Overexpression of Protease-Deficient DegP_{S210A} Rescues the Lethal Phenotype of *Escherichia coli* OmpF Assembly Mutants in a *degP* Background

RAJEEV MISRA,^{1,2*} MARIA CASTILLOKELLER,^{1,2} AND MING DENG^{2,3}

Department of Microbiology,¹ and Molecular and Cell Biology Program,² Arizona State University, Tempe, Arizona 85287, and Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142³

Received 17 February 2000/Accepted 9 June 2000

Replacement of OmpF's conserved carboxy-terminal phenylalanine with dissimilar amino acids severely impaired its assembly into stable trimers. In some instances, interactions of mutant proteins with the outer membrane were also affected, as judged by their hypersensitivity phenotype. Synthesis of all mutant OmpF proteins elevated the expression of periplasmic protease DegP, and synthesis of most of them made its presence obligatory for cell viability. These results showed a critical role for DegP in the event of aberrant outer membrane protein assembly. The lethal phenotype of mutant OmpF proteins in a *degP* null background was eliminated when a protease-deficient DegP_{S210A} protein was overproduced. Our data showed that this rescue from lethality and a subsequent increase in mutant protein levels in the envelope did not lead to the proper assembly of the mutant proteins in the outer membrane. Rather, a detergent-soluble and thermolabile OmpF species resembling monomers accumulated in the mutants, and to a lesser extent in the parental strain, when DegP_{S210A} was overproduced. Interestingly, this also led to the localization of a significant amount of mutant polypeptides to the inner membrane, where DegP_{S210A} also fractionated. These results suggested that the DegP_{S210A}-mediated rescue from toxicity involved preferential sequestration of misfolded OmpF monomers from the normal assembly pathway.

The proper functioning of proteins requires their correct localization to predetermined cellular locations. In the gram-negative bacterium *Escherichia coli* K-12, a special class of proteins known as porins (24) must be properly targeted and assembled in the outer membrane to form channels that facilitate the diffusion of hydrophilic solutes. The atomic structure showed that porins primarily consist of antiparallel β strands that are arranged in a pseudocyclic β -barrel structure, which encloses a water-filled channel (6, 29). On the periplasmic side, β strands are adjoined by short turns, whereas long loops provide connections on the medium-exposed side. Although the majority of loops are surface exposed, one or more loops fold inward thus restricting the channel. Surface-exposed loops are often utilized by bacteriophages as their receptors (35, 36, 37).

Outer membrane proteins (OMPs) are synthesized in the cytoplasm as precursors with an amino-terminal signal sequence that assists in exiting the cytoplasm (26). Cleavage of the signal sequence on the periplasmic side yields mature molecules that contain the necessary information for outer-membrane targeting (2, 8). It is increasingly recognized that the targeting of mature porin molecules to the outer membrane is associated with folding events that allow monomers to gain structures crucial for assembly into protease- and heat-resistant trimers (11, 19, 22, 27, 38). Thus, targeting and folding are likely to be overlapping events. The issue of whether or not trimerization occurs prior to outer membrane insertion has not been fully resolved.

Besides intragenic information, proper assembly of OMPs relies on several extragenic factors. The periplasmic proteins that catalyze correct disulfide bond formation (DsbA) and peptidyl prolyl *cis-trans* isomerization (SurA and FkpA) have

profound effects on OMP assembly (for reviews see references 8 and 23). Periplasmic protein Skp shows affinity to unfolded soluble OMPs, thus suggesting its role as a general chaperone (5, 28). Besides proteins, the lipid components of the outer membrane, phospholipids and lipopolysaccharide (LPS), also play an important role in OMP assembly (1, 13, 14, 30). As LPS is exclusively localized in the outer membrane, it is possible that OMPs reach the outer membrane through their interactions with LPS.

Alterations within the primary sequence or extragenic factors can lead to defective OMP assembly. Assembly-incompetent intermediates tend to misfold and are diverted to aggregation and degradation pathways. Major protease DegP, whose activity resides in the periplasmic space, is responsible for the degradation of misfolded envelope proteins (16, 33). DegP's activity is essential when bacterial cultures are grown in rich media at 42°C. The reason for this essentiality is not entirely clear, but presumably a greater number of misfolded proteins accumulate under these growth conditions. In an exciting development concerning DegP, a recent study has shown that it contains both proteolytic and general chaperone activities (32). Aberrant OMP assembly in the envelope leads to elevated DegP levels (8, 9, 17, 23). Presumably a rise in proteolytic or chaperone activity assists cells in coping with the physiological stress by minimizing the population of misfolded polypeptides. Elevated DegP levels rely on the activation of an alternative sigma factor, σ^E , which controls *degP* transcription (17).

In this study, we carried out an *in vivo* analysis of OmpF mutants with altered carboxyl termini, which are occupied by phenylalanine residues in most OMPs. Tommassen's group first reported the significance of this conserved residue in PhoE biogenesis; its replacement or deletion affected PhoE's outer membrane incorporation and assembly into trypsin- and sodium dodecyl sulfate (SDS)-resistant trimers (7, 34). Re-

* Corresponding author. Mailing address: Department of Microbiology, Arizona State University, Tempe, AZ 85287-2701. Phone: (480) 965-3320. Fax: (480) 965-0098. E-mail: rajeev.misra@asu.edu.

FIG. 1. SDS-PAGE analysis of whole-cell envelopes obtained from strains expressing chromosomally encoded OmpF (lanes 1 and 2), chromosomally encoded OmpF205 (lanes 3 and 4), and plasmid-encoded OmpF205 under noninducing (without IPTG) growth conditions (lanes 5 and 6). Some strains also expressed OmpC (lanes 1, 3, and 5) or OmpG (lane 6).

placement of OmpF's terminal phenylalanine with unrelated residues affected one or more steps of assembly. Expression of assembly-defective OmpF proteins compelled DegP's presence, thus pointing to a critical role for DegP in mutant OmpF assembly.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. PLB3260 (Δ lamB106 *ompF-lacZ* fusion) and RAM831 (Δ lamB106 *ompF80 cog192* [OmpG⁺] *ompC-lacZ* fusion) were used as host strains for pTrc99A (Pharmacia) and its recombinant derivatives. A *degP::Km^r* null allele was obtained from KS474 (33). PND2000 (MC4100 *degP-lacZ* fusion) (9) was used to assess *degP*'s regulation. Luria broth and M63 salt-based minimal medium were prepared as described by Silhavy et al. (31). When cultures were grown on minimal medium, 0.4% glycerol was the supplemented carbon source. Maltodextrins were purchased from Pfansthil Laboratories and were further purified as described previously (21). When required, ampicillin (50 μ g/ml), chloramphenicol (25 μ g/ml), and kanamycin (25 μ g/ml) were added to the growth medium. Presoaked antibiotic discs were purchased from Difco. [³⁵S]methionine Expre protein labeling mixture was purchased from DuPont-New England Nuclear. Heat-killed *Staphylococcus aureus* cells (Pansorbin) were purchased from Calbiochem. DNA-modifying enzymes were purchased from Promega. All other chemicals were of analytical grade.

Cloning, mutagenesis, and other genetic manipulations. The *ompF205* gene (19) without its native promoter was amplified from the chromosome using primers complementary to the start (5'-GAGGGTAATAAACCATTGGTGAA GCGCAATATTCTGGCAGTG-3') and end (5'-GACGAGGATCCATTATG GTTACAGAAGG-3') of the gene. Restriction sites for *Nco*I (start of the gene; underlined) and *Bam*HI (end of the gene, underlined) were incorporated for cloning into a pTrc99A vector plasmid where an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter controlled the expression of *ompF205*. The first two residues of the terminal phenylalanine codon of *ompF* (UUC) were randomly altered in PCR by utilizing a mutagenic reverse primer (5'-GAGGG ATCCTATTAGNNCTGGTAAACGATACCCAC-3') and the forward *Nco*I primer listed above. The reverse primer incorporated a *Bam*HI site (underlined) and randomized nucleotides (NN) at positions corresponding to the first two residues of the terminal UUC codon of *ompF*. PCR fragments were restricted with *Nco*I and *Bam*HI and cloned into pTrc99A. DNA sequence analysis of several independent clones was carried out to reveal alterations at *ompF*'s terminal codon. The *ompF* gene and its IPTG-regulated promoter from plasmid clones were recombined first onto a lambda vector and then to the chromosome by a method described by Boyd et al. (4). Plasmid isolation, transformation, and P1 transduction were carried out utilizing standard laboratory protocols.

Protein methods. Whole-cell envelopes from cultures grown overnight were obtained by the French press lysis procedure (19). Envelope samples were analyzed by SDS-11% polyacrylamide gel electrophoresis (PAGE). For better resolution of OmpF from nearby protein bands, urea (4 M) was added to the running gel. Protein bands were visualized after staining gels with Coomassie blue. When required, envelope samples were treated with sodium sarcosyl (1% final concentration) at room temperature for 30 min. Sarcosyl-insoluble material was pelleted by centrifuging samples at 50,000 rpm for 30 min at 4°C in a tabletop ultracentrifuge (TLA120.2 rotor; Beckman).

Envelopes were separated into outer and inner membranes by centrifugation through sucrose density gradients. Sucrose gradients were prepared by layering in order the following percentages of sucrose into Beckman ultraclear ultracentrifuge tubes (13 by 5 mm): 600 μ l of 55% sucrose, 800 μ l of 50% sucrose, 800 μ l of 45% sucrose, 800 μ l of 40% sucrose, 800 μ l of 35% sucrose, and 800 μ l of 30% sucrose. Envelopes in 15% sucrose were loaded onto sucrose gradients, and tubes were centrifuged in a Beckman L8-80 ultracentrifuge using an SW 55Ti rotor at 50,000 rpm for 6 h at 4°C. After centrifugation, fractions were collected and the refractive index for each fraction was determined at 25°C. Densities for the fractions were determined by measuring the respective refractive indices.

For Western blot analysis, protein samples were analyzed on mini (7- by 8-cm)-SDS-polyacrylamide gels and transferred onto Immuno-Lite membranes (Bio-Rad) using a Mini Trans-blot electrophoretic transfer cell (Bio-Rad). Blots

were incubated with primary antibodies raised against denatured OmpF (1:10,000 dilution) for 90 min and then with the secondary antibody (goat anti-rabbit immunoglobulin G) for 1 h. Detection was carried out in accordance with the manufacturer's protocol (Bio-Rad).

Pulse-chase labeling and trimer assays. Protein-labeling experiments were carried out with cells that also produced OmpG (10). Constitutive expression of the OmpG porin provided identical growth patterns of strains expressing the various forms of OmpF. Pulse-chase labeling and trimer extractions were carried out as described previously (19, 22). Equal amounts of trichloroacetic acid (TCA)-precipitable radioactive counts were used for immunoprecipitating OmpF with polyclonal antibodies raised against trimers or denatured OmpF (19). Immunocomplexes were removed by heat-killed *Staphylococcus aureus* cells, solubilized in sample buffer, and analyzed by SDS-PAGE after heating at 62.5 or 100°C for 5 min. Gels were fluorographed, dried, and exposed to X-ray films at -70°C. Radioactive protein bands were quantified by a Bio-Rad gel analyzer.

