

Protease-Deficient DegP Suppresses Lethal Effects of a Mutant OmpC Protein by Its Capture

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The expression of assembly-defective outer membrane proteins can confer lethality if they are not degraded by envelope proteases. We report here that the expression of a mutant OmpC protein, OmpC_{2Cys}, which forms disulfide bonds in the periplasm due to the presence of two non-native cysteine residues, is lethal in cells lacking the major periplasmic protease, DegP. This lethality is not observed in *dsbA* strains that have diminished ability to form periplasmic disulfide bonds. Our data show that this OmpC_{2Cys}-mediated lethality in a *degP::Km^r dsbA⁺* background can be reversed by a DegP variant, DegP_{S210A}, that is devoid of its proteolytic activity but retains its reported chaperone activity. However, DegP_{S210A} does not reverse the lethal effect of OmpC_{2Cys} by correcting its assembly but rather by capturing misfolded mutant OmpC polypeptides and thus removing them from the assembly pathway. Displacement of OmpC_{2Cys} by DegP_{S210A} also alleviates the negative effect that the mutant OmpC protein has on wild-type OmpF.

OmpC and OmpF represent a major class of *Escherichia coli* outer membrane proteins (OMPs). These proteins form water-filled channels that permit the diffusion of small solutes into the cell. Because of this property, OmpC and OmpF are called porins or sometimes “classical” porins, which refer to their stable β -barrel structure and nonspecific solute diffusion properties (17). Each porin monomer consists of a 16-stranded antiparallel β -barrel (22). These porins exist as homotrimers, in which three barrels assemble to produce three separate channels (22).

Besides their atomic structures, the assembly and targeting of the outer membrane β -barrel proteins have been an intense topic of study (for a recent review, please see reference 6). Based on studies conducted in several laboratories, it has been shown that the assembly of porin proteins begins with the proteolytic cleavage of an amino-terminal signal sequence that yields soluble nascent monomers. Rapid folding of these monomers is followed by their oligomerization into protease- and heat-sensitive trimers called metastable trimers, which slowly mature into protease- and heat-resistant trimers, the final product of assembly.

Although porins *in vitro* can be fully folded into their native trimeric structures from denatured monomers without the need of an accessory protein (7, 27), assembly *in vivo* is highly dependent on several cellular folding factors. Among them are specific periplasmic foldases such as SurA and FkpA, which catalyze *cis-trans* peptidyl-prolyl isomerization (16, 20), although it has never been demonstrated that this enzymatic activity is actually involved in OMP assembly. Consistent with this notion, Behrens et al. (3) have recently shown that SurA assists in OMP assembly independent of its isomerase activity. Another periplasmic foldase, DsbA, is involved in promoting

correct disulfide bond formation of certain OMPs (2). The wild-type sequence of the classical porins, however, lacks cysteine residues and thus they assemble independently of DsbA's activity. In contrast to wild-type OmpC, the assembly of a mutant OmpC protein, OmpC_{2Cys}, containing two non-native cysteine residues (C74 and C154) is drastically influenced by DsbA (11). Since OmpC_{2Cys} is the subject of the present study, it is further discussed below.

Another periplasmic protein, Skp, is thought to assist in OMP assembly by acting as a general chaperone (5, 21). However, the *skp*-null mutant phenotype showing a dramatic reduction in OMP levels could not be repeated in other laboratories since it was first reported. Recent genetic studies have shown that the presence of *skp*- and *surA*-null alleles in a strain confers a synthetic lethal phenotype (19). Based on these studies, it has been proposed that Skp and SurA are components of separate folding pathways providing chaperone activities (19). Nonproteinaceous components, including phospholipids and lipopolysaccharide (which is exclusively localized on the outer leaflet of the outer membrane), are also important for OMP assembly (1, 9, 18).

Periplasmic and envelope proteases become particularly important when the machinery that ensures correct OMP assembly malfunctions or the primary sequence of an OMP endures an unacceptable alteration. One periplasmic protease, DegP, has received the most attention in connection with OMP assembly. *degP*-null mutants do not grow at temperatures above 39°C (10, 25, 26), presumably because of a greater need for the removal of misfolded proteins at elevated temperatures. However, expression of certain misfolded OMPs renders the DegP's presence essential even at lower growth temperatures (14). Elegant work carried out by Spiess et al. (25) showed that DegP, in addition to proteolysis, assists in the correct folding of a periplasmic protein. A recent genetic study also argued for DegP's involvement in providing periplasmic chaperone activities (19); however, this contention was not backed by direct biochemical evidence.

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We have previously described the isolation of a novel class of OmpC mutants that affected either the channel or phage receptor property of the protein (13, 28). One such OmpC mutant carrying a R74C substitution functionally enlarged the channel to permit the entry of large sugars such as maltodextrins, which are normally excluded from the OmpC channel (13). This isolate was subsequently used for the isolation of phage-resistant mutants under conditions that demanded the retention of a functional OmpC channel but no phage receptor activity. A group of OmpC mutants from this selection contained a G154C substitution while retaining the original R74C alteration (11). This mutant OmpC protein, OmpC_{2Cys}, which now had two non-native cysteine residues in its mature sequence, conferred phage resistance due to a biogenesis defect (11).

The OmpC_{2Cys} biogenesis defect was shown to stem from the protein's ability to form disulfide bonds in DsbA⁺ cells (30); OmpC_{2Cys} assembles normally in a genetic background lacking DsbA. OmpC_{2Cys} produces a novel transdominant effect on OmpF (11). Since the effect on OmpF is specific, this suggests that either the inhibition was due to the mixing of mutant OmpC assembly intermediates with that of OmpF or that the two proteins share a common assembly factor.

Just as noted for several assembly-defective OmpF mutants (14), it was discovered that the expression of OmpC_{2Cys} in a DsbA⁺ background obligated the presence of DegP at all growth temperatures. As anticipated, in a background lacking DsbA, and hence with a diminished disulfide forming ability in the periplasm, the absence of DegP produced no lethal effects at growth temperatures of $\leq 37^\circ\text{C}$.

We investigated here the effect of a protease-deficient DegP, DegP_{S210A}, on OmpC_{2Cys}-mediated lethality in a *degP*-null strain. Our findings provide direct biochemical evidence showing that DegP_{S210A} alleviates OmpC_{2Cys}'s lethal effects not by improving its folding or assembly but rather by sequestering misfolded mutant OmpC polypeptides and thus removing them from the normal assembly pathway.

MATERIALS AND METHODS

Media, reagents, and bacterial strains. Luria broth medium, Luria agar (1.5% [wt/vol]) medium, and M63 salt-based minimal medium were prepared according to the method of Silhavy et al. (23). When cultures were grown on minimal medium, 0.4% glycerol and 0.1 mM amino acid mixture lacking methionine were added. When required, ampicillin (50 $\mu\text{g/ml}$), kanamycin (25 $\mu\text{g/ml}$), chloramphenicol (25 $\mu\text{g/ml}$), tetracycline (25 $\mu\text{g/ml}$), and IPTG (isopropyl- β -D-thiogalactopyranoside; 0.4 mM) were added to the growth medium. Heat-killed *Staphylococcus aureus* (Pansorbin) was purchased from Calbiochem. [³⁵S]methionine Expre protein labeling mixture was purchased from Du Pont-New England Nuclear. All other chemicals were of analytical grade. The bacterial strains used in the present study are listed in Table 1.

Pulse-chase labeling. Pulse-chase labeling was performed essentially as described previously (14). Cells were grown overnight at 37°C in glycerol minimal medium supplemented with 0.1 mM amino acid mixture. After a 1-in-30 dilution of overnight culture into fresh medium supplemented with 0.4 mM IPTG, cells were grown to an optical density at 600 nm of 0.3. Cells were pulse-labeled with [³⁵S]methionine (20 $\mu\text{Ci/ml}$) for 20 s and then chased with cold methionine (20 mM) in the presence of a protein synthesis inhibitor, spectinomycin (200 $\mu\text{g/ml}$). Samples (0.5 ml) were taken 0.5, 1, 2, 5, 10, and 20 min postchase and immediately mixed with 0.5 ml of ice-cold 15% trichloroacetic acid. Cells were centrifuged at $12,000 \times g$ for 5 min at 4°C . Pellets were washed once with 0.5 ml of 5% trichloroacetic acid and once with 95% ethanol and then dried under a vacuum. Dried pellets were resuspended in 200 μl of sodium dodecyl sulfate (SDS) buffer (20 mM Tris-HCl, pH 7.5; 10 mM EDTA; 1% SDS) and boiled for 5 min. Cell

TABLE 1. Strains used in this study

Strain	Relevant characteristics ^a	Source or reference
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>) <i>U139 rpsL150</i> <i>flbB5301 ptsF25 deoC1 thi-1 rbsR relA</i>	4
PLB3261	MC4100 Δ <i>lamB106</i> ϕ <i>ompC'</i> :: <i>lacZ</i> ⁺	S. Benson
RAM412	PLB3261 <i>ompC234</i> (OmpC _{1Cys}) <i>zei</i> ::Tn10 (50% linkage to <i>ompC</i> by P1 transduction)	12
RAM415	PLB3261 <i>ompC402</i> (OmpC _{2Cys}) <i>zei</i> ::Tn10	12
RAM1015	MC4100 <i>lamB677</i> (LamB amber)	Lab strain
RAM1025	RAM1015 <i>degP</i> ::Km ^r	Lab strain
RAM1120	RAM1025 pCS10 (DegP _{S210A})	This study
RAM1121	RAM1025 pACYC184	This study
RAM1122	RAM1015 pCS10	This study
RAM1123	RAM1015 pACYC184	This study
RAM1124	PLB3261 <i>degP</i> ::Km ^r pCS10	This study
RAM1125	RAM412 <i>degP</i> ::Km ^r pCS10	This study
RAM1126	RAM415 <i>degP</i> ::Km ^r pCS10	This study
RAM1127	RAM415 <i>recA</i> ::Km ^r	This study
RAM1128	RAM415 <i>recA</i> ::Km ^r pCS21 (DegP _{S210A-6His})	This study

^a Km^r, kanamycin resistance.

lysates (100 μl) were transferred into tubes containing 1 ml of the immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5; 20 mM NaCl; 1% Triton X-100) and MalE and LamB antibodies. Tubes were incubated overnight at 4°C on a rocker. To bring down immunocomplexes, heat-killed *S. aureus* cells (50 μl) were added, and tubes were incubated at 4°C on a rocker for at least 4 h. Immunocomplexes were recovered by centrifugation and washed twice in immunoprecipitation buffer, twice in Tris buffer (10 mM Tris-HCl; pH 7.5), and once in water. Washed pellets were resuspended in sample buffer, boiled, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were fluorographed, dried, and exposed to X-ray films at -85°C . Radioactive protein bands were quantified by using a Bio-Rad gel analyzer.

