in accordance with previous results (47)—the immunoglobulin light-chain variable domain REL, were detected, indicating the cell surface exposure of these passenger proteins. No immunofluorescence was detected upon cell staining with an antibody directed against a Send epitope carboxy terminal to the calmodulin, BLIP, or β-lactamase passenger domain. These data indicate that fusion of the passenger domains to C-terminally truncated intimin prevented neither the outer membrane localization of the C-terminally truncated intimin core domain nor the cell surface exposure of the immunoglobulin-like C-terminally truncated intimin extracellular domain, while the outer membrane translocation of the calmodulin, BLIP, and β-lactamase passenger domains failed.

Since an anti-E epitope immunofluorescent label was detected for all fusion proteins investigated, it can be assumed that Cal, Bla, and BLIP passenger domains are trapped in the periplasmic space or the outer membrane. In order to test this assumption, cells expressing the REL, Bla, and Cal fusion proteins were incubated with trypsin prior to being stained with anti-E epitope antibody. After trypsin treatment, periplasmic proteins were isolated by osmotic shock treatment, and the cleared osmotic shock fluid was subjected to SDS-PAGE and Western blot analysis with anti-E epitope antibody (Fig. 4B). Only for cells expressing Cal and Bla fusion proteins could bands be detected. Cell permeabilization by the addition of EDTA prior to trypsin treatment resulted in the complete digestion of the periplasmic fragments (data not shown), corroborating the notion that these fragments are susceptible to trypsin cleavage but are shielded from enzyme attack by the presence of an intact outer membrane. The sizes of these bands corresponded to those of fragments that would result from cleavage of the proteins in surface-exposed domain D0 (Fig. 4C). Our findings indicate that these passenger domains reside within the periplasmic space or the outer membrane, are detached from the membrane anchor by trypsin cleavage, and are subsequently found within the periplasm. For REL, however, no periplasmic fragment could be detected, in compliance with the finding that REL is located on the cell surface and therefore is susceptible to trypsin cleavage.

Klausner et al. showed that cell surface exposure of CtxB—a subunit of cholera toxin which contains a single intramolecular disulfide bond—fused to the N. gonorrhoeae IgA protease autotransporter domain was observed only when disulfide bond formation of CtxB in the E. coli periplasm prior to translocation was interfered with either by the addition of 2-ME to the growth medium or by the use of an E. coli dsbA mutant lacking a major periplasmic oxidoreductase which promotes periplasmic disulfide bond formation (23). In order to investigate the influence of periplasmic disulfide bond formation on the cell surface exposure of passenger domains fused to C-terminally truncated intimin, strains 71-18 and 71-18dsbA harboring the respective pASKInt100 derivatives were grown in the absence or presence of 20 mM 2-ME, induced with anhydrotetracycline, and analyzed for cell surface exposure of the respective passenger domains by immunofluorescence staining as described above. With the exception of BLIP (Fig. 5), none of the passenger domains displayed elevated levels of cell surface exposure.

FIG. 2. Western blot analysis with anti-Send epitope antibody of a whole-membrane preparation of induced recombinant 71-18 cells harboring pASKInt100-REI, pASKInt100-Ubi, pASKInt100-Bla, pASKInt100-BLIP, pASKInt100-Cal, or pASKInt100-ΔP and grown in the presence or absence of 20 mM 2-ME or 20 mM EGTA. M, marker proteins (pencil marked after Ponceau S staining); sizes (in thousands) are indicated. Arrowheads indicate full-length proteins.

FIG. 3. C-terminally truncated intimin-mediated cell surface display of passenger proteins. FACS histograms of E. coli 71-18 cells harboring no plasmid (A), pASKInt100-ΔP (B), pASKInt100-REI (C), pASKInt100-Ubi (D), pASKInt100-Bla (E), pASKInt100-Cal (F), or pASKInt100-BLIP (G) are shown. Induced cells were incubated with anti-Send epitope antibody (S) or anti-E epitope antibody (E), biotinylated anti-mouse antibody, and streptavidin–R-PE conjugate. Unlabeled 71-18 cells served as a control (−) in panels B to D.
exposure (data not shown). The addition of 20 mM 2-ME to the growth medium of strain 71-18(pASKInt100-BLIP) resulted in a slight improvement in the extracellular exposure of BLIP, a protein that contains two intramolecular disulfide bonds. The same results were obtained when 1 mM tris(2-carboxyethyl)phosphine (TCEP) was used as a reducing agent (data not shown). The use of 71-18dsbA as an expression host resulted in a further increase in the number of surface-exposed BLIP molecules per cell, and with the addition of 20 mM 2-ME, the surface display of BLIP was substantially improved (Fig. 5).

**Export of calmodulin.** The results obtained with BLIP could be interpreted as indicating that there is a correlation between export and prevention of periplasmic folding of the passenger domain. To test this supposition, we chose as a passenger domain calmodulin (Cal), which does not contain disulfide bonds but whose folding stability is strongly dependent on the presence of calcium ions. The apo form of calmodulin is significantly unfolded at normal temperatures, while the calcium-loaded form of calmodulin has been found to be exceptionally stable (17), because it can be exposed to temperatures of >90°C or to a 9 M urea solution without a marked change in its tertiary structure. It is important to note that the secretion of calmodulin through the cytoplasmic membrane is unaffected by the presence or absence of calcium ions (33). This property allowed us to probe the influence of periplasmic folding of the

![Diagram of calmodulin secretion](http://example.com/diagram.png)
calmodulin passenger domain on surface display by varying the periplasmic concentrations of Ca\(^{2+}\) ions.

For this experiment, *E. coli* cells containing pASKInt100-Cal were grown in the presence or absence of 20 mM EDTA. As shown in Fig. 2, the expression of a full-length C-terminally truncated intimin–Cal fusion protein could be detected in the presence or absence of EGTA, a result which could be verified by immunofluorescence staining with an anti-E epitope antibody; however, calmodulin surface display was observed only in the presence of 20 mM EDTA. The same results were obtained with EGTA, a chelator that is more Ca\(^{2+}\) specific, or by growing cells in M9 minimal medium supplemented with 20 mM EDTA (data not shown). Furthermore, when cells that produced the C-terminally truncated intimin–Cal fusion protein in the presence of 20 mM EGTA were washed and resuspended in buffer containing 50 mM calcium chloride, the surface-exposed calmodulin regained its native fold and was able to bind the natural substrate molecule Myk (20), a short peptide from light-chain myosin kinase, since calmodulin-presenting cells were immunofluorescently stained with a Myk-Bla\(^{Send}\) fusion protein (Fig. 6D). No enhancement of cell surface net accumulation was observed upon cell growth in the presence of 20 mM EDTA for any of the other passenger domains (data not shown), supporting the theory that the successful export of calmodulin in the absence of calcium ions is due to the low folding stability of apo-calmodulin rather than to the destabilization of the outer membrane by chelating compounds.

**Surface display of β-lactamase variants.** β-Lactamase (Bla) has been used as a reference passenger domain for surface display based on the IgA protease (Kolmar, unpublished) and AIDA (28) autotransporter domains or a shortened OmpA porin (15). Surface display of Bla fused to the C-terminally truncated intimin translocator, however, was unsuccessful under all conditions tested. As a working hypothesis, we assumed that periplasmic folding and/or disulfide bond formation might prevent the outer membrane translocation of Bla. In this situation, it might be possible to isolate Bla variants that have folding characteristics different from those of the wild-type enzyme and that are able to pass through the outer membrane barrier when fused to C-terminally truncated intimin.

In a search for such Bla variants, we randomly mutagenized pASKInt100-Bla by propagating the plasmid in strain 71-18 mutS(pZA22-mutD5). This hypermutator strain is deficient in DNA mismatch repair because it carries an insertion of Tn10 (tetracycline resistance) in the mutS locus. In addition, it contains the low-copy-number plasmid pZA22-mutD5, carrying the dnaQ gene and a mutD5 mutation encoding a dominant-negative ε subunit of the proofreading exonuclease of DNA polymerase III under lac promoter control (42). Cell surface-exposed Bla variants were isolated from the mutant cell population by cell staining with mouse anti-Send epitope antibody and FACS with the single-cell deposition unit of the MoFlo cell sorter. After verification of the surface display of Bla by immunostaining with anti-Send epitope antibody (Fig. 7), the complete bla genes of seven clones were sequenced. All clones carried a mutation at either codon 52 or codon 98, which encodes a cysteine residue in wild-type Bla (Table 3). Notably, we found the cysteine residues exchanged with comparably bulky arginine and tyrosine residues, a finding which may mean that apart from eliminating disulfide bonds, further destabilization of the protein fold may be required for Bla surface display.