β -Galactosidase assays. Assays were carried out as described by Miller (18).

RESULTS

Isolation of OmpF mutants with substitutions at F340. A variant *ompF* allele, *ompF205*, was utilized to obtain mutants with substitutions at the carboxy-terminal phenylalanine residue (F340). The single alteration of R82 to S in OmpF205 increases its channel size, thus permitting the transport of larger sugars such as maltodextrins (3). The Dex⁺ phenotype (ability to grow on maltodextrins) of OmpF205 thus provides a powerful tool for mutant isolation and characterization (19). The *ompF205* gene was cloned into plasmid vector pTrc99A, where an IPTG-inducible promoter controlled its expression. A comparison of envelope protein profiles from strains expressing OmpF205 either from the chromosome or from the plasmid under noninducing (without IPTG) conditions showed that they produced similar levels of the OmpF205 protein (Fig. 1; compare lanes 3 and 5).

The terminal F340 codon (UUC) of OmpF was altered through PCR in which the reverse primer had randomized nucleotides complementing the first two bases of the F340 codon. PCR-amplified products were cloned into pTrc99A, and recombinant plasmids were transformed into an OmpF⁻ LamB⁻ (Dex⁻) strain. Transformants were initially screened on the basis of their Dex phenotypes. Nucleotide sequence analysis of 36 plasmids with various Dex phenotypes revealed a total of 11 different codon substitutions placing eight different amino acid residues at the terminal position (Table 1).

Effects of F340 substitutions on OmpF levels and channel activities. Examination of envelopes obtained from cultures

TABLE 1. Summary of OmpF mutants with substitutions at the carboxy-terminal phenylalanine residue

Codon 340 substitution for UUC	OmpF340 residue	OmpF level ^a	Dex phenotype ^b	EOP (K20) ^c
None ^d	Phenylalanine	1.00	+++	1.00
UAC	Tyrosine	0.96	+++	0.94
CUC	Leucine	0.88	++	0.92
CCC	Proline	0.38	++	0.64
CAC	Histidine	0.24	+++	0.70
ACC	Threonine	0.14	+	0.65
UCC	Serine	0.13	+	0.48
CGC	Arginine	0.08	++	0.50
GCC	Alanine	0	-	>10 ⁻⁶

^a OmpF levels were determined from envelopes of cultures grown overnight without IPTG. Levels relative to that of the parental proteins are shown.

^b The Dex phenotype was scored on maltodextrin minimal medium after 24 h of incubation at 37°C. Colony sizes of mutants were compared with those of the parental strain.

^c EOP (K20), efficiency of plaquing for OmpF-specific bacteriophage K20.

^d Parental codon.

FIG. 2. Relative levels of newly synthesized total and trimeric OmpF. Cells expressing the various OmpF proteins were labeled for 20 s with [35 S]methionine and chased for 30 s with 20 mM nonradioactive methionine and chloramphenicol (200 μ g/ml). Immunoprecipitation conditions are described in the text. Immunocomplexes were solubilized in SDS sample buffer, heated for 5 min at 100°C, and analyzed by SDS-PAGE. OmpF-specific bands were quantified from two independent experiments, and values relative to the parental protein bands were graphed.

grown overnight revealed the extent of the defects in various mutants (Table 1). The F340Y and F340L substitutions produced relatively little effect on OmpF levels. Substitutions involving histidine and proline produced an intermediate effect, while those involving arginine, serine, and threonine greatly reduced the protein level. A substitution involving alanine produced the severest effect, as no OmpF could be detected.

As OmpF is the only means by which maltodextrins can enter these strains, the ability to grow on maltodextrin minimal medium reflected the channel properties of various OmpF proteins. Mutants expressing OmpF with the F340S or F340T substitution grew poorly, while no growth was registered by the mutant expressing OmpF with the F340A substitution (Table 1). The Dex⁻ or Dex-down phenotype of these three mutants correlated well with the level of OmpF in the membrane (Table 1). However, the protein level and Dex phenotype did not always correlate, as a mutant expressing OmpF with the F340R substitution had significantly reduced levels of OmpF in the envelope (8% of the parental level) but produced a Dex phenotype quite comparable to that of the parental strain (Table 1). The remaining mutant strains produced colonies with sizes very similar to that of the parental strain on maltodextrin minimal medium. With the exception of the mutant with an F340A substitution, all others allowed efficient infection by OmpF-specific bacteriophage K20 (Table 1).

Substitutions of F340 affect trimerization and not synthesis. To determine the levels of newly synthesized mature OmpF molecules and trimers, cells were labeled with [35 S]methionine for 20 s and chased for 30 s in the absence of new protein synthesis. This period was sufficient to complete the processing of any residual OmpF precursor molecules into mature forms. OmpF was solubilized from labeled cells by utilizing a method that efficiently extracts soluble and insoluble forms of the protein including SDS-resistant metastable and stable trimers (19, 22). Prior to immunoprecipitation, labeled protein extracts were divided into two halves, one of which was boiled to convert the various species of OmpF into a denatured form. Aliquots of denatured (boiled) or native (unboiled) cell extracts corresponding to an equal number of TCA-precipitable counts

were then used for immunoprecipitation with antibodies raised against denatured OmpF or native trimers, respectively. The data presented in Fig. 2 showed that levels of OmpF synthesis in all mutants were very similar to that from the parental strain. The slightly reduced levels of OmpF detected in the case of the proline, histidine, threonine, and alanine substitutions may reflect degradation immediately after synthesis. These results showed that the various substitutions at the terminal OmpF residue did not substantially interfere with the protein's synthesis and precursor processing.

