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Effect of OmpA Signal Peptide Mutations on OmpA Secretion, Synthesis, and Assembly

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In previous investigations, we have examined the effect of OmpA signal peptide mutations on the secretion of the two heterologous proteins TEM β-lactamase and nuclease A. During these studies, we observed that a given signal peptide mutation could affect differentially the processing of precursor OmpA-nuclease or precursor OmpA-lactamase. This observation led us to further investigate the influence of the mature region of a precursor protein on protein export. Preexisting OmpA signal peptide mutations of known secretion phenotype when directing heterologous protein export (nuclease A or β-lactamase) were fused to the homologous mature OmpA protein. Four signal peptide mutations that have previously been shown to prevent export of nuclease A and β-lactamase were found to support OmpA protein export, albeit at reduced rates. This remarkable retention of export activity by severely defective precursor OmpA signal peptide mutants may be due to the ability of mature OmpA to interact with the cytoplasmic membrane. In addition, these same signal peptide mutations can affect the level of OmpA synthesis as well as its proper assembly in the outer membrane of *Escherichia coli*. Two signal peptide mutations dramatically stimulate the rate of precursor OmpA synthesis three- to fivefold above the level observed when a wild-type signal peptide is directing export. The complete removal of the OmpA signal peptide does not result in increased OmpA synthesis. This finding suggests that the signal peptide mutations function positively to stimulate OmpA synthesis, rather than bypass a down-regulatory mechanism effected by a wild-type signal peptide. Overproduction of wild-type precursor OmpA or precursors containing signal peptide mutations which lead to relatively minor kinetic processing defects results in accumulation of an improperly assembled OmpA species (imp-OmpA). In contrast, signal peptide mutations which cause relatively severe processing defects accumulate no or only small quantities of imp-OmpA. All mutations result in equivalent levels of properly assembled OmpA. Thus, a strong correlation between imp-OmpA accumulation and cell toxicity was observed. A mutation in the mature region of OmpA which prevents the proper outer membrane assembly of OmpA was suppressed when export was directed by a severely defective signal peptide. These findings suggest that signal peptide mutations indirectly influence OmpA assembly in the outer membrane by altering both the level and rate of OmpA secretion across the cytoplasmic membrane.

The major outer membrane protein OmpA is synthesized as a precursor protein with a signal peptide 21 amino acid residues in length. The structural requirements of the OmpA signal peptide needed for optimal function in protein export have been examined in great depth (9, 16, 17). The signal peptide may aid in the maintenance of the nascent precursor in a conformation capable of interaction with a chaperone (20). Alternatively, the signal peptide may serve as a recognition site for the chaperone SecB (25). It is now widely accepted that proper interaction of precursor with the membrane requires insertion of the signal peptide as a loop into the lipid bilayer (13). Strong evidence indicates that the signal peptide assumes a conformation which is essential for proper proteolytic cleavage of the precursor (6). It seems clear that the signal peptide must perform several of these functions.

Mutations in the OmpA signal peptide can also lead to increased levels of precursor protein synthesis (9). Mutations in the signal peptides of other exported proteins have also been observed to influence the level of protein production (2, 10). The cause of this effect is not known. In one case, the signal peptide mutation has been shown to cause stabilization of the secondary structure of transcribed mRNA (21). For this mutation, the effect appears to be at the level of initiation of translation. However, many of the other signal peptide mutations which alter protein synthetic levels are not predicted to alter mRNA structure (2, 10). These results point to a possible role for the signal peptide in translational regulation.

Following translocation to the periplasm, mature OmpA inserts into the outer membrane. The first 170 N-terminal amino acid residues are believed to form a membrane domain consisting of eight antiparallel β strands which form a β barrel (7). The remaining C-terminal domain is periplasmic. A small region between residues 154 to 180 may serve as a sorting signal for proper localization of OmpA to the outer membrane (15). Outer membrane OmpA interacts with lipopolysaccharide (22), but this interaction does not appear to be required for assembly in the membrane (7).

In this study, we have determined the effects of various OmpA signal peptide mutations on the kinetics of precursor protein processing. Our findings demonstrate the importance of the mature region of a precursor protein in the secretion process. In addition, we observed that OmpA signal peptide mutations could influence both the level of precursor protein synthesis and assembly of mature OmpA in the outer mem-

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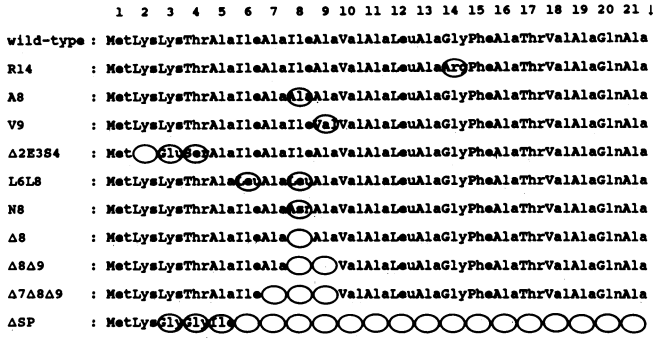


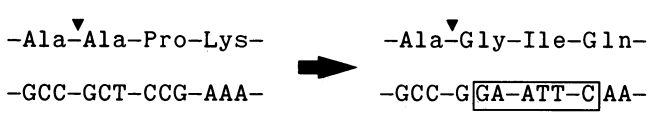
FIG. 1. Amino acid sequences of the wild-type and 10 mutant OmpA signal peptides. The deleted amino acid residues are represented by an empty circle. Substituted amino acid residues are circled. An arrowhead indicates the signal peptide cleavage site.

brane. In this report, we document these effects and discuss their possible cause and significance.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* W620-7 (F^- *ompA thi-1 pyrD36 gltA6 galK30 strA129* λ^- *supE44*) (26) was used as the host strain. Plasmid pAM103 (4) was used as the template for site-specific mutagenesis of the OmpA protein. The high-level expression vector pYM007 containing the *lacI*^{q} gene to reduce uninduced protein expression was used as the vector in the construction of pYT200. pYM007 is a derivative of the high-level expression vector pINII-A1 (18). To construct the OmpA signal peptide deletion mutant (ΔSP), pINIII-A3 (18) was used.

Construction of plasmids. To fuse the mutated OmpA signal peptides previously isolated to the OmpA protein, an *Eco*RI site was first introduced at the beginning of the OmpA mature region by using pAM103 as follows:



Arrowheads indicