Folding-Based Suppression of Extracytoplasmic Toxicity Conferred by Processing-Defective LamB

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We have utilized processing-defective derivatives of the outer membrane maltoporin, LamB, to study protein trafficking functions in the cell envelope of *Escherichia coli*. Our model proteins contain amino acid substitutions in the consensus site for cleavage by signal peptidase. As a result, the signal sequence is cleaved with reduced efficiency, effectively tethering the precursor protein to the inner membrane. These mutant porins are toxic when secreted to the cell envelope. Furthermore, strains producing these proteins exhibit altered outer membrane permeability, suggesting that the toxicity stems from some perturbation of the cell envelope (J. H. Carlson and T. J. Silhavy, *J. Bacteriol.* 175:3327–3334, 1993). We have characterized a multicopy suppressor of the processing-defective porins that appears to act by a novel mechanism. Using fractionation experiments and conformation-specific antibodies, we found that the presence of this multicopy suppressor allowed the processing-defective LamB precursors to be folded and localized to the outer membrane. Analysis of the suppressor plasmid revealed that these effects are mediated by the presence of a truncated derivative of the polytopic inner membrane protein, TetA. The suppression mediated by TetA' is independent of the CpxA/CpxR regulon and the σ^E regulon, both of which are involved in regulating protein trafficking functions in the cell envelope.

In *Escherichia coli*, proteins are first synthesized in the cytoplasm and may be subsequently targeted to the inner membrane, outer membrane, or periplasmic space, which are collectively referred to as the cell envelope. Little is known concerning the mechanisms by which envelope proteins are folded and assembled after their export from the cytoplasm. Presumably, the envelope should contain its own array of chaperones, protein-folding catalysts, and proteases.

The question of how integral outer membrane proteins are properly targeted, folded, and assembled is a unique one. These proteins are significantly more hydrophilic than their inner membrane counterparts and, in general, are believed to assemble in the outer membrane bilayer as amphipathic β -barrels (8, 9, 35). The monomeric OmpA protein and the trimeric porins OmpF, PhoE, and LamB have been used extensively as models to study outer membrane protein assembly. These proteins have been particularly useful because they are generally resistant to denaturation in sodium dodecyl sulfate (SDS) when heated to moderately high temperatures (33, 45), and furthermore, they remain intact during SDS-polyacrylamide gel electrophoresis (SDS-PAGE), allowing them to be distinguished from the denatured species. For the trimeric porins, assembly of heat- and protease-resistant trimers serves as a hallmark of proper outer membrane assembly.

There have been two fundamentally distinct models proposed to explain how proteins are targeted to the outer membrane. First, the protein may be transported from the inner membrane to the outer membrane via zones of membrane

adhesion (3). Alternatively, the protein may pass through the periplasmic compartment en route to the outer membrane. This second model is currently favored, as several labs have provided evidence for the existence of periplasmic intermediates. For example, studies by Sen and Nikaido (36) demonstrated that *E. coli* spheroplasts secreted a soluble, monomeric form of the OmpF porin and that this protein could be assembled into mature trimers in the presence of detergent and outer membranes. In another study, partially assembled OmpF has been localized to the periplasm by osmotic shock (20). A related issue is the mechanism by which lipopolysaccharide (LPS) is assembled into the outer membrane. Since LPS has been shown to be important for porin assembly both in vivo (2, 4, 23) and in vitro (37), it has been suggested that these components may be assembled by a common mechanism.

Several assembly intermediates have been proposed to exist prior to formation of the native trimeric porins, including a folded monomer, a dimer, and a metastable trimer. Evidence for a folded monomeric intermediate was first provided by using in vitro-synthesized PhoE (13). This protein was shown to possess elements of native structure, as determined by its ability to be recognized by conformation-specific monoclonal antibodies. However, studies with OmpF suggested that the folded monomer was actually an off-pathway product that retained elements of the native structure, an artifact of the in vitro system (38). More recently, however, in vivo studies have shown the existence of a folded monomeric intermediate that appears to chase into trimers, lending support to the idea that the folded monomer represents a true intermediate (34, 44). In addition, in vivo experiments have detected both a dimeric intermediate (31) and a trimeric intermediate that is more thermolabile than native trimers (45). Conversion of this metastable trimer to the native trimer occurs slowly, with a half-life of 5.7 min (45).

In order to further understand the mechanisms of outer membrane protein targeting, we have utilized derivatives of the

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LamB porin which are defective for signal sequence processing. The *lamBA23D* mutation (5) specifies a protein that has an Ala-to-Asp substitution in the signal sequence, at position -3 relative to the cleavage site (see Fig. 1A). While this mutation does not interfere with translocation of the LamBA23D precursor via the secretion machinery, it does interfere with recognition of the signal sequence by signal peptidase. As a result, the precursor protein becomes effectively tethered to the inner membrane via the signal sequence. Export of this mutant porin to the cell envelope is toxic, and strains harboring the *lamBA23D* mutation are inducer (maltose) sensitive and exhibit increased sensitivity to SDS and amikacin, suggesting that the precursor protein alters outer membrane permeability (5, 7). However, since the processing defect conferred by the *lamBA23D* mutation is leaky, the protein is eventually processed at the correct site, and the resulting wild-type porin is then assembled into the outer membrane.

Previously, we used suppressor analysis of *lamBA23D* to search for protein-targeting factors in the cell envelope (7). In our analysis, we identified a two-component signal transduction pathway, consisting of the CpxA histidine kinase and the CpxR response regulator. We proposed that this pathway senses protein trafficking stresses in the envelope and regulates expression of its targets in response to these stresses (7, 12, 40). Thus far, several genes that encode envelope protein trafficking factors have been identified as transcriptional targets of CpxR (11, 12, 28). In addition, a second mechanism for responding to protein-folding stresses in the cell envelope has been described. Activity of the alternative heat shock σ factor, σ^E , has been shown to increase in the presence of extracytoplasmic stimuli, such as excess production of outer membrane proteins (26). Like CpxR, σ^E regulates synthesis of factors involved in protein trafficking in the envelope (11, 17).

During our studies of the Cpx pathway, we identified a multicopy suppressor of *lamBA23D* that is described here. Normally, precursor LamBA23D is either processed or degraded. However, pulse-chase analysis revealed that the LamBA23D precursor was stabilized in the presence of the suppressor (7). This observation suggested that the suppressor must be acting by some novel mechanism to neutralize the toxicity conferred by the LamB precursor. In the present study, we determined the identity of the suppressor and characterized its effects on several processing-defective LamB proteins. Evidence presented here demonstrates that the presence of this multicopy suppressor results in release of the precursor proteins from the inner membrane, as well as their folding and targeting to the outer membrane.