Substitutions involving F340Y and F340L did not affect the protein's ability to form trimers, but the remaining mutants showed various degrees of defect (Fig. 2). The mutant with a F340A substitution produced no detectable trimers even though it produces mature monomeric species at levels similar to that of the parental protein (Fig. 2). These results showed that the primary effect of certain substitutions at F340 was at the level of trimer formation.

Substitutions of F340 affect trimerization kinetics. Trimerization kinetics were examined by labeling cells with [35 S]methionine for 20 s and chasing for 1, 2, 4, 6, and 8 min in the absence of new protein synthesis. Trimer-specific antibodies were used to immunoprecipitate trimers from aliquots of labeled cell extracts corresponding to an equal number of TCA-precipitable counts. After immunoprecipitation, precipitates were boiled to convert various trimeric species into denatured forms, which were then analyzed by SDS-PAGE. The results presented in Fig. 3 showed that, for the parental protein, trimers accumulated in two distinct steps: almost two-thirds of the total SDS-resistant trimers rapidly assembled within 1 min of chase, while the remaining one-third assembled with a slower kinetic pathway lasting 5 to 7 min. Mutants with an F340Y or F340L substitution had levels of trimers similar to that of the parental protein after 1 min of chase and displayed kinetics of trimerization very similar to that observed for the parental protein (Fig. 3).

Mutants with an F340T or F340S substitution amassed lower levels of trimers (66% of that of the parental protein) after 1 min of chase, with no further increase during subsequent chase periods. Significantly lower levels of trimers were present after 1 min of chase in mutants with an F340P, F340H, or F340R

FIG. 3. Kinetics of OmpF trimerization. Cells expressing the various OmpF proteins were labeled with [35 S]methionine for 20 s and chased with nonradioactive methionine in the presence of chloramphenicol. OmpF trimers extracted from samples withdrawn after various chase periods were immunoprecipitated with OmpF trimer-specific antibodies. Immunocomplexes were heated at 100°C for 5 min and analyzed by SDS-PAGE. OmpF-specific bands were quantified and plotted.

TABLE 2. *degP* expression and hypersensitivity of OmpF mutants

OmpF340 residue ^a or vector	Relative <i>degP</i> expression ^b	Growth inhibition ^c by:	
		Rifampin (5 µg)	Erythromycin (15 µg)
Vector	1.00	9.00	8.50 ^d
F340 (parent)	1.00	9.50	8.50 ^d
Y340	1.60 ± 0.06	10.75	8.75 ^d
L340	1.44 ± 0.07	10.25	8.25 ^d
P340	1.29 ± 0.10	16.50	14.00
H340	2.02 ± 0.16	14.00	10.50
T340	1.31 ± 0.15	11.25	8.75 ^d
S340	1.85 ± 0.18	14.00	11.00
R340	1.89 ± 0.06	15.00	11.75
A340	1.77 ± 0.01	11.25	9.00 ^d

^a OmpF proteins were expressed from plasmids (without IPTG) in a genetic background lacking the chromosomal OmpF protein.

^b *degP* expression was determined by utilizing a *degP::lacZ*⁺ construct. β-Galactosidase assays were done in triplicate. Cells were grown in Luria broth with ampicillin. Similar data were obtained from exponential cultures and cultures grown overnight.

^c Experiments to determine inhibition zones were carried out in triplicate. The diameter of presoaked antibiotic paper disks was 7 mm.

^d Fuzzy inhibition zones.

substitution. These mutants displayed an increase in trimers during further chase, but the trimer levels remained lower than that of the parental protein. Taken together, these results showed that all the mutants except the ones bearing an F340Y or F340L substitution were defective in their ability to form trimers from mature monomers. The assembly defect observed in several mutants leads to the degradation of mutant proteins, as a significantly reduced level of OmpF (F340P, F340H, F340T, F340S, F340R) or no OmpF (F340A) was detected from envelopes of cultures grown overnight (Table 1).

Up-regulation of *degP* and destabilization of the outer-membrane permeability barrier by mutant OmpF proteins. The expression of *degP* is known to be up-regulated in response to either the presence of misfolded proteins in the envelope or overexpression of OMPs (8, 9, 17). Plasmids expressing various OmpF proteins were introduced into a strain containing *degP::lacZ*⁺ so that the effect on *degP* expression could be analyzed by measuring β-galactosidase activity. The presence of a vector plasmid or a plasmid expressing the parental OmpF205 protein did not alter basal β-galactosidase activity and hence *degP* expression (Table 2). In contrast to what was found for the parental protein, the presence of plasmids expressing mutant proteins elevated *degP* expression (Table 2). As the mutant proteins were not overproduced, the elevated *degP* expression observed was likely due to the transient accumulation of misfolded assembly intermediates. It is interesting to note that the OmpF mutants with an F340Y or F340L substitution, which appeared normal in trimer assays (Fig. 3), also up-regulated *degP* transcription.