Membrane analysis. Whole-cell envelopes were extracted by the French cell lysis method as described previously (12). The detergent solubility of membrane proteins was tested by mixing an equal amount of membrane and 2% Sarkosyl in 20 mM Tris-HCl (pH 7.5). The mixture was incubated for 30 min at room temperature and then centrifuged in a Beckman benchtop ultracentrifuge at $105,000 \times g$ for 30 min at 4°C with a TLA 120 rotor. Both the supernatant and the detergent-insoluble pellet were recovered for protein analysis.

Separation of inner and outer membranes from whole-cell envelopes was achieved by sucrose (30 to 55%) density gradients according to the method of Misra et al. (14). After centrifugation, fractions were collected and the refractive index for each fraction was determined at 25°C . Densities for each fraction were calculated by their respective refractive index. Samples from each fraction were analyzed by SDS-PAGE.

Interaction of OmpC_{2Cys} with DegP_{S210A}. In vivo interactions between OmpC_{2Cys} and DegP_{S210A} were examined by purifying the soluble His tag variant of DegP_{S210A} (DegP_{S210A-6His}) through Ni²⁺ affinity chromatography. Subsequently, fractions containing DegP_{S210A-6His} were examined for the presence of OmpC_{2Cys} by Western blot analysis. Briefly, 5 ml of cells containing pCS21 (in which the expression of DegP_{S210A-6His} is inducible by IPTG) were grown to an optical density at 600 nm of 0.2 in the presence of 0.4 mM IPTG. Cells were pelleted and resuspended in 2 ml of 10 mM imidazole containing 20 μl of freshly prepared lysozyme (100 mg/ml). Cells were lysed by a small French pressure cell and, after the removal of unlysed cells by low-speed centrifugation, lysates were centrifuged at $105,000 \times g$ in a 50 Ti rotor for 1 h at 4°C to pellet the membranes. DegP_{S210A-6His} was purified from the supernatant by affinity chromatography in a Ni²⁺-nitrilotriacetic acid column according to the Pharmacia Biotech HisTrap purification protocol, except that the elution of DegP_{S210A-6His} was carried out in several steps involving a gradual increase in the imidazole concentration from 20 to 200 mM. The DegP_{S210A-6His} protein eluted with 200 mM imidazole.

Proteins were analyzed by SDS-PAGE and transferred into Immuno Lite membranes by using a Mini Transblot electrophoretic transfer cell (Bio-Rad). Blotted proteins were incubated for 90 min with primary antibodies raised against DegP (1:10,000 dilution) and OmpC (1:10,000 dilution) and then with the secondary antibody (goat anti-rabbit immunoglobulin G) for 1 h. Detection was carried out according to the manufacturer's protocol (Bio-Rad). Anti-DegP antibodies were kindly provided by Jon Beckwith (Harvard).

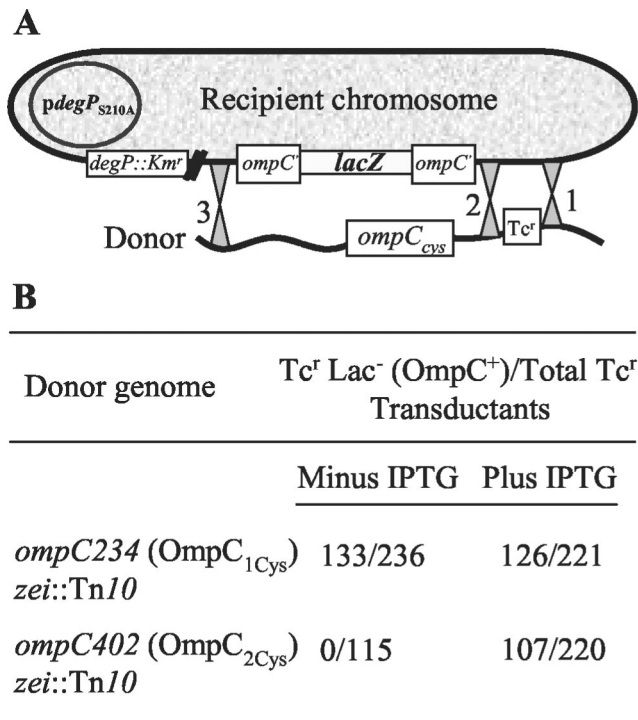


FIG. 1. (A) Diagram showing two possible P1-mediated crossover events between the donor (*ompC234* [OmpC_{1Cys}] *zei::Tn10* or *ompC402* [OmpC_{2Cys}] *zei::Tn10*) and the recipient (*ompC'::lacZ'* *degP::Km^r/pdegP_{S210A}*) DNA. The cotransductional linkage between the *zei::Tn10* (the selectable marker) and *ompC* is 50%. A crossover between 1 and 2 will yield tetracycline-resistant (Tc^r) Lac⁺ (*ompC'::lacZ'*) transductants, whereas a crossover between 1 and 3 will yield Tc^r Lac⁻ (*ompC*) transductants. (B) P1 transduction results drawn from diagram presented in panel A.