MATERIALS AND METHODS

Media and chemicals. Media and growth conditions have been described (39), with the following exceptions. M63 liquid minimal medium was supplemented with sugars at a final concentration of 0.4% (wt/vol), and in addition, glycerol minimal medium was supplemented with Luria-Bertani broth at a concentration of 0.5% (vol/vol). Antibiotics were used in the following concentrations: chloramphenicol, 20 μ g/ml in rich media and 5 to 10 μ g/ml in minimal media; ampicillin, 125 μ g/ml in rich media and 50 μ g/ml in minimal media; kanamycin, 50 μ g/ml in rich media and 125 μ g/ml in minimal media; spectinomycin, 50 μ g/ml in rich media; and tetracycline, 25 μ g/ml in rich media and 10 μ g/ml in minimal media. [³⁵S]methionine was purchased from DuPont NEN Research Products. Amikacin antibiotic discs (30 μ g) were purchased from Difco. Maltose-binding protein (MBP) and LamB antisera are from our laboratory stock (27). Formalin-fixed *Staphylococcus aureus* (Immuno-Precipitin; Bethesda Research Laboratories) was used for immunoprecipitations. ECL Western blotting reagents were purchased from Amersham Life Science.

Bacterial strains, plasmids, and microbiological techniques. All strains are derivatives of *E. coli* K-12 strain MC4100 [F⁻ *araD139* Δ (*argF-lac*)U169 *rpsL150* *relA1* *flbB5301* *deoC1* *ptsF25* *rbsR*] (39). pK17 and pK12 are from K. Strauch and have been described (43). pBST324 is a pACYC177 derivative carrying the *tetR* gene (1). pPW100 carries the *degP* gene under the control of the *trc* pro-

motor (46). pHP45 Ω was the source of a spectinomycin resistance Ω cassette (18). Plasmids pKBZ9000 (30) and pDJA100 (5) are described below.

Standard microbiological techniques for P1 transduction and transformations have been described previously (39). Maltose sensitivity was measured by streaking on maltose minimal agar and incubation at 30°C. Degrees of sensitivity were determined by examining colony size, density of growth in the primary streak, and the presence or absence of pseudorevertant (Mal⁺) colonies.

Pulse-labeling and immunoprecipitations. Standard pulse-labeling and immunoprecipitations were performed as described previously (7). However, for labeled samples that were to be characterized by native immunoprecipitation, cells were labeled as described previously (7), but cell lysate preparation and immunoprecipitation were performed as described by Misra et al. (27). Samples were resuspended in sample buffer containing 3% (wt/vol) SDS, 10% (vol/vol) glycerol, and 5% (vol/vol) β -mercaptoethanol in 70 mM Tris-HCl (pH 6.8). Resuspended samples were allowed to solubilize at room temperature for 3 h prior to removal of *S. aureus*. Samples were then aliquoted into separate tubes before heating and electrophoresis.

Cell fractionation. Strains to be fractionated were grown overnight in glycerol minimal medium containing chloramphenicol. Cells were then subcultured into 50 ml of fresh medium in a 250-ml flask and were grown with aeration at 30°C to an A_{600} between 0.3 and 0.4. Maltose was added to a final concentration of 0.4% (wt/vol), and the cells were grown for an additional hour. The final A_{600} was noted for further calculations (see below). From this point, all solutions and manipulations were conducted on ice or at 4°C unless otherwise noted. Cells were harvested by centrifugation at 2,500 $\times$ g for 10 min. The cell pellet was washed in 25 ml of 50 mM Tris-HCl, pH 7.5. Cells were then resuspended in this same buffer, containing 2 μ g of RNase A per ml, 1 μ g of DNase per ml, and protease inhibitors (5 μ g of leupeptin per ml, 20 μ g of aprotinin per ml, 500 μ M pepstatin A, and 1 mM phenylmethylsulfonyl fluoride), at a volume equal to $A_{600}/5$ ml (thus normalizing the number of cells per milliliter for all samples). Cells were lysed by two passes through a French pressure cell at 15,000 lb/in². Unbroken cells were removed by centrifugation at 2,500 $\times$ g for 15 min. A 200- μ l aliquot of the supernatant (whole-cell lysate) was saved for analysis, and 1.6 ml was subjected to centrifugation in a TLA100.2 rotor in a Beckman Optima TL ultracentrifuge at 100,000 rpm for 20 min. The supernatant, containing the soluble cytoplasmic and periplasmic fractions, was saved for analysis. The membrane pellet was resuspended overnight in 100 μ l of 50 mM Tris-HCl, pH 7.5, on ice. Membranes were gently resuspended, and a sample of this total membrane fraction was added to a suitable volume of sample buffer and set aside for further analysis. Inner and outer membrane fractions were separated on a two-step sucrose gradient as follows: 750 μ l of 53% (wt/vol) sucrose (in 50 mM Tris-HCl, pH 7.5) was layered on top of 300 μ l of 70% (wt/vol) sucrose (in 50 mM Tris-HCl, pH 7.5). The 90- μ l membrane sample was placed at the top of this gradient, and the samples were centrifuged as above at 100,000 rpm for 65 min. Acceleration and deceleration were set to 5. Inner and outer membrane bands were identified by inspection and removed. An equal volume of 2 $\times$ sample buffer (minus glycerol) was added. Proteins from whole-cell lysate and soluble fractions were precipitated with trichloroacetic acid and were resuspended in a suitable volume of sample buffer. Samples were boiled prior to electrophoresis.

Electrophoresis, autoradiography, and immunoblot analysis. Electroelution and immunoblotting have been described previously (42), except that horseradish peroxidase-linked goat anti-rabbit immunoglobulin secondary antibody was used. SDS-PAGE (22) and autoradiography (6) have also been described.

Site-directed mutagenesis. The *degP15*(Oc)17(Am) allele, which contains an ochre mutation at codon 15 and an amber mutation at codon 17, was constructed by using a double-stranded site-directed mutagenesis protocol described by Deng and Nickoloff (16). The mutagenic primer degPSTOP, 5' CTCTGAGTTAAGGTTAGGCGTTATCTC 3', and the unique site elimination primer dgtpn⁻, 5' ATAACTGGGACCTACGCG 3', were used to mutagenize the *degP* allele present on pK17. After two rounds of digestion with *KpnI* to linearize plasmids that had not incorporated the unique site elimination primer, plasmid DNA was transformed into CLC224 (MC4100 *recA::kan degP::Tn10*). Plasmid DNA was prepared from transformants that displayed a temperature-sensitive phenotype at 42°C, and the presence of the *degP15*(Oc)17(Am) allele was confirmed by sequence analysis.