Normally the outer membrane provides an effective barrier against the entry of hydrophobic antibiotics, such as rifampin and erythromycin (25). However, the insertion and assembly of mutant OMPs can disrupt this barrier, causing hypersensitivity to hydrophobic antibiotics (15, 20). Cells expressing the various OmpF proteins were tested for sensitivity to rifampin and erythromycin. Expression of the parental OmpF205 as well as of two variants, one with an F340Y substitution and one with an F340L substitution, produced no significant change in antibiotic sensitivity compared to that of cells that only contained the vector plasmid DNA (Table 2). In contrast, the remaining OmpF mutants displayed significant increases in sensitivity

(Table 2). These results showed that the insertion and assembly of several OmpF F340 mutant proteins affected the outer-membrane permeability barrier.

DegP's involvement in the biogenesis of mutant OmpF proteins. The DegP protease has been shown to degrade misfolded proteins in the envelope (22, 33). We examined the role of DegP in the biogenesis of mutant OmpF proteins produced under noninducing (without IPTG) growth conditions. It was reasoned that if DegP is responsible for degrading mutant OmpFs, their levels might rise in cells devoid of DegP. On the other hand, if the accumulation of mutant OmpF proteins is toxic, the absence of DegP may cause lethality. Plasmids encoding OmpF205 and three variants with an F340Y, F340L, or F340A substitution were successfully introduced into a *degP* null strain at 30°C but not those expressing an OmpF with an F340P, F340H, F340R, F340S, or F340T substitution (Table 3). We surmise from these observations that DegP mediates the degradation of certain mutant proteins that become toxic if left to accumulate. Curiously, the expression of OmpF with an F340A substitution in a DegP[−] strain conferred temperature sensitivity at 37°C, suggesting an exaggerated assembly defect at the elevated growth temperature.

The inability to construct strains expressing certain mutant OmpFs in a background devoid of DegP could be due to the toxic accumulation of misfolded OmpF polypeptides. Since the plasmid constructs allowed for leaky expression of mutant proteins even in the absence of IPTG, the *ompF* alleles, together with their IPTG-inducible promoters, were recombined from plasmids in the chromosome via a lambda InCh phage vehicle (4). OmpF expression from the recombinant chromosomal alleles was drastically reduced, as now it could only be detected from cultures grown in the presence of IPTG (data not shown). As anticipated, lowering of OmpF expression well below physiological levels permitted the introduction of a *degP* null allele into all the OmpF mutant strains in the absence of IPTG at 30°C (Table 3). This was also the case at 37°C with the exception of the strain encoding OmpF with an F340R substitution. The presence of IPTG at 30°C produced normal growth, but at 37°C lethality was observed in strains expressing OmpF with an

TABLE 3. Growth of OmpF mutants without DegP and complementation by DegP_{S210A}^a

OmpF-340 residue	Growth without DegP at indicated temp of cells with:						Complementation by DegP _{S210A} in cells with Chr- OmpF in the pres- ence of IPTG ^b at:	
	Plasmid- OmpF		Chr-OmpF					
			No IPTG		With IPTG ^b		30°C	37°C
	30°C	37°C	30°C	37°C	30°C	37°C		
F340 (wt ^c)	+	+	+	+	+	+	+	+
Y340	+	+	+	+	+	+	ND	ND
L340	+	+	+	+	+	+	ND	ND
P340	(−)	(−)	+	+	+	−	+	+
H340	(−)	(−)	+	+	+	−	+	+
T340	(−)	(−)	+	+	+	−	+	+
S340	(−)	(−)	+	+	+	−	+	+
R340	(−)	(−)	+	−	−	−	+	+
A340	+	−	+	+	+	−	+	+

^a Plus sign, growth on all parts of the streak; minus sign, either no growth at all or no growth past the first streak on Luria agar medium; minus sign in parentheses, inability to construct OmpF mutants lacking DegP; Chr-OmpF, recombinant chromosomal alleles from which OmpF expression was controlled by an IPTG-inducible plasmid promoter; plasmid-OmpF, plasmid encoding OmpF; ND, not determined.

^b IPTG concentration was 0.4 mM.

^c wt, wild type.

FIG. 4. Effects of DegP_{S210A} on OmpF levels (A), detergent solubility (B), and membrane localization (C). OmpF was examined by Western blot analysis utilizing antibodies that recognized denatured OmpF and OmpA and an unknown protein migrating between OmpF and OmpA. All strains expressed wild-type DegP. Samples from strains overproducing DegP_{S210A} are indicated by a plus sign, an upward arrow, or the protein designation. Inner-membrane (IM) and outer-membrane (OM) fractions corresponding to buoyant densities of 1.15 and 1.22, respectively, were analyzed.

F340P, F340H, F340T, F340S, F340R, or F340A substitution (Table 3). As OmpF proteins were not overproduced, their aberrant assembly is the likely reason for the observed lethality. These results showed a critical role for DegP in cells expressing certain assembly-defective OmpFs.

Overproduction of protease-deficient DegP reverses the lethal phenotype of OmpF mutants. Recently it has been shown that DegP contains both protease and chaperone activities (32). Since the *degP* null allele utilized in this study disrupts both activities, it could not be concluded which of the two activities is essential in strains expressing mutant OmpF proteins. To address this, we utilized a variant DegP protein in which an S210A substitution abolished the protease activity without affecting its proposed chaperone function (32). Overproduction of DegP_{S210A} in a *degP* null background permitted the growth of all strains expressing mutant OmpF proteins at 37°C (Table 3), thus showing a protective role for protease-deficient DegP in overcoming the lethal effects of mutant OmpFs.