Protein density levels from Coomassie blue-stained gels and Western blots were determined by using Scion Image Analysis software.

RESULTS AND DISCUSSION

Overexpression of a protease-deficient DegP_{S210A} eliminates the toxic effect of OmpC_{2Cys}. We have previously observed that the expression of assembly-defective OmpF mutants is lethal in a DegP⁻ background (14). This is presumably because DegP degrades misfolded proteins, thus eliminating their toxic effects. As found for the assembly-defective OmpF mutants, the expression of OmpC_{2Cys} also demanded the presence of DegP for cell viability. Initially, this conclusion was reached due to our inability to introduce, through P1 transduction, the *ompC402* (OmpC_{2Cys}) allele into a *degP::Km^r* strain. Subsequently, direct evidence for this synthetic lethality was obtained by demonstrating that the *ompC402* allele could be transduced into a *degP::Km^r* background only when the expression of a protease-deficient DegP_{S210A} protein was simultaneously induced from a plasmid replicon (Fig. 1). In contrast, the parental *ompC234* (OmpC_{1Cys}) allele was transducible into a *degP::Km^r* background at the expected frequency independent of DegP_{S210A}'s expression (Fig. 1B).

DegP_{S210A} does not stimulate OmpC_{2Cys} degradation. Since substitution of the only serine residue of DegP to alanine

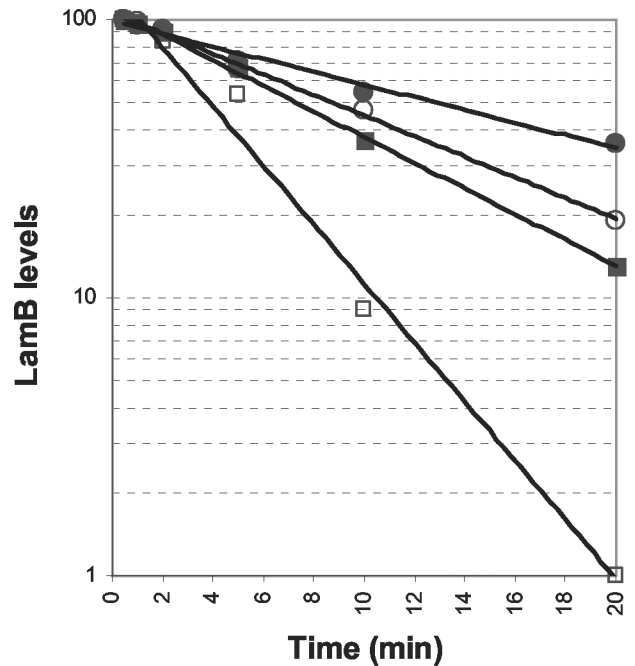


FIG. 2. In vivo degradation of a LamB amber fragment in various genetic backgrounds. Freshly grown cells were subjected to pulse-chase analysis as described in Materials and Methods. LamB and MalE (as a gel loading control) were immunoprecipitated from [³⁵S]methionine-labeled protein extracts obtained from DegP⁺ (□), DegP⁻ (■), DegP⁺/DegP_{S210A} (○), and DegP⁻/DegP_{S210A} (●). The highest LamB level was taken as 100, and the other LamB values were adjusted accordingly.

(S210A) is known to make it proteolytically inactive (29), it is unlikely that the rescue from OmpC_{2Cys}-mediated lethality by DegP_{S210A} involved degradation of the mutant protein. To substantiate this, we first confirmed that the overexpression of DegP_{S210A} did not directly or indirectly induce proteolysis of mutant envelope proteins. For this we utilized a LamB amber fragment whose in vivo degradation is largely dependent on DegP (Fig. 2). Pulse-chase experiments showed that overexpression of DegP_{S210A} did not increase the rate of degradation of the LamB amber fragment (Fig. 2). Instead, overexpression of DegP_{S210A} in a DegP⁺ background increased the half-life of the LamB amber fragment (Fig. 2), thus suggesting a protective rather than a destructive role for the variant DegP_{S210A} protein. This protective role of DegP_{S210A} was most apparent in a background devoid of wild-type DegP. These results clearly demonstrated that the overexpression of DegP_{S210A} did not involve increased proteolytic activity in vivo.