Construction of new LamB processing mutants. Plasmid pKBZ9000 expresses the Φ (*lamB-lacZ*)Hyb42-1 gene fusion from a weak, uncharacterized promoter (30). Plasmid pDJA100 is a derivative of pKBZ9000 that contains an amber mutation at codon 24 of *lamB-lacZ*, within the *lamB* sequence (5). Signal sequence mutations in *lamB* were first introduced onto the *lamB-lacZ* gene fusion carried on pDJA100 by using a double-stranded site-directed mutagenesis protocol similar to that described above, except that only the mutagenic primer was used and linearization was not employed. Primers used simultaneously revert the amber mutation at codon 24 and introduce the desired mutations at codons 23 and 25. Primer A23DA25D was used to generate allele *lamBA23D/A25D*, primer A23DA25Y was used to generate allele *lamBA23D/A25Y*, and primer A23YA25Y was used to generate allele *lamBA23Y/A25Y*: A23DA25D, 5' CCG TGG AAA TCA ACA TCC ATA TCC TGA GCA GAC ATT AC 3'; A23DA25Y, 5' CCG TGG AAA TCA ACA TAC ATA TCC TGA GCA GAC ATT AC 3'; and A23YA25Y, 5' CCG TGG AAA TCA ACA TAC ATA TCC TGA GCA GAC ATT AC 3'. Mutagenized plasmid DNA was transformed into ECB594 (MC4100 *malBA15*/F⁺:Tn10), and transformants harboring the desired

mutated plasmids were detected by the acquisition of a Lac⁺ phenotype on medium containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). The presence of the new *lamB* signal sequence mutations was confirmed by sequence analysis.

The processing mutations were introduced onto the chromosome as follows. The *malBΔ1* mutation contains a deletion of the 3' end of *malK* through the 5' end of *lamB*, thus rendering cells Mal⁻. pDJA100 contains the 3' end of the *malK* gene, followed by *lamB-lacZ*. The amount of *malK* and *lamB* present on the plasmid is sufficient to repair the *malBΔ1* mutation by recombination, thus restoring a Mal⁺ phenotype and simultaneously introducing any mutations from the signal sequence of *lamB-lacZ* onto the *lamB* gene in the chromosome. Strains NT1001 (MC4100 *malBΔ1*) and MDC3 (MC4100 *malBΔ1 cpxA101*) were transformed with pDJA100 and its derivatives carrying the *lamB*-processing mutations. The resulting strains were plated on maltose minimal medium to select for Mal⁺ recombinants. In the NT1001 background, Mal⁺ colonies arose at a frequency of 10⁻⁴ in the presence of pDJA100; however, no colonies were obtained with any of the processing mutant derivatives. In the MDC3 background, Mal⁺ colonies arose at a frequency of 10⁻⁴ in the presence of pDJA100 or any of its derivatives. The presence of the processing-defective *lamB* mutations was confirmed by sequence analysis of the chromosomal loci, and the strains were transduced back to *cpxA*⁺ in the absence of maltose.

RESULTS

New processing-defective mutations in LamB. The partial processing defect conferred by the *lamBA23D* mutation has proved useful in our genetic analyses; however, some experiments have been complicated by the presence of two species of LamB protein, the precursor and the mature proteins. To supplement our analysis, we used site-directed mutagenesis to generate additional mutations in *lamB* and recombined them onto the chromosome as described in Materials and Methods. Alleles *lamBA23D/A25D*, *lamBA23D/A25Y*, and *lamBA23Y/A25Y* direct the production of LamB proteins with Ala→Asp or Ala→Tyr substitutions at residues 23 (position -3 relative to the cleavage site) and 25 (position -1 relative to the cleavage site), as shown in Fig. 1A. Pulse-chase analysis demonstrated that all of these mutations completely block signal sequence cleavage by signal peptidase (Fig. 1B).

The new mutations were tested for the phenotypes previously observed with *lamBA23D*. Similar to the original mutation, each of the new alleles confers inducer (maltose) sensitivity. Indeed, these mutations appear to be even more toxic than *lamBA23D*. Specifically, *lamBA23D/A25D* strains are more maltose sensitive than *lamBA23D* strains, and strains harboring *lamBA23D/A25Y* or *lamBA23Y/A25Y* are the most sensitive of the group. Like the parent mutation, *lamBA23D/A25D* also appears to confer SDS sensitivity in the presence of maltose (data not shown); however, growth on maltose minimal medium is so poor as to preclude a measurement of SDS sensitivity for *lamBA23D/A25Y* and *lamBA23Y/A25Y*. In contrast to *lamBA23D* strains, which are λ^s and Dex⁺ (but inducer sensitive), strains carrying any of the new alleles are completely λ^r and Dex⁻. This observation suggests that the presence of processed LamB protein in the *lamBA23D* strain is responsible for the λ sensitivity and the ability to import maltodextrins and that the presence of a signal sequence interferes with assembly or function of the native porin.

Gain-of-function mutations in the gene encoding the CpxA histidine kinase have been shown to suppress the toxicity of *lamBA23D*, resulting in a Mal⁺ and Mal^r phenotype (7). Our ability to recombine the new mutations into the chromosome of a *cpxA101* strain, but not a *cpxA*⁺ strain, in the presence of maltose suggested that activation of the *cpx* pathway suppresses the new mutations as well (see Materials and Methods). Indeed, these recombinants were Mal^r, and the Mal^s phenotype was restored upon replacement of the *cpxA101* locus with *cpxA*⁺.

A multicopy suppressor of precursor LamB toxicity. We have previously demonstrated that plasmid pKS17 serves as a

A LamB Processing Mutants

LamB protein	SS cleavage site
Wild-type	...AQAMA VDF... ↓ 23
LamBA23D	...AQDMA VDF... ↓ *
LamBA23DA25D	...AQDMD VDF...
LamBA23DA25Y	...AQDMY VDF...
LamBA23YA25Y	...AQYMY VDF...

B

FIG. 1. Mutations in *lamB* that confer a processing defect. (A) The amino acid sequences of residues 21 to 28 of wild-type and processing-defective LamB proteins are shown. The arrow indicates the normal site of signal sequence (SS) cleavage. (B) Pulse-chase analysis of strains carrying various *lamB* alleles was performed as described previously (7). Lanes 1 and 2, MC4100 (*lamB*⁺); lanes 3 and 4, CLC567c (MC4100 *lamBA23D*); lanes 5 and 6, MDC11 (MC4100 *lamBA23D/A25D*); lanes 7 and 8, MDC14 (MC4100 *lamBA23D/A25Y*); and lanes 9 and 10, MDC15 (MC4100 *lamBA23Y/A25Y*). Samples were immunoprecipitated with anti-LamB monomer and anti-MBP sera. The autoradiograph of an SDS-PAGE gel is shown, with chase time points indicated at the bottom. p, precursor; m, mature.

multicopy suppressor of the toxicity conferred by LamBA23D (7). Furthermore, we find that this plasmid also suppresses the toxicity of the new processing-defective LamB proteins. pKS17 is a derivative of a larger plasmid, pKS12, which contains an 8-kb *Bam*HI fragment inserted into the *Bam*HI site of pACYC184, thus interrupting the *tetA* gene (43) (Fig. 2). A 4-kb *Sal*I-*Stu*I fragment was dropped from pKS12 to generate pKS17. Like pKS17, the parent plasmid, pKS12 also behaves as a multicopy suppressor of *lamBA23D*. The insert contained within pKS17 carries two complete open reading frames: *dgt*, which encodes a dGTPase (29), and *degP*, which encodes a heat-inducible periplasmic protease (24, 43).