Results presented in Fig. 4A showed that overproduction of DegP_{S210A} increased the level of assembly-defective mutant OmpF proteins in envelopes compared to that observed in a DegP⁺ background. Interestingly, this was not the case for the assembly-proficient parental OmpF protein or OmpA. This showed that the DegP_{S210A}-mediated rescue from lethality did not result from the degradation of mutant OmpFs. Next we examined whether the elevated levels of mutant OmpFs coincided with their proper insertion in the outer membrane. This was tested by examining their solubility in sarcosyl, which normally does not solubilize properly inserted porin proteins. In a DegP⁺ background, OmpF proteins remained insoluble in sarcosyl, showing that they were inserted in the outer membrane (Fig. 4B). In contrast, a significant amount of mutant OmpFs became sarcosyl soluble when DegP_{S210A} was overproduced (Fig. 4B). Curiously, under these conditions even the parental OmpF protein was partially solubilized. It should be noted, however, that the ratio of the insoluble population of parental

proteins to the soluble population was significantly greater than those observed for mutant proteins. Interestingly, SDS-PAGE analysis revealed that OmpF from unheated samples of the sarcosyl-soluble fraction, but not from the sarcosyl-insoluble fraction, migrated at the denatured monomeric position (data not shown). This showed that a species resembling OmpF monomers accumulated when DegP_{S210A} was overproduced.

The sarcosyl solubility of mutant proteins suggested their aberrant insertion or localization to the envelope. To decide between these two possibilities, envelopes were fractionated into inner and outer membranes by sucrose density gradients. In this analysis we included envelopes from the parental strain and two different assembly-defective mutants expressing OmpF_{R340} or OmpF_{T340} in a DegP⁺ or DegP_{S210A}-overproducing background. The results showed that in a DegP⁺ background, all OmpF molecules were exclusively localized to the outer membrane (Fig. 4C). However, in a DegP_{S210A}-overproducing background, the parental protein localized primarily to the outer membrane, while mutant OmpFs fractionated with both membranes (Fig. 4C). Thus, overproduction of DegP_{S210A} promoted the localization of a significant population of mutant proteins to the inner membrane. It is important to note that under these conditions the localization of OmpA, like that of the assembly-proficient parental OmpF, remained unaltered.

DISCUSSION

The fact that phenylalanine occupies the carboxyl-terminal position of nearly all OMPs points to a role for this residue in biogenesis (34). Support for this notion initially came from mutant analysis in which the deletion or substitution of phenylalanine impaired PhoE's assembly (34). Curiously the assembly defect was observed only when the mutant PhoE proteins were overproduced (7). The *in vivo* data presented in this study provided many useful insights concerning the assembly and physiology of mutant OmpF proteins. Unlike what was found for PhoE, defects in OmpF assembly were observed even when OmpF was synthesized at a level similar to that produced from the chromosomal allele.

Assembly defects of OmpF F340 mutants. Substitutions of F340 with dissimilar amino acids drastically affected the assembly and stability of SDS-resistant metastable and stable trimers. The extent of assembly defect was dependent on the substituting amino acid residue. As the synthesis of mature monomers was virtually unaffected by the various substitutions, the lack of trimers was primarily due to a defect in an assembly step involving monomer trimerization. This defect was distinct from the one displayed by previously described OmpF assembly mutants, where a late assembly step involving the conversion of metastable trimers to stable trimers was conditionally blocked (19). The reduced ability to produce trimers suggested that substitutions at the terminal position might have affected events such as the correct folding of monomers or their proper interactions with assembly-promoting factors LPS and Skp.

Data presented here showed that the parental OmpF protein assembled into SDS-resistant trimers in two distinct steps. Assembly of about two-thirds of the trimers occurred through a fast-assembly pathway completed in around 1 min, whereas a slower pathway, requiring about 7 min, was utilized for the biogenesis of the remaining one-third of trimers. These observations suggested the existence of either two separate pathways for trimer assembly or two different steps of the same pathway. Several OmpF mutants studied here showed defects in one or both the steps or pathways. Replacement of F340 with either an aromatic (Y) or a hydrophobic (L) residue did not affect trimerization kinetics, suggesting that phenylalanine per se is

not critical at the terminal position. Yet its conservation among many OMPs suggests a particular role for phenylalanine that cannot be readily played by similar amino acids.

Physiological effects of mutant OmpF proteins. The data presented in this study showed that the expression of all mutant OmpF proteins elevated *degP* transcription, which is known to be up-regulated via σ^E in the event of aberrant envelope protein assembly (9, 17). Interestingly, although OmpF_{Y340} and OmpF_{L340} showed parental trimerization kinetics, they too induced *degP* expression to about the same extent as did the assembly-defective mutant OmpF proteins. So the amplitude of *degP* up-regulation did not truly reflect the degree of assembly defect observed. It is conceivable that, even though a greater pool of misfolded assembly intermediates accumulates in severely defective mutants than in those with no defect or a less severe defect, the rapid turnover of the signal for *degP* up-regulation causes the signal to be about the same in all cases. The hypersensitivity phenotypes of assembly mutants were more reflective of their assembly defects than was *degP* up-regulation. It could be that molecules which are not degraded as rapidly as those that induce *degP* upregulation confer the hypersensitive phenotype. Additionally, different threshold levels of molecules may be needed to produce diverse phenotypes.