Next, we tested the effect of DegP_{S210A} overexpression on mutant OmpC and wild-type OmpF. As found for the LamB amber fragment, the level of OmpC_{2Cys} in the envelope increased when DegP_{S210A} was overexpressed (Fig. 3). This result confirmed that the rescue from lethality did not involve degradation of the mutant protein. As mentioned earlier, the expression of OmpC_{2Cys} imposes a negative effect on the assembly of the wild-type OmpF protein, presumably by interfering with the oligomerization of OmpF monomers or sequestering a common assembly factor (11). Under conditions in which DegP_{S210A} overexpression elevated OmpC_{2Cys} levels, it

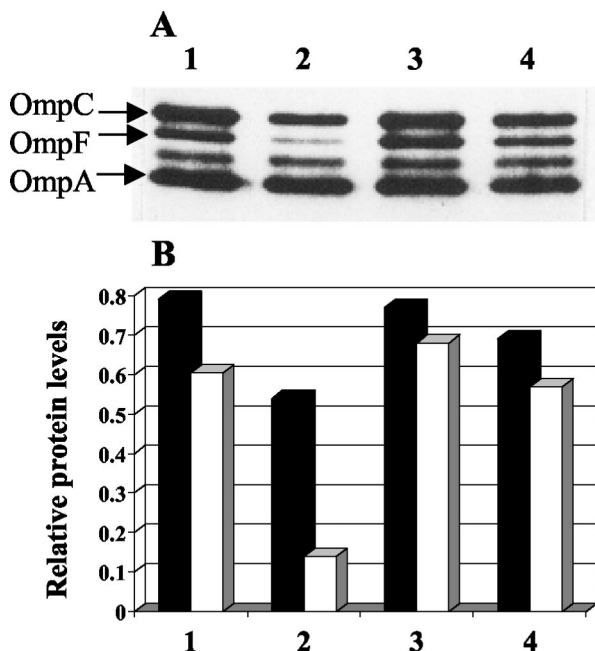


FIG. 3. Effect of DegP_{S210A} on OmpC and OmpF. (A) Envelopes obtained from DegP⁺ OmpC_{1Cys} (lane 1), DegP⁺ OmpC_{2Cys} (lane 2), DegP_{S210A} OmpC_{1Cys} (lane 3), and DegP_{S210A} OmpC_{2Cys} (lane 4) were examined by SDS-PAGE. OmpC, OmpF, and OmpA bands were identified by Western blot analysis. (B) OmpC (solid bars) and OmpF (open bars) levels from panel A were quantified relative to OmpA.

also elevated OmpF levels, thus abolishing the negative effect of OmpC_{2Cys} on OmpF (Fig. 3, compare lanes 2 and 4).

DegP_{S210A} increases the level of improperly assembled OmpC. The elevation of OmpC_{2Cys} levels and a concomitant increase in wild-type OmpF levels suggested that overexpression of DegP_{S210} improved the envelope's assembly or folding environment. Consistent with this notion, it was demonstrated by Spiess et al. (25) that the protease-deficient DegP_{S210A} protein promoted proper folding of a periplasmic protein MalS. Also, based on genetic data, it has been recently argued that DegP may be involved as a general chaperone in the folding and assembly of OMPs (19). We therefore tested the possibility that the elevated OmpC_{2Cys} levels caused by DegP_{S210A} overexpression may be due to its improved assembly. For this we examined the solubility of membrane-bound OmpC in the anionic detergent Sarkosyl, which is known to solubilize inner membrane proteins and proteins that are loosely bound to the outer membrane. Since improperly assembled OMPs may not be correctly inserted in the outer membrane, they could be solubilized by Sarkosyl.

In the absence of DegP_{S210A} overexpression, the parental and mutant OmpC proteins fractionated with the membrane pellet from untreated envelopes (Fig. 4, lanes 1 and 2). However, when DegP_{S210A} was overexpressed, a significant portion of OmpC_{2Cys}, and to some extent OmpC_{1Cys}, became soluble even without detergent treatment (Fig. 4, lanes 3 and 4). When membranes obtained from cells not overexpressing DegP_{S210A} were treated with Sarkosyl, OmpC_{2Cys}, like the parental OmpC_{1Cys} protein, was present primarily in the Sarkosyl-insoluble membrane pellet (Fig. 4, lanes 5 and 6 [top panel]),

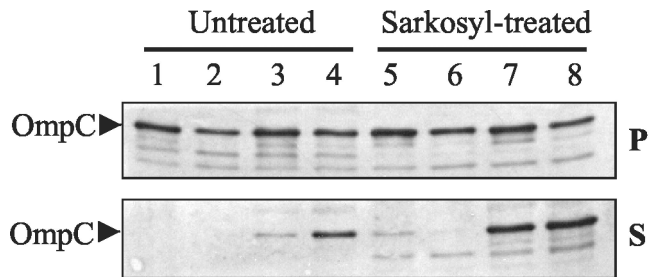


FIG. 4. Sarkosyl solubility of membrane-associated OmpC. Purified whole-cell envelopes were treated either with just buffer (Untreated) or with Sarkosyl as indicated. After incubation, samples were centrifuged to separate Sarkosyl-soluble proteins (S, supernatant) from envelopes containing Sarkosyl-insoluble proteins (P, pellet). OmpC from each fraction was detected by Western blot analysis with polyclonal antibodies specific to OmpC. Lanes: 1 and 5, DegP⁺ OmpC_{1Cys}; 2 and 6, DegP⁺ OmpC_{2Cys}; 3 and 7, DegP_{S210A} OmpC_{1Cys}; 4 and 8, DegP_{S210A} OmpC_{2Cys}.