Since the multicopy suppressor carries the *degP* gene, we suspected that its presence might accelerate degradation of precursor LamB, thus leading to suppression. To address this possibility, we performed pulse-chase analysis and found that the toxic LamBA23D precursor was not degraded in this background. In contrast, precursor LamBA23D is stabilized and persists in the cell even as late as 3 h postchase (6a, 7; also, see below). Clearly the suppressor activity conferred by pKS17 must neutralize the toxicity of precursor LamB without eliminating the protein.

Presence of the multicopy suppressor causes precursor LamB to fractionate with the outer membrane. Previous results by Carlson and Silhavy (5) demonstrated that precursor LamB_{A23D} fractionates to the inner and outer membranes in roughly equal proportions. Therefore, we wished to determine the cellular localization of precursor LamBA23D protein in the presence of the suppressor plasmid. *lamBA23D* strains carrying either the control (pACYC184) or the suppressor

FIG. 2. Suppressor plasmids and their derivatives. Representation of the constructs used in this study. Open reading frames, shown as arrowheads, are identified only in the first construct in which they appear. All constructs have been lined up starting with a common *StyI* site for comparison. Blank areas indicate regions relative to pKS12 that have been deleted. All genes and origins of replication are shown in gray except for *tetA* and its derivatives, which are shown in black. Genes and their products: *tetA*, tetracycline transporter; *cat*, chloramphenicol acetyltransferase; *ori*, plasmid origin of replication; *dgt*, dGTP triphosphohydrolase; *degP*, DegP periplasmic protease; *spc^R/str^R*, Ω cassette conferring spectinomycin and streptomycin resistance; *bla*, β -lactamase. Restriction sites: Sa, *SaI*; St, *StuI*; B, *Bam*HI; Sy, *StyI*; Bx, *Bst*XI; E, *Eco*NI; C, *Cla*I. The large X on the *degP* open reading frame indicates the presence of the *degP15*(Oc)17(Am) allele.

(pKS17) were grown to mid-logarithmic phase in glycerol minimal medium and were subjected to fractionation analysis as described in Materials and Methods. Figure 3 shows a Western immunoblot of whole-cell, soluble, total membrane, inner membrane, and outer membrane fractions. In agreement with previous results (5), LamBA23D precursor localized in roughly equal proportions to the inner and outer membrane fractions. In contrast, the precursor fractionates predominantly with the outer membrane in the presence of the suppressor. As expected, mature LamB localizes predominantly to the outer membrane fraction.

Precursor LamB chases into intermediates distinct from mature trimers. As discussed in the introduction, porins such as LamB typically form heat- and detergent-stable trimers in the outer membrane. The formation of these trimers is considered to be a hallmark of outer membrane localization. Therefore, we wished to determine whether the processing-defective precursors form such trimers in the presence of the suppressor plasmid. Figure 4 shows a type of pulse-chase experiment called a trimer assay (27). Cells are labeled with [³⁵S]methionine, and their proteins are then subjected to native immunoprecipitations. These conditions fail to dissociate either native or metastable porin trimers or to remove LPS from any assembly intermediates (27). Following immunoprecipitation, samples are resuspended in sample buffer and are

heated to 70°C to strip LPS molecules from the trimers and to denature all assembly intermediates. Finally, SDS-PAGE allows the resolution of stable trimers from the denatured monomeric species. Figure 4 (left) shows that in the presence of the control plasmid, precursor LamBA23D is eventually processed and goes on to form stable trimers. However, in the presence of the suppressor plasmid, very little trimer is formed. While it is not surprising that the precursor LamB has difficulty forming stable trimers, it appears that to some degree, the processed species also fails to form stable trimers, suggesting that the precursor may interfere with assembly of the processed species.

One possible explanation for these seemingly contradictory results is that the precursor LamBA23D is localized to the outer membrane but is incapable of forming trimers or trimers of sufficient stability to be resolved in the above assay (i.e., stable at 70°C). To determine whether the precursor was capable of forming other, more thermolabile assembly intermediates, we used the same immunoprecipitated samples from the previous experiment but instead heated them to 40°C prior to electrophoresis (Fig. 4, right). Under these conditions, the fully assembled native porin and the metastable trimer run near the top of the separating gel (45). This is presumably due to their failure to be dissociated from LPS at the lower temperature (32, 45). Furthermore, a folded monomeric intermediate can be distinguished by its increased electrophoretic mobility on SDS-PAGE gels relative to that of the fully denatured monomer (34). Figure 4 (right) shows that in the control strain, the various high-molecular-weight species do not form very efficiently, and a significant proportion of the precursor protein remains as a denatured monomer. This observation is consistent with the idea that signal sequence processing is required for assembly into higher-order species. However, in the presence of pKS17, this requirement appears to be bypassed, as both the precursor and mature species quickly chase into the folded monomer intermediate and the high-molecular-weight species. In addition, bands identified as LPS-associated material were excised, boiled, and reexamined by SDS-PAGE to determine whether they contained the processed or precursor forms of LamB. The bands isolated from control samples contained only mature LamB, while the bands isolated from suppressor strain samples contained both species (data not shown). Thus, it appears that the suppressor plasmid allows precursor LamBA23D to assemble into oligomeric intermediates and to associate with the outer membrane, even though it does not form stable native trimers.

We also performed the identical experiment to examine the kinetics of LamBA23D/A25D assembly. In the samples heated

FIG. 3. LamBA23D precursor localizes to the outer membrane in the presence of suppressor plasmid pKS17. Western immunoblot of an SDS-PAGE gel of cellular fractions derived from CLC279 (MC4100 *lamBA23D zjb-1::Tn10 degP41::kan/pACYC184*) and CLC300 (MC4100 *lamBA23D zjb-1::Tn10/pKS17*). Blots were probed with anti-LamB monomer and anti-MBP sera. Note that the *degP41::kan* allele (43) was used to stabilize precursor LamBA23D enough that it could be detected at steady state. Previous results by Carlson and Silhavy (5) showed the same fractionation pattern for precursor LamBA23D in a *degP⁺* background. Furthermore, the presence of a *degP* null allele in the pKS17 strain does not affect fractionation results (data not shown). Fractions: W, whole cell; S, soluble; T, total membrane; I, inner membrane; O, outer membrane. p, precursor; m, mature.

FIG. 4. The pKS17 suppressor plasmid allows assembly of LamBA23D. Strains CLC279 and CLC300 (see the legend to Fig. 3) were subjected to a pulse-chase labeling followed by native immunoprecipitations with anti-LamB monomer, anti-LamB trimer, and anti-MBP sera (27). Autoradiographs of SDS-PAGE gels are shown, with chase time points indicated at the bottom. Samples were heated to 70 or 40°C prior to electrophoresis. p, precursor; m, mature.

to 70°C, no stable trimers were observed in the presence of either plasmid (Fig. 5, top gel). This observation is consistent with the idea that precursors cannot be assembled into stable trimers. However, in the samples that were heated to 40°C, the presence of the suppressor plasmid allowed LamBA23D/A25D to form the same high-molecular-weight species and the putative folded monomeric species that were observed with LamBA23D (Fig. 5, bottom gel).