Critical role for DegP in mutant OmpF assembly. As stated above, several mutant OmpF proteins were synthesized normally yet their levels in the envelope were significantly lower than that of the parental protein. This reflected their degradation prior to assembling into stable trimers. The degradation of mutant OmpF proteins appeared to be mediated by the DegP protease whose activity resides in the periplasm (33). DegP has been previously shown to play an important role during the biogenesis of envelope proteins in *E. coli* (22, 32, 33). Here we have shown that the requirement for DegP for the viability of cells expressing OmpF bearing an F340P, F340H, F340R, F340T, or F340S substitution was absolute. Initially, this conclusion was reached due to our inability to construct strains lacking DegP and simultaneously expressing the aforementioned mutant OmpF proteins from plasmids under noninducing conditions. However, when *ompF* genes from plasmids were recombined into the chromosome, the further lowering of protein expression allowed DegP to become dispensable. Interestingly, the requirement for DegP under these conditions became conditional, i.e., cellular toxicity was observed when mutant OmpF expression was induced by IPTG. It should be emphasized that induction with IPTG did not lead to overexpression of OmpF proteins from the recombinant chromosomal alleles; rather, their levels simply reached a point where the presence of DegP became mandatory. It is conceivable that in a DegP⁻ background, the accumulated mutant polypeptides acted as a "sink" for folding factors whose depletion resulted in disarrayed protein assembly and ultimately cell death. The results presented here thus provided genetic evidence for the crucial role of DegP in the event of aberrant OMP assembly.

Role of protease-deficient DegP in mutant OmpF assembly. Elevated expression of a variant DegP_{S210A} protein which is deficient in protease activity reversed the lethal effects of several OmpF mutants in a *degP* null background. This rescue from toxicity was not accomplished by promoting degradation of mutant proteins or correcting their assembly. Rather, it appeared that a significant population of mutant polypeptides was sequestered from the normal assembly pathway. While the level of correctly assembled (detergent-insoluble, thermostable, and outer-membrane-localized) molecules did not increase, a detergent-soluble and highly thermolabile species accumulated when DegP_{S210A} was overproduced. Curiously,

this overproduction of DegP_{S210A} also preferentially promoted the localization of mutant polypeptides to the inner membrane, where DegP_{S210A} itself is localized. This raises the possibility that the inner-membrane localization of mutant polypeptides occurs through their binding to DegP_{S210A}. Recent electron microscopic and cross-linking studies showed that DegP consists of a hexameric double-layered ring structure with a central cavity that has a higher affinity for unfolded than for folded polypeptides (12). In light of this, it is tempting to suggest that misfolded OmpF polypeptides are captured within the double-ring structure of DegP_{S210A}. This "molecular capturing" of misfolded polypeptides would clear the assembly pathway for OMP molecules that fold correctly and assemble into detergent-insoluble thermostable forms that are localized to the outer membrane.

The conditional and hypersensitive phenotypes of mutant OmpF proteins studied here open the door for further genetic and biochemical analyses that will assist us in better understanding the targeting and assembly of outer membrane proteins. Additionally, molecular and biochemical bases for the role of DegP_{S210A} in eliminating the toxic effect of mutant OMPs can now be studied.

ACKNOWLEDGMENTS

We thank Dana Boyd for providing lambda InCh strains and to Michael Ehrmann for a plasmid expressing DegP_{S210A}. We are grateful to Leanne Misra for critically reading the manuscript.

This work was supported by a grant from the National Institutes of General Medical Sciences (GM48167) to R.M.

REFERENCES

- Ames, G. F.-L., E. N. Spudich, and H. Nikaido. 1974. Protein composition of the outer membrane of *Salmonella typhimurium*: effect of lipopolysaccharide mutations. *J. Bacteriol.* **117**:406-416.
- Baker, K., N. Mackman, and I. B. Holland. 1987. Genetics and biochemistry of the assembly of proteins into the outer membrane of *E. coli*. *Prog. Biophys. Mol. Biol.* **49**:89-115.
- Benson, S. A., J. L. Occi, and B. A. Sampson. 1988. Mutations that alter the pore function of the OmpF porin of *Escherichia coli* K-12. *J. Mol. Biol.* **203**:961-970.
- Boyd, D., D. S. Weiss, J. C. Chen, and J. Beckwith. 2000. Towards single-copy gene expression systems making gene cloning physiologically relevant: lambda InCh, a simple *Escherichia coli* plasmid-chromosome shuttle system. *J. Bacteriol.* **182**:842-847.
- Chen, R., and U. Henning. 1996. A periplasmic protein (Skp) of *Escherichia coli* selectively binds a class of outer membrane proteins. *Mol. Microbiol.* **19**:1287-1294.
- Cowen, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghose, R. A. Paupit, J. N. Jansonius, and J. P. Rosenbusch. 1992. Crystal structures explain functional properties of two *E. coli* porins. *Nature (London)* **358**:727-733.
- de Cock, H., M. Struyvé, M. Kleerebezem, T. van der Krift, and J. Tommasen. 1997. Role of the carboxy-terminal phenylalanine in the biogenesis of outer membrane protein of PhoE of *Escherichia coli* K-12. *J. Mol. Biol.* **269**:473-478.
- Denese, P. N., and T. Silhavy. 1999. Targeting and assembly of periplasmic and outer membrane proteins in *Escherichia coli*. *Annu. Rev. Genet.* **32**:59-94.
- Denese, P. N., W. B. Snyder, C. L. Cosma, L. J. B. Davis, and T. Silhavy. 1995. The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the genes specifying the stress-inducible periplasmic protease, DegP. *Genes Dev.* **9**:387-398.
- Fajardo, D. F., J. Cheung, C. Ito, E. Sugawara, H. Nikaido, and R. Misra. 1998. Biochemistry and regulation of a novel *Escherichia coli* K-12 porin, OmpG, which produces unusually large channels. *J. Bacteriol.* **180**:4452-4459.
- Fouriel, D., S. Mizushima, and J.-M. Pages. 1992. Dynamics of the exposure of epitopes on OmpF, an outer membrane protein of *Escherichia coli*. *Eur. J. Biochem.* **206**:109-114.
- Kim, K. I., S.-C. Park, S. H. Kang, G.-W. Cheong, and C. H. Chung. 1999. Selective degradation of unfolded proteins by the self-compartmentalizing HtrA protease, a periplasmic heat-shock protein in *Escherichia coli*. *J. Mol. Biol.* **294**:1363-1374.
- Kloser, A. W., M. W. Laird, M. Deng, and R. Misra. 1998. Modulation in lipid A and phospholipid biosynthesis pathways influence outer membrane