suggesting that at least as far as detergent solubility is concerned it was properly inserted in the outer membrane. Detergent treatment of membranes obtained from DegP_{S210A}-overexpressing strains dislodged a large quantity of both OmpC species from the envelope (Fig. 4, lanes 7 and 8 [bottom panel]). These results showed that the DegP_{S210A}-mediated increase in OmpC levels did not lead to the proper assembly or insertion of OmpC in the envelope. This finding was further supported by the observation that the increase in the OmpC_{2Cys} level did not make cells sensitive to OmpC-specific bacteriophages, reflecting the continued presence of a conformationally defective OmpC_{2Cys}. The observation that overexpression of DegP_{S210A} causes the parental OmpC_{1Cys} protein to become detergent soluble suggests that porin assembly intermediates in general can be targeted by DegP_{S210A}. Consistent with this notion we found that a fraction of the assembly-competent wild-type OmpC protein was also subjected to DegP_{S210A}-mediated detergent solubilization (data not shown). The lack of this effect on OmpA by DegP_{S210A} overexpression may reflect distinct folding properties of its assembly intermediates compared to that of the trimeric porin proteins.

DegP_{S210A} alters OmpC localization. Since an increase in the mutant OmpC level by DegP_{S210A} overexpression did not lead to its proper assembly or insertion, we were curious as to its localization in the cell envelope. This was examined by fractionating whole-cell envelopes into inner and outer membranes by using sucrose density gradients (Fig. 5). OmpC and OmpA (as a control protein) levels were quantified from the inner (buoyant densities of 1.15 to 1.16) and outer membrane (buoyant densities of 1.21 to 1.22) fractions (Table 2).

Remarkably, overexpression of DegP_{S210A} caused the localization of some OmpC_{2Cys} molecules to the inner membrane (Fig. 5) such that approximately four times more OmpC_{2Cys} was present there compared to that in a DegP⁺ background (Table 2). This increase in the inner membrane was not due to an overall increase in mutant protein levels because, in contrast to the inner membrane, the level of OmpC_{2Cys} decreased in the outer membrane (Table 2). Interestingly, the parental OmpC_{1Cys} protein was also subjected to this effect of