Precursor LamB is folded in the presence of pKS17. We then wished to demonstrate that the presence of the suppressor plasmid caused an actual conformational change in precursor LamB. To do this, we utilized two different preparations of anti-LamB polyclonal sera. One preparation was raised against denatured, monomeric LamB and recognizes this form exclusively (anti-LamB monomer), while the sera raised against purified LamB trimers (anti-LamB trimer) recognize the trim-

ers, metastable trimers, a folded monomeric species, and the denatured monomer to a lesser degree (6a, 27). Cells were labeled with [³⁵S]methionine for 30 s and were chased for 20 min with excess cold methionine. Cell lysates were prepared as described for the trimer assay, and labeled proteins were subjected to native immunoprecipitation either with the anti-LamB monomer sera alone or with both sera. The final immunoprecipitated samples were aliquoted into three tubes that were heated to 40, 70, or 100°C prior to electrophoresis.

Figure 6 shows immunoprecipitated proteins from wild-type (lanes 1 to 6), *lamBA23D* (lanes 7 to 18), and *lamBA23D/A25D* (lanes 19 to 30) strains. It is expected that wild-type LamB will be completely folded at 20 min postsynthesis, and in agreement with this prediction is our observation that the wild-type protein is not immunoprecipitated by the anti-LamB monomer sera (Fig. 6, lanes 1 to 3). It is, however, efficiently immunoprecipitated by the anti-LamB trimer sera (Fig. 6, lanes 4 to 6). In contrast, LamBA23D precursor (Fig. 6, lanes 7 to 9) and LamBA23D/A25D precursor (lanes 19 to 21) are immunoprecipitated by the anti-LamB monomer sera. Furthermore, the presence of both sera (Fig. 6, lanes 10 to 12 and 22 to 24) does not significantly increase the amount of precursor protein immunoprecipitated, suggesting that the precursor LamB is predominantly unfolded, even as late as 20 min postsynthesis. Finally, precursor LamB that is isolated from strains harboring the suppressor plasmid is recognized by the anti-LamB trimer sera (Fig. 6, lanes 16 to 18 for LamBA23D and lanes 28 to 30 for LamBA23D/A25D) and not by the anti-LamB monomer sera (lanes 13 to 15 for LamBA23D and lanes 25 to 27 for LamBA23D/A25D). Thus, we conclude that the presence of the suppressor plasmid, pKS17, results in a conformational change in precursor LamB from a denatured state to a folded state that is no longer recognized by sera specific for denatured LamB monomer but is instead recognized by sera raised against LamB trimers.

Multicopy suppression of precursor LamB toxicity by pKS17 does not require *degP* or *dgt*. We next sought to identify the component(s) of this plasmid relevant for suppression and for the relocalization and folding of precursor LamB. Figures 2 and 7 and Table 1 summarize the salient features of our analysis. Three components of pKS17 that code for protein products were considered to be candidates: the *degP* locus, the *dgt* locus, and the truncated *tetA'* locus that was generated by

FIG. 5. The pKS17 suppressor plasmid allows assembly of LamBA23D/A25D. Strains MC4100 and MDC11 (MC4100 *lamBA23D/A25D*) transformed with either pACYC184 or pKS17 were subjected to a pulse-chase labeling followed by native immunoprecipitations with anti-LamB monomer, anti-LamB trimer, and anti-MBP sera (27). Autoradiographs of SDS-PAGE gels are shown, with chase time points indicated at the bottom. Samples were heated to 70°C (top panel) or 40°C (bottom panel) prior to electrophoresis. p, precursor; m, mature.

FIG. 6. The presence of the suppressor plasmid allows the folding of precursor LamB. Strains CLC457 (MC4100 *zjb-1::Tn10kan/pKS17*), CLC397 (MC4100 *lamBA23D zjb-1::Tn10kan degP::Tn10/pACYC184*), CLC420 (MC4100 *lamBA23D zjb-1::Tn10kan/pKS17*), MDC24 (MC4100 *lamBA23D/A25D/pACYC184*), and MDC27 (MC4100 *lamBA23D/A25D/pKS17*) were subjected to 30 s of pulse-labeling followed by a 20-min chase. Samples were prepared and immunoprecipitated as described previously (27) with anti-MBP sera (all lanes), anti-LamB monomer sera (all lanes), and anti-LamB trimer sera (lanes indicated by the check marks). An autoradiograph of an SDS-PAGE gel is shown. The temperature to which each sample was heated prior to electrophoresis is shown at the bottom. An additional band is observed in lanes 24 and 30 (100°C) that runs slightly slower than the folded LamB monomer. We believe that this band does not represent LamB and that it may be OmpF/C, which occasionally cross-reacts with anti-LamB trimer sera. p, precursor; m, mature.

the initial insertion of chromosomal sequence into the *tetA* gene of pACYC184. As shown in Table 1, the bulk of the *dgt* locus could be deleted (pCLC8) without affecting the suppression of precursor LamB toxicity. Furthermore, the stabilization of precursor LamBA23D was likewise unaffected (data not shown). Similarly two stop codons could be introduced early in the *degP* coding sequence, *degP15(Oc)I7(Am)* (pCLC10), without eliminating suppression (Table 1) or stabilization (Fig. 7) of the precursor protein.

TABLE 1. Multicopy suppression of maltose sensitivity conferred by *lamBA23D*

<i>lamB</i> allele	Plasmid	Maltose sensitivity ^a
<i>lamB</i> ⁺	pACYC184	R
<i>lamBA23D</i>	pACYC184	S ^b
	pKS12	R
	pKS17	R
	pCLC8	R
	pCLC10	R
	pCLC11	R
	pCLC14	S
	pCLC19	R
	pBR322	S ^b
	pCLC20	R

^a Maltose sensitivity (determined as described in Materials and Methods) was scored in MC4100 or CLC567c (MC4100 *lamBA23D*). S, sensitive; R, resistant.

^b The presence of full-length *tetA* in pBR322 and pACYC184 enhances *lamBA23D* toxicity somewhat compared with isogenic plasmids that have had the entire *tetA* open reading frame removed (data not shown).