*E. coli* cells exposing the Bla variants on their cell surface displayed markedly reduced Bla activity and were able to grow only at ampicillin concentrations of <100 μg/ml. Wild-type Bla and the variant Bla-C98Y were further characterized by cloning of the respective genes into expression vector pBRB22HI, which allows the expression of β-lactamase with six additional carboxy-terminal histidine residues under T7 promoter control. Purified Bla-C98Y displayed markedly reduced β-lactamase activity on the chromogenic substrate PADAC (Table 3). Furthermore, while the relative enzymatic activity of wild-type β-lactamase was reduced to 80% in the presence of 1 M urea, the activity of Bla-C98Y was reduced to 10% under these conditions; these results indicate drastically reduced stability of Bla-C98Y compared to that of wild-type Bla. Taken together, these data suggest that β-lactamase can be translocated across the bacterial outer membrane via fusion to C-terminally truncated intimin only under conditions in which folding and disulfide bond formation of the β-lactamase passenger domain in the periplasm are prevented.

**DISCUSSION**

In this study, we investigated the transport of passenger proteins to the surface of *E. coli* cells by fusion to C-terminally truncated intimin, an *E. coli* adhesin. Our data provide experimental evidence that outer membrane translocation of passenger domains depends on periplasmic folding of the passenger domains and that the formation of disulfide bridges prior to outer membrane translocation may completely prevent cell surface exposure of passenger proteins.

The present study was initiated by the finding that passenger domains like the immunoglobulin domain RE1v, the cysteine knot peptide EETI-II, and interleukin-4 (47) are translocated across the outer membrane when fused to C-terminally truncated intimin, albeit with markedly different yields. We constructed *E. coli* vectors for the expression of additional C-terminally truncated intimin–passenger fusion proteins in which the respective passenger domain is tagged with flanking epitopes. This strategy allowed us to detect the translocation of the passenger domain by immunofluorescence staining of the producing cells. While the immunoglobulin domain RE1, ubiquitin are exported with high yields, the outer membrane translocation of BLIP is poor and that of calmodulin and β-lactamase is completely absent. All constructs, irrespective of the successful surface exposure of the passenger domain, displayed immunofluorescence staining of the epitope sequence located at the junction of C-terminally truncated intimin and the passenger domain. This finding indicates that the C-terminally truncated intimin translocator resides in the outer membrane and exposes the extracellular immunoglobulin-like domain. A full-length C-terminally truncated intimin–passenger fusion protein was detected by Western blot analysis for all constructs whether or not they became surface exposed. Although extensive proteolysis of the fusion proteins did occur, it most likely was not the reason for the failure of the surface display of Cal and BLIP, since we could demonstrate a similar level of proteolysis under conditions permitting the surface localization of these proteins. Furthermore, we could show...
FIG. 6. C-terminally truncated intimin-mediated cell surface display of human calmodulin. (A and B) *E. coli* 71-18 cells harboring pASKInt100-Cal were grown in the absence (A) or presence (B) of 20 mM EDTA. Induced cells were harvested, successively incubated with anti-Send epitope antibody (S) or anti-E epitope antibody (E), biotinylated anti-mouse antibody, and streptavidin–R-PE conjugate, and analyzed by FACS. Unlabeled 71-18 cells served as a control (−). (C and D) Binding of an MykBla<sub>Send</sub> fusion protein to surface-exposed calmodulin. Induced cells grown in the absence (C) or presence (D) of 20 mM EGTA were washed, resuspended in 50 mM CaCl<sub>2</sub>, and incubated with MykBla<sub>Send</sub>, consisting of a calmodulin-binding segment of myosin kinase fused to β-lactamase and tagged with a Send epitope. Binding of the fusion protein to calmodulin was detected by fluorescence microscopy with anti-Send epitope antibody, biotinylated anti-mouse antibody, and streptavidin–R-PE conjugate for cell staining.
that Bla and Cal passenger proteins were found in the periplasmic fraction after trypsin treatment of induced cells grown in unsupplemented dYT. These data provide experimental evidence for the assumption that passenger domains that do not become surface exposed through fusion with C-terminally truncated intimin are trapped in the periplasm. A model of a trapped passenger domain is depicted in Fig. 4A.

Surface display of BLIP could be drastically enhanced under reducing conditions in the periplasmic space. However, surface exposure of the disulfide bond-containing passenger Bla remained unaffected. Disulfide bond formation is a late step in protein folding. Supplementation of growth media with reducing agents may delay or prevent passenger domain folding in the periplasm and thereby enhance the export of BLIP, which contains two intramolecular disulfide bonds. Most likely, the observed enhancement of surface display by the addition of 2-ME is the result of a direct effect on the disulfide bond formation of the passenger domain and is not due to destabilization of the outer membrane, since the export of Cal, which requires calcium ions to adopt its stable native fold, occurs only in the presence of EDTA or EGTA, with 2-ME having no effect.