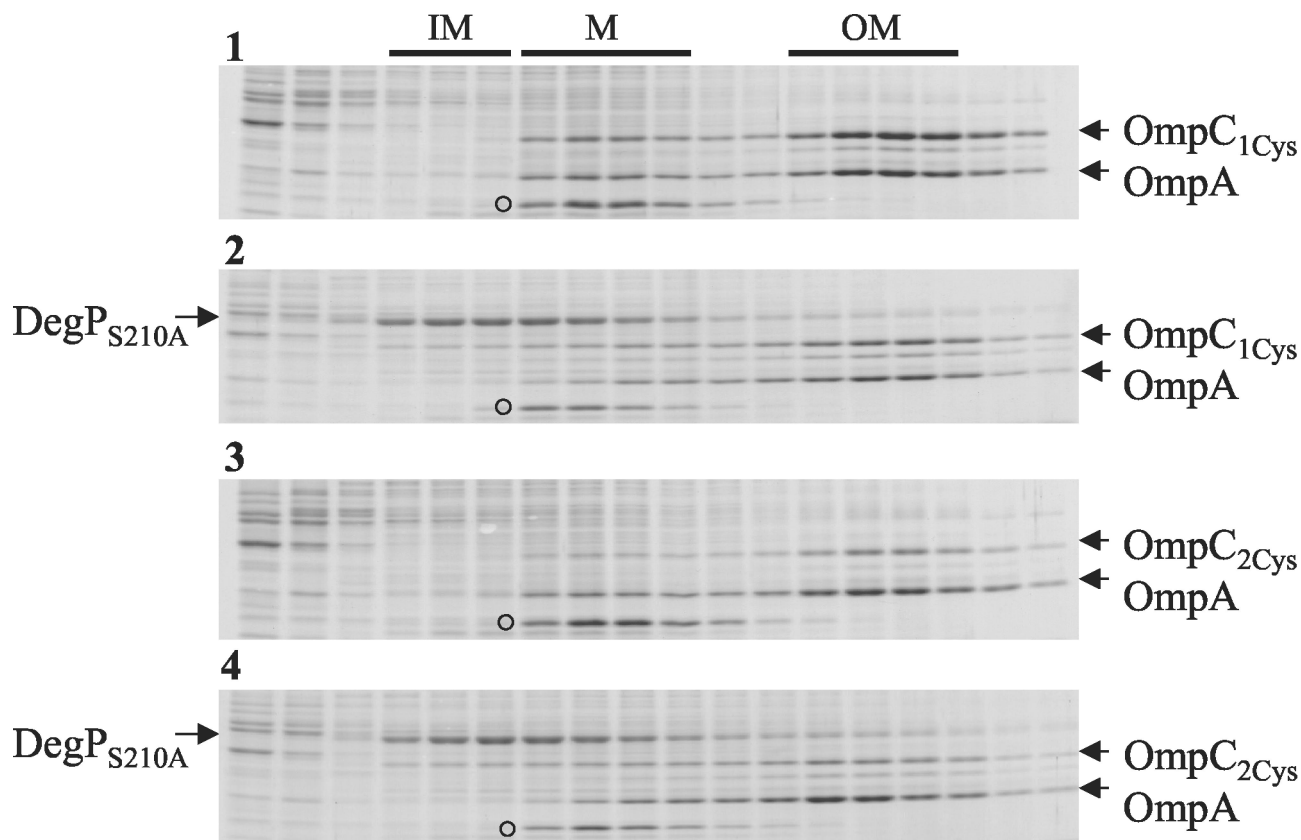


FIG. 5. Sucrose density gradient analyses of whole-cell envelopes. Envelopes obtained from DegP⁺ OmpC_{1Cys}, DegP_{S210A} OmpC_{1Cys}, DegP⁺ OmpC_{2Cys}, and DegP_{S210A} OmpC_{2Cys} strains (panels 1 to 4, respectively) were fractionated into inner and outer membranes by sucrose density gradients. After centrifugation, fractions were collected from top and analyzed by SDS-PAGE. Protein bands were visualized by Coomassie blue staining. Fractions corresponding to inner membrane (IM), intermediate density material (M), and outer membrane (OM) are shown on top. Positions of OmpC, OmpA, DegP_{S210A}, and a protein band (○) that primarily localizes with the intermediate-density material are indicated.

DegP_{S210A} overexpression (Fig. 5), although a greater influence was observed for OmpC_{2Cys} (Table 2). Unlike these variant OmpC proteins, overexpression of DegP_{S210A} had no effect on OmpA's localization (Fig. 5 and Table 2); thus, this DegP_{S210A}-mediated relocalization was specific to the mutant proteins.

Interestingly, fractionation studies revealed that a significant portion of DegP_{S210A} was also localized in the inner membrane (Fig. 5). The inner membrane localization of DegP_{S210A} is consistent with a detailed fractionation analysis carried out previously by Skorko-Glonek et al. (24). The colocalization of DegP_{S210A} and OmpC in the inner membrane raises the pos-

sibility of direct interactions between these proteins. It is important to mention here that our fractionation studies showed that significant amounts of both DegP_{S210A} and mutant OmpC proteins were also present in the soluble fraction; the data showing enhanced solubility of mutant OmpC due to increased DegP_{S210A} expression are presented in Fig. 4. Experiments to examine a possible direct interaction between DegP_{S210A} and OmpC_{2Cys} from soluble fractions are described below.

Inner membrane-localized OmpC molecules have characteristics of unassembled polypeptides. Properly assembled porin molecules are resistant to heat denaturation and detergent solubility. Heat sensitivity assays (Fig. 6) showed that the inner membrane-localized OmpC_{1Cys} and OmpC_{2Cys} denatured into monomers even when analyzed at room temperature (25°C). On the other hand, outer membrane-localized OmpC proteins displayed heat resistance that is characteristic of properly folded stable trimers (Fig. 6). Additionally, the inner membrane-localized OmpC molecules, in contrast to those localized in the outer membrane, could be completely solubilized by Sarkosyl (data not shown). Together, these results indicated that the inner membrane-localized OmpC molecules most likely represent unassembled polypeptides.

TABLE 2. Distribution of OmpC and OmpA in outer and inner membrane fractions

Relevant protein composition	Distribution of OmpC (%)		Distribution of OmpA (%)	
	Outer membrane	Inner membrane	Outer membrane	Inner membrane
OmpC _{1Cys} DegP ⁺	94	6	91	9
OmpC _{1Cys} DegP _{S210A}	82	18	94	6
OmpC _{2Cys} DegP ⁺	92	8	90	10
OmpC _{2Cys} DegP _{S210A}	69	31	92	8

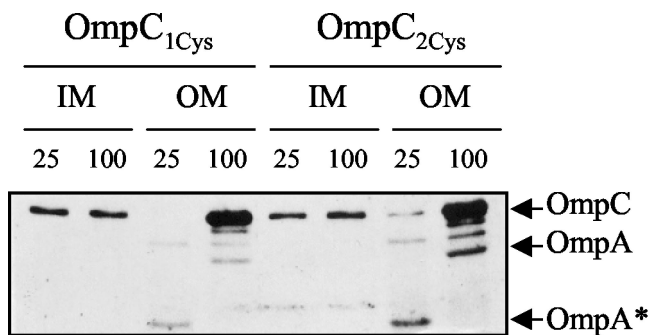


FIG. 6. Heat sensitivity of OmpC_{1Cys} and OmpC_{2Cys}. Equal amounts of samples from the inner membrane (IM) and outer membrane (OM) were incubated at 25 or 100°C as indicated for 15 min prior to SDS-PAGE. OmpC was detected by Western blot analysis with a mixture of antibodies specific to OmpC and OmpA. OmpA* is a heat-modifiable form of OmpA.

DegP_{S210A} directly interacts with OmpC_{2Cys}. As stated above, colocalization of subpopulations of OmpC_{2Cys} and a fraction of DegP_{S210A} in both the inner membrane and soluble fractions raised the possibility that these two proteins engage in direct interactions. To examine this, a variant DegP_{S210A} containing a His₆ tag was utilized. The His₆-tagged DegP_{S210A} protein (DegP_{S210A-6His}) was expressed in a background also expressing OmpC_{2Cys}, and the soluble tagged DegP protein was purified through affinity chromatography with Ni²⁺ columns. Affinity-bound DegP_{S210A-6His} was eluted with imidazole, and the eluted material was tested for the presence of mutant OmpC.