The truncated *tetA* locus is required for the folding of precursor LamB. We next sought to determine if the fusion joint generated by the insertion of a *Bam*HI fragment into pACYC184 was responsible for the multicopy suppression. Removal of the truncated *tetA'* locus (pCLC11) also failed to eliminate the suppression of maltose sensitivity conferred by the processing-defective LamB precursors. However, when this deletion is combined with the *degP15(Oc)I7(Am)* mutation (pCLC14), suppression is eliminated. Pulse-chase analysis of strains carrying these constructs provides an explanation for the phenotypes. As can be seen in Fig. 7, deletion of the *tetA'* fragment alone (pCLC11) eliminates stabilization of precursor LamBA23D and allows its accelerated degradation (compare with pACYC184). This degradation occurs via the DegP protease encoded by the plasmid because combining both mutations (pCLC14) results in wild-type stability of LamBA23D precursor (compare with pACYC184). Furthermore, we find that a plasmid that overproduces the DegP protease from a heterologous promoter, pPW100 (46), suppresses the toxicity conferred by LamBA23D but in a proteolysis-dependent manner (data not shown). Thus, while it is clear that DegP overproduction is sufficient to suppress the toxicity conferred by the processing-defective precursors, it is not the mechanism at work in pKS17. Rather, a novel mechanism appears to be dependent on the presence of the truncated *tetA'* locus and, furthermore, is epistatic to DegP-mediated degradation of LamB precursors. DegP protease produced by pKS17 is known to be active, as it complements a null mutation (43), and has been shown to enhance degradation of a LamB-LacZ-PhoA fusion protein in vivo (6a, 41).

FIG. 7. The truncated *tetA'* locus is required for stabilization of precursor LamBA23D. Strain JHC285Kan (MC4100 *lamBA23D zjb-1::Tn10kan*) was transformed with pACYC184 (CLC419), pKS17 (CLC420), pCLC11 (CLC491), pCLC10 (CLC448), and pCLC14 (CLC499). Pulse-chase analysis of strains carrying various multicopy suppressor constructs was performed as described previously (7). Samples were immunoprecipitated with anti-LamB monomer and anti-MBP sera. The autoradiograph of an SDS-PAGE gel is shown, with chase time points indicated at the bottom. p, precursor; m, mature.

FIG. 8. Expression of TetA' is required for stabilization of precursor LamBA23D. Strain JHC285 (MC4100 *lamBA23D zjb-1::Tn10*) was transformed with pBR322 (CLC504), with pCLC20 (CLC510), or with both pCLC20 and pBST324 (CLC511). Pulse-chase analysis was performed as described previously (7) in the absence (CLC504, CLC510, and CLC511) or presence (CLC511) of 10 μ g of tetracycline per ml. Samples were immunoprecipitated with anti-LamB monomer and anti-MBP sera. The autoradiograph of an SDS-PAGE gel is shown, with chase time points indicated at the bottom. The presence of pBST324 has no effects on the processing or stability of precursor LamBA23D in a pBR322 background (data not shown). p, precursor; m, mature.

Expression of *tetA'* is necessary and sufficient to facilitate precursor LamB folding. In order to demonstrate that the truncated *tetA'* locus was indeed necessary and sufficient for the folding-based suppression of precursor LamB, we attempted to show that the suppression phenotype could be reconstructed de novo. To do this, we introduced a spectinomycin resistance cassette (18) into the *Bam*HI site of plasmid vectors pACYC184 and pBR322, to generate plasmids pCLC19 and pCLC20, respectively (Fig. 2). The truncated polypeptide produced by these plasmids consists of the amino-terminal 98 amino acids of TetA, followed by a single arginine residue. Our sequence analysis of the fusion joint in pKS17 predicts a similar truncation consisting of the amino-terminal 98 amino acids of TetA followed by Pro-Met. These new constructs confer maltose resistance to *lamBA23D* and *lamBA23D/A25D* strains. Thus, the insert present in pKS17 is not required for the suppression phenotype, but rather it is the interruption of the *tetA* gene that appears to be responsible for this activity.

Next, we took advantage of the inducible nature of the *tetA* promoter to determine if transcription of this locus was required for stabilization of LamBA23D. In order to do this we utilized a plasmid (pBST324) that constitutively expresses the *tet* repressor (TetR) protein (1). Figure 8 shows the results of a pulse-chase analysis of *lamBA23D* strains carrying either a control plasmid (pBR322) or the suppressor plasmid (pCLC20). As expected, in the control strain carrying pBR322, precursor LamBA23D is unstable. In the presence of a suppressor plasmid carrying the truncated *tetA* locus (pCLC20), the precursor is stabilized. Addition of the TetR plasmid (pBST324) eliminates transcription from the *tetA* promoter as well as stabilization of precursor LamBA23D. Induction of the *tetA* promoter by addition of tetracycline reverses this effect, thus restoring precursor LamBA23D stabilization. Finally, pCLC19 and pCLC20 were found to confer folding of precursor LamB in experiments identical to those whose results are presented in Fig. 6. In the case of pCLC20, folding was dependent on expression of *tetA'* (data not shown). Based on these observations, we conclude that expression of the first 98 amino acids of the polytopic inner membrane protein, TetA, leads to the suppression of the toxicity conferred by precursor LamB by a novel folding-based mechanism.

Folding-based suppression mediated by TetA' does not involve the Cpx pathway or σ^E . We considered the possibility that the TetA'-mediated suppression of the processing-defective LamB might involve one of the two recently described pathways for responding to extracytoplasmic stresses, the Cpx pathway or the σ^E pathway. Therefore, we tested whether the presence of the suppressor plasmids, pKS17 and pCLC19, would induce either of these pathways. Induction of the Cpx pathway can be assayed by scoring the activity of a *lac* operon fusion to the *cpxP* gene, which is completely dependent on CpxR for its transcription (10). Similarly, *fkpA*, which encodes

a putative periplasmic peptidyl-prolyl isomerase that is activated by the σ^E RNA polymerase, serves as a reporter of σ^E activity (11). The activities of *cpxP-lacZ* and *fkpA-lacZ* operon fusions are unaffected by the presence of the suppressor plasmids as measured by colony color on MacConkey agar or tetrazolium indicator agar or by β -galactosidase assays. Therefore, it does not appear that expression of *tetA'* is altering the activity of either of these two extracytoplasmic-stress signalling pathways.

While it does not appear that the suppression of *lamBA23D* occurs by induction of either the Cpx or the σ^E pathway, the possibility remained that the target(s) of these pathways may be required for the folding-based suppression. Therefore, we transformed isogenic strains JHC285 (MC4100 *lamBA23D zjb-1::Tn10*) and CLC534 (JHC285 *cpxR::spc*) with the control (pACYC184) and multicopy suppressor (pKS17 and pCLC19) plasmids and found that the suppressors retain their ability to alleviate maltose sensitivity, even in the absence of the Cpx proteins or their downstream targets. We attempted this same experiment with an *rpoE::cam* allele, but we were unable to build a reliable *rpoE::cam lamBA23D* doubly mutant strain. It has recently been suggested that *rpoE* is an essential gene at all growth temperatures and that the *rpoE::cam* allele is tolerated only in the presence of an extragenic suppressor mutation (15). Since the processing-defective *lamB* mutations confer an extracytoplasmic stress, it would not be surprising if they are incompatible with an *rpoE* null mutation, even in the presence of the extragenic *rpoE* suppressor. Thus, while CpxR target loci are unlikely to be involved, we cannot exclude the possibility that factors regulated by σ^E are involved, even though *tetA'* expression does not appear to affect σ^E activity.