In this respect, C-terminally truncated intimin-mediated outer membrane translocation is quite similar to the transport of proteins through the cytoplasmic and mitochondrial membranes. Eilers and Schatz showed that methotrexate, a folate antagonist, blocks import into mitochondria of dihydrofolate reductase fused to a mitochondrial presequence due to stabilization of the dihydrofolate reductase moiety prior to secretion (11). Likewise, Randall and Hardy established a correlation between competence for export and lack of stable tertiary structure for the secretion of maltose-binding protein into the periplasmic space (36).

However, as with Bla, the export of passenger domains that contain intramolecular disulfide bonds in their native state is neither generally enhanced in the presence of reducing agents nor generally hampered in their absence, because REI, and the protease inhibitor EETI-II (47) are efficiently exported despite the fact that they contain one disulfide bond and three disulfide bonds, respectively, in their native structure.

What is the reason for the observed large differences in passenger protein export? Studies on the folding of REI, indicated that disulfide bond formation is the rate-limiting step, with an intrathio half-life of refolding of 200 s (41). This time window may be sufficient for the outer membrane passage of REI, in an unfolded state. The same may hold true for EETI-II, in which disulfide bond formation is also a rather slow process (48). The situation for β-lactamase export is quite different. Like REI, β-lactamase contains a single disulfide bond. However, the outer membrane export of wild type β-lactamase is completely precluded under all conditions tested. It has been shown that in contrast to the situation for REI, and EETI-II, the in vitro folding of β-lactamase is a rapid process, with a half-life of refolding of less than 1 min (27). During the de novo folding in the periplasm of E. coli, the disulfide bond is introduced into β-lactamase by the DsbA protein (2, 3, 14). However, reduced and oxidized β-lactamasenses fold at identical rates to native-like conformations with similar stabilities (14). Hence, Bla reaches its native, enzymatically active conformation in the presence of 2-ME and/or in the absence of DsbA, and the rapid folding of both the reduced and the oxidized forms of Bla may efficiently compete with the export of the unfolded molecule. Our screening experiment for β-lactamase variants that were able to pass through the outer membrane only returned variants with one or the other cysteine residue replaced by arginine or tyrosine. These residues are rather bulky, and it is not astonishing that these Bla variants are less active and more prone to denaturation than wild-type Bla, features which may facilitate their export. However, the finding that, except for cysteine substitutions, no other destabilizing mutations have been discovered indicates that the absence of intramolecular disulfide bonds is required for the outer membrane translocation of this protein.

### TABLE 3. Single-base-pair substitutions in variant bla genes isolated by selection on surface exposure and relative Bla activity

<table>
<thead>
<tr>
<th>Clone(s)</th>
<th>Amino acid exchange</th>
<th>Codon&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Additional mutation</th>
<th>Relative Bla activity with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No urea</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>C98Y</td>
<td>TGC → TAC</td>
<td>Conservative T84 (ACA → ACG)</td>
<td>0.02</td>
</tr>
<tr>
<td>1, 3, 5, 7</td>
<td>C98R</td>
<td>TGC → CGC</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>C98R</td>
<td>TGC → CGC</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>4</td>
<td>C53R</td>
<td>TGT → CGT</td>
<td></td>
<td>0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Change is shown in bold type.
We chose RTEM1-β-lactamase as a model passenger domain to study C-terminally truncated intimin-mediated E. coli surface display, since it could be directed to the E. coli cell surface via fusion to a shortened version of OmpA (13) or the AIDA autotransporter domain (28). According to the current model of autotransporter function, an outer membrane pore is formed by homooligomerization of the autotransporter core domain, and it is through this pore that the outer membrane passage of the passenger domain occurs (46). Recent data indicate that this pore can accommodate passenger domains that are conformationally constrained by intramolecular disulfide bonds, albeit with a reduced efficiency of surface exposure (6, 46). Differences in efficiency between the C-terminally truncated intimin-mediated E. coli surface display of Bla and the autotransporter pathway may be due to different geometries of the translocation channel—if it exists—and/or to differences in the kinetics of folding of the passenger domain when fused to the respective translocator domain. A recent work demonstrated that intimin forms a ring-shaped structure with a 7-nm diameter and a channel with a conductance of 50 pS (44). The findings of our study are in accordance with the presence of a channel which can accommodate only peptide chains with a certain diameter and not fully folded passenger domains.

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