- protein assembly in *Escherichia coli* K-12. *Mol. Microbiol.* **27**:1003–1008.
14. Laird, M. W., A. Kloser, and R. Misra. 1994. Assembly of LamB and OmpF in deep rough lipopolysaccharide mutants of *Escherichia coli* K-12. *J. Bacteriol.* **176**:2259–2264.
 15. Lathron, J. T., B. Y. Wei, G. A. Touchie, and R. J. Kadner. 1995. Sequences of the *Escherichia coli* BtuB protein essential for its insertion and function in the outer membrane. *J. Bacteriol.* **177**:6810–6819.
 16. Lipinska, B., O. Fayet, L. Baird, and C. Georgopoulos. 1989. Identification, characterization, and mapping of the *Escherichia coli* *htrA* gene, whose product is essential for growth only at elevated temperatures. *J. Bacteriol.* **171**:1574–1584.
 17. Mecsas, J., P. E. Rouviere, J. W. Erickson, T. J. Donohue, and C. A. Gross. 1993. The activity of sigma E, an *Escherichia coli* heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. *Genes Dev.* **7**:2618–2628.
 18. Miller, J. H. 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 19. Misra, R. 1993. OmpF assembly mutants of *Escherichia coli* K-12: isolation, characterization, and suppressor analysis. *J. Bacteriol.* **175**:5049–5056.
 20. Misra, R. 1993. A novel *ompC* mutation of *Escherichia coli* K-12 that reduces OmpC and OmpF levels in the outer membrane. *Mol. Microbiol.* **10**:1029–1035.
 21. Misra, R., and S. A. Benson. 1988. Isolation and characterization of OmpC porin mutants with altered pore properties. *J. Bacteriol.* **170**:528–533.
 22. Misra, R., A. Peterson, T. Ferenci, and T. J. Silhavy. 1991. A genetic approach for analyzing the pathway of LamB assembly into the outer membrane of *Escherichia coli*. *J. Biol. Chem.* **266**:13592–13597.
 23. Missiakas, D., and S. Raina. 1997. Protein folding in the bacterial periplasm. *J. Bacteriol.* **179**:2465–2471.
 24. Nikaido, H. 1994. Porins and specific diffusion channels in bacterial membranes. *J. Biol. Chem.* **269**:3905–3908.
 25. Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* **49**:1–32.
 26. Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* **57**:50–108.
 27. Reid, J., H. Fung, K. Gehring, P. E. Klebba, and H. Nikaido. 1988. Targeting of porin to the outer membrane of *Escherichia coli*: rate of trimer assembly and identification of a dimer intermediate. *J. Biol. Chem.* **263**:7753–7759.
 28. Schäfer, U., K. Beck, and M. Müller. 1999. Skp, a molecular chaperone of gram-negative bacteria, is required for the formation of soluble periplasmic intermediates of outer membrane proteins. *J. Biol. Chem.* **274**:24567–24574.
 29. Schirmer, T. 1998. General and specific porins from bacterial outer membrane. *J. Struct. Biol.* **121**:101–109.
 30. Sen, K., and H. Nikaido. 1991. Lipopolysaccharide structure required for in vitro trimerization of *Escherichia coli* OmpF porin. *J. Bacteriol.* **173**:926–928.
 31. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 32. Spiess, C., A. Bell, and M. Ehrmann. 1999. A temperature-dependent switch from chaperones to protease in a widely conserved heat shock protein. *Cell* **97**:339–347.
 33. Strauch, K. L., K. Johnson, and J. Beckwith. 1989. Characterization of *degP*, a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. *J. Bacteriol.* **171**:2689–2696.
 34. Struyvé, M., M. Moons, and J. Tommassen. 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. *J. Mol. Biol.* **218**:141–148.
 35. Traurig, M., and R. Misra. 1999. Identification of bacteriophage K20 receptor binding regions of OmpF and lipopolysaccharide in *Escherichia coli* K12. *FEMS Microbiol. Lett.* **181**:101–108.
 36. Vakharia, H., and R. Misra. 1996. A genetic approach for analyzing surface-exposed regions of the OmpC protein of *Escherichia coli* K-12. *Mol. Microbiol.* **19**:881–889.
 37. van der Ley, P., and J. Tommassen. 1987. PhoE protein structure and function, p. 159–163. In A. Torriani-Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil. (ed.), Phosphate metabolism and regulation in microorganisms. American Society for Microbiology, Washington, D.C.
 38. vos-Scheperkeuter, G. H., and B. Witholt. 1984. Assembly pathway of newly synthesized LamB protein, an outer membrane protein of *Escherichia coli* K-12. *J. Mol. Biol.* **175**:511–528.
 39. Xiong, X., J. N. Deeter, and R. Misra. 1996. Assembly-defective OmpC mutants of *Escherichia coli* K-12. *J. Bacteriol.* **178**:1213–1215.