DISCUSSION

The folding and assembly of outer membrane proteins are complex, multistep processes. The mechanism(s) by which these proteins reach the outer membrane has been a topic of intensive investigation, and the cellular players involved in this process are presently being sought. In this study, we have characterized mutations in the gene encoding the outer membrane protein LamB, which we hope will shed light on the mechanism of outer membrane protein targeting. One of these mutations, *lamBA23D*, encodes a derivative of LamB that is partially defective for signal sequence processing (5). In addition, we have generated three new mutations that encode proteins that are not processed at all. As a result of the processing block, these proteins are tethered to the inner membrane via their signal sequences. In this study, we describe experiments that further clarify the source of the toxicity conferred by these porins, as well as a multicopy suppressor of processing-defective LamB that appears to act by a novel mechanism.

Why is precursor LamB toxic? Previous studies with *lamBA23D* strains provided evidence that the processing-defective porin was exerting an extracytoplasmic toxicity (5). While translocation of the precursor protein was required for this toxicity, it did not appear to affect the translocation process directly, as induction of *lamBA23D* does not result in the accumulation of precursors of other exported proteins (5). Fikes and Bassford (19) constructed a mutation in the gene for MBP that is analogous to the *lamBA23D* mutation. Like precursor LamBA23D, precursor MBP24-1 is tethered to the inner membrane via its signal sequence. However, unlike LamBA23D, the tethered MBP is never processed, and it is not toxic. In addition, precursor MBP24-1 localizes exclusively to the inner membrane, while precursor LamBA23D was observed to fractionate with both the inner and outer membranes. The unusual fractionation pattern of precursor LamBA23D is difficult to interpret. While it is possible that the protein localizes to both membranes simultaneously, it is also possible that some exists in the inner membrane while the rest is in the outer membrane. Based on these observations and comparison with the MBP24-1 protein, Carlson and Silhavy (5) concluded that LamBA23D is targeted to the outer membrane. They suggested that the mature portion of the protein might be associated with the outer membrane while the signal sequence remained attached to the inner membrane, thus facilitating an abnormal contact between the two membranes that leads to increased membrane permeability. However, the alternative possibility, that the presence of precursor protein in the outer membrane is toxic, could not be excluded.

During our analysis of the multicopy suppressor that is the subject of this communication, we studied the conformational status of the processing-defective LamB proteins in various backgrounds. As part of this analysis, we found that these precursors are recognized by polyclonal antiserum that is specific for denatured LamB (Fig. 6). This result demonstrated that in a wild-type background, the processing-defective precursors are unfolded. However, in the presence of the suppressor, the precursor fractionates exclusively to the outer membrane (Fig. 3), is folded (Fig. 6), and is not toxic. Based on these observations, we can exclude the possibility raised by Carlson and Silhavy (5), that the presence of precursor protein in the outer membrane is toxic. However, the alternative possibility, that precursor LamB is toxic because it is targeted to the outer membrane while still tethered to the inner membrane, remains valid. Indeed, in light of our finding that the precursor is unfolded, this view is supported by a study using a LamB porin with a temperature-sensitive assembly defect, which suggested that porins target the outer membrane at an early stage in their biogenesis, as unfolded monomers (27).

Our results raise the additional possibility that the presence of a denatured protein tethered to the inner membrane is the true source of toxicity. The fractionation of precursor LamB to both membranes might be explained as an artifact stemming from the fact that unfolded and/or aggregated proteins occasionally localize with the outer membrane in fractionation experiments. A recent study by Jones et al. (21) provides precedence for such a model. They find that when the P-pilus subunit protein, PapG, is overproduced in the absence of its corresponding chaperone, PapD, it remains in a denatured conformation in the cell envelope. Specifically, the denatured PapG protein appears to remain tethered to the inner membrane via a hydrophobic segment at the C terminus, although it is not yet clear whether this hydrophobic segment actually inserts into the membrane or whether it interacts with the membrane surface. Similar to the processing-defective LamB proteins, PapG overproduction is toxic. Furthermore, PapG

overproduction stimulates the stress-responsive Cpx pathway, as does overproduction of the processing-defective LamB proteins (6a). However, this model for precursor LamB toxicity does not readily account for the increase in outer membrane permeability observed when these proteins are overproduced. In order to distinguish between the alternative models, a higher-resolution analysis of precursor localization *in vivo* will be required.

Multicopy suppression mediated by truncated TetA' occurs by folding of precursor LamB and its localization to the outer membrane. The processing-defective LamB proteins are unfolded in a wild-type background. In contrast, we find that they are folded and localize to the outer membrane in the presence of the multicopy suppressor described here. This was demonstrated by several observations. First, precursor LamBA23D localizes almost exclusively to the outer membrane in the presence of the suppressor, suggesting that the tethered protein must have been released from the inner membrane. Second, precursor LamBA23D and LamBA23D/A25D are clearly recognized by polyclonal antiserum directed against folded LamB trimers and not by antiserum specific for denatured LamB, when cells contain the suppressor plasmids. This observation suggests that the precursor interacts with the outer membrane in a native-like fashion and that the fractionation result is not an artifact caused by aggregation. Third, native immunoprecipitations and SDS-PAGE of samples prepared at low temperatures reveal that these proteins fold into several previously described intermediates, in the presence of the suppressor plasmid. For example, these proteins are observed to assemble into a thermolabile high-molecular-weight species that has been shown to be associated with LPS and to represent metastable trimers (32, 45). In addition, a portion of the protein appears as a fast-migrating, folded monomer (34). Based on these observations, we propose that in the presence of the multicopy suppressor, the processing-defective precursors are folded and are assembled into the outer membrane in a pseudo-wild-type conformation. This conformation is not detrimental to cell growth, and thus, toxicity of the processing-defective LamB proteins is relieved by the suppressor plasmid. Interestingly, precursor assembly may also explain its increased stability in the suppressor background. Once assembled, this species becomes resistant to signal sequence processing and/or degradation. It is important to note that even when localized to the outer membrane, precursor LamB is not functional, as strains carrying the tight processing-defective *lamB* alleles are still λ^+ and Dex^- in the presence of the multicopy suppressor.