Four equal aliquots of the fraction containing eluted DegP_{S210A-6His} were analyzed in separate lanes on a polyacrylamide gel and, after electroblotting, the filter was cut into strips corresponding to individual lanes and then hybridized with antibodies raised against DegP, OmpC, DegP plus OmpC, or OmpC plus OmpA (Fig. 7). The results showed unequivocally that the mutant OmpC_{2Cys} polypeptides coeluted with the DegP_{S210A-6His} protein (Fig. 7, lane 2). The absence of OmpA

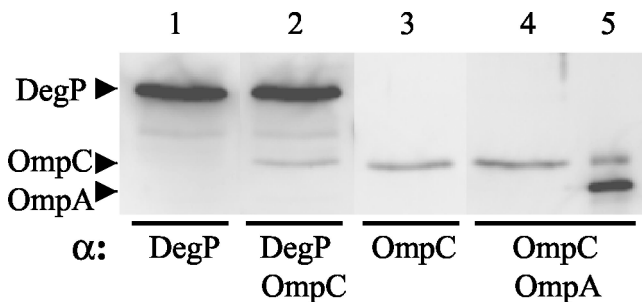


FIG. 7. Direct interaction between DegP_{S210A-6His} and OmpC_{2Cys}. Freshly grown cells expressing DegP_{S210A-6His} and OmpC_{2Cys} were lysed as described in Materials and Methods. Membrane-free cell lysates were subjected to Ni²⁺-nitrilotriacetic acid column chromatography to purify DegP_{S210A-6His}. Four identical samples (lanes 1 to 4) of the eluted fraction containing DegP_{S210A-6His} were subjected to SDS-PAGE and Western blot analyses with the indicated antibodies. An aliquot of outer membrane containing OmpC and OmpA proteins was analyzed in lane 5.

from the eluted DegP_{S210A} signified the specificity of the DegP_{S210A}-OmpC interaction (Fig. 7, lane 4). These results are consistent with the fractionation analysis (Table 2), which also showed a specific effect of DegP_{S210A} on OmpC_{2Cys} but not on OmpA.

These results corroborated the notion that overexpression of DegP_{S210A} relieved the toxicity of the mutant OmpC protein not by correcting assembly but rather by sequestering it. Capturing of the mutant OmpC_{2Cys} protein by DegP_{S210A} also alleviated the transdominant effect of mutant OmpC on OmpF's assembly (Fig. 3).

DegP and OMP assembly. Both genetic (14, 15, 19) and biochemical (25) studies support the notion that DegP plays an important role in the assembly of envelope proteins. Initially, this was thought to be solely due to the degradation of mutant envelope proteins. However, the finding of Spiess et al. (25) suggested that DegP is also involved in the biogenesis of at least one periplasmic protein, MalS, as a general chaperone. Despite this exciting discovery, no other *E. coli* protein has been shown to be dependent on DegP's chaperone activity for its assembly; thus, the notion that DegP can act as a "general" chaperone still awaits further biochemical verification.

The data presented here and published earlier (14) showed that a protease-deficient DegP_{S210A}, which retains its chaperone activity (25), stabilizes assembly-defective OMPs without actually improving their assembly. As demonstrated for OmpC_{2Cys} in the present study, the stabilization of mutant proteins likely involves their sequestration by the polypeptide-binding cavity of DegP_{S210A} (8). Such binding could improve the assembly of other OMPs indirectly by eliminating misfolded polypeptides that could directly interfere with the assembly of normal polypeptide chains. Also, misfolded polypeptides could potentially sequester folding factors and thus indirectly influence the assembly of normal proteins, so their removal by DegP_{S210A} could prevent such a negative effect on overall assembly in the periplasm.

It can be argued that the overexpression of DegP_{S210A} failed to improve OmpC_{2Cys} assembly because the covalent disulfide bond formation would intrinsically disrupt normal assembly. Indeed, the absence of these bonds in a *dsbA* background allows normal OmpC_{2Cys} assembly (11, 30). However, the assembly of OmpF mutants, in which the defect originates from noncovalent disruptions, was also not improved by DegP_{S210A} overexpression (14). It thus remains to be seen whether DegP_{S210A} assists OMP assembly by acting as a general chaperone. Elegant genetic studies carried out in Silhavy's lab showed that the synthetically lethal phenotype of *degP::Tn10 surA::Km^r* could be reversed by a plasmid expressing protease-deficient DegP_{S210A} at a growth temperature of ≤37°C (19). Whether the observed reversibility by DegP_{S210A} is through actually promoting assembly or sequestering misfolded polypeptides, as we have shown here, remained to be determined.

The recently solved crystal structure of protease-deficient DegP provides some exciting insights into the functioning of this protein (8). It suggests that partially folded polypeptides may be fed into DegP's central cavity which, in an open conformation, is accessible through the lateral pores. The presence of hydrophobic phenylalanine residues in the central cavity is likely to play an important role in substrate interaction. It

is proposed that at lower temperatures, when the protease activity is dormant, unfolded substrates are released from DegP for refolding. In the case of substrates such as OmpC_{2Cys}, where correct folding may be unattainable due to the covalent disulfide bond formation, the protein appears to be trapped eternally in this binding-release cycle.

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