In a wild-type background, LamBA23D is eventually processed, and as a result, trimers are formed from the mature species. In contrast, we find a noticeable lack of LamB trimers in strains carrying the multicopy suppressor despite the fact that there is abundant processed protein present to support the formation of trimers (Fig. 4 and 6). We propose that the reason for the lack of trimers is that the precursor and mature LamB species are interacting to form mixed metastable trimers in this background. As a result, fewer trimers are formed from three processed subunits. Any metastable trimers containing one or more precursor molecules would be denatured at 70°C and would not be resolved in the experiments shown in Fig. 4 and 6. These metastable trimers appear as LPS-associated, high-molecular-weight bands at the top of the separating gel when the samples are heated to 40°C. We examined whether complexes composed of both precursor and mature LamB could be stripped of LPS and be identified as trimers at temperatures between 40 and 70°C. However, no such species was ever observed, and the LPS-associated complexes melt directly into the denatured monomeric species at temperatures of 55 to

FIG. 9. Diagrams of LamB assembly showing the folded monomer as an assembly intermediate (A) and as a product of dissociation of metastable trimers (B).

60°C (data not shown), reminiscent of the metastable trimer intermediate proposed by Vos-Scheperkeuter and Witholt (45). Like LamBA23D, the LamBA23D/A25D processing-deficient mutant forms a similar LPS-associated complex in a suppressor background that can be resolved when samples are heated to 40°C prior to electrophoresis (Fig. 5 and 6). This result demonstrates that the mature species is not required for an oligomeric association. These observations support a model in which the multicopy suppressor allows for the folding and outer membrane assembly of the unfolded toxic precursor protein into native-like metastable trimers, although the formal possibility remains that they may form another type of oligomer.

An alternative explanation for the lack of trimers might be that the multicopy suppressor serves to fold the tethered LamB protein into a conformation that is native-like but is incompetent for proper oligomerization and maturation. Some of the tethered protein is processed, but at a time at which it is too late to be properly assembled. By using an *in vitro* approach, de Cock et al. (14) have proposed that porins actually exist in an assembly-competent state for only a short time before folding into an off-pathway product that possesses elements of the native tertiary structure.

Is the folded monomer a bona fide assembly intermediate?

As discussed above, several labs have proposed that porins exist as folded monomeric intermediates prior to being assembled into dimers or metastable trimers. In the experiments whose results are shown in Fig. 4, 5, and 6, we too have observed the presence of a folded monomeric form of LamB. However, from the results shown in Fig. 4 and 5, our folded monomer does not appear to chase into the higher-molecular-weight species. We offer two possible explanations for this observation. First, it is possible that this folded monomeric species reflects a true assembly intermediate that is inefficiently chased into metastable trimers and is never chased into stable trimers due to the presence of a signal sequence (Fig. 9A). In the absence of the suppressor, the protein remains tethered to the inner membrane and cannot proceed beyond the denatured monomer.

An alternative explanation for the presence of the folded monomer in these experiments is that it is not an intermediate but rather a product of the *in vivo* or *in vitro* dissociation of the

mixed metastable trimers that never chase into stable trimers (Fig. 9B). Since these mixed metastable trimers are not formed in the nonsuppressed *lamBA23D* strains, the folded monomeric species would not be observed in those backgrounds.

In our studies, we have observed the folded monomer only with the processing-defective LamB proteins in the presence of the multicopy suppressor. We do not observe a folded monomeric species in the presence of a wild-type *lamB*⁺ allele (Fig. 5). Rouviere and Gross (34) detected a LamB folded monomer in wild-type strains using a pulse-chase assay identical to ours. The discrepancy between our results and theirs appears to be due to subtle strain differences between MC1061, used by those authors, and MC4100, used in our study (31a). If metastable trimers are less stable (either *in vivo* or during extraction) in MC1061 than they are in MC4100, the folded monomer would be observed and would still be expected to chase into mature trimers even though it is not a bona fide assembly intermediate, since wild-type metastable trimers will eventually become stable trimers (Fig. 9B). Alternatively, the folded monomer may be a true assembly intermediate that is immediately or concurrently assembled into an LPS-associated or oligomeric intermediate more efficiently in MC4100 than in MC1061, thus preventing its detection in MC4100. Since both metastable trimers and folded monomers appear to possess similar melting temperatures, it is difficult to distinguish between these possibilities. Perhaps the key to understanding the authenticity of this intermediate lies in the differences between MC4100 and MC1061.

Two mechanisms of suppression in the pKS17 suppressor.

In *lamBA23D* strains, the precursor protein is eventually processed or degraded. It was this instability that allowed us to first identify pKS17 as an interesting multicopy suppressor of LamBA23D. This suppressor plasmid was found to contain two discrete entities that can confer multicopy suppression. First, this plasmid contains a copy of the *degP* locus, which encodes a heat-inducible periplasmic protease. Indeed, pKS17 has been shown to suppress the toxicity of another envelope protein, the LamB-LacZ-PhoA fusion protein, by causing its degradation in a DegP-dependent fashion (6a, 41). Similarly, expression of DegP from a heterologous promoter, or from a derivative of pKS17, pCLC11 (Fig. 2), suppresses the toxicity conferred by processing-defective LamB by degradation. However, a second mode of suppression appears to be present in pKS17. Expression of the amino-terminal 98 residues of the TetA protein were shown to be sufficient for the folding-based suppression conferred by pKS17. Indeed, it appears as though this mechanism of suppression is epistatic to degradation by DegP. We propose that in the absence of the *tetA'* multicopy suppressor, precursor LamBA23D is unfolded, leaving it susceptible to DegP-dependent degradation. However, in the presence of the TetA' protein, precursor LamBA23D is folded and assembled into the outer membrane, thus protecting it from DegP. This result suggests that the factor(s) responsible for permitting precursor folding must shield it from proteolytic attack. Interestingly, we observed that plasmid constructs that carry only the truncated *tetA'* allele are mildly attenuated in their suppression of maltose sensitivity, compared with pKS17. This observation suggests that DegP may provide some indirect contribution to the relief of precursor LamB toxicity.

What is the actual mechanism by which TetA' mediates the folding and targeting of the processing-defective precursors? While there are a number of formal possibilities, we favor the explanation that expression of this truncated inner membrane protein induces a response that mediates the folding-based suppression. We envision two possible types of responses. First, since TetA' is itself a defective inner membrane protein,

its expression could induce a response that clears defective membrane proteins, with the processing-defective LamB protein being one such substrate. Once released from the inner membrane, the precursor porin would then be available for folding and membrane insertion by the normal cellular machinery. A second type of response might be one that increases the activity or alters the specificity of the machinery responsible for folding and assembly of outer membrane proteins. In this situation, precursor LamB would be folded and released from the inner membrane under circumstances in which it would normally be ignored by the cellular machinery. Indeed, it has been shown that the outer membrane lipoprotein chaperone, p20, removes substrates from the inner membrane (25). Similarly, PapD removes PapG from the inner membrane by binding the C-terminal peptide used by PapG to associate with the inner membrane (21).

We believe that the multicopy suppression of processing-defective LamB mediated by TetA' will provide a valuable tool for understanding the assembly of outer membrane proteins. Furthermore, since the suppressor plasmids do not appear to affect signalling by either of the two known pathways for responding to extracytoplasmic stress, we suggest that yet another stress-responsive regulon exists for protein trafficking in the bacterial envelope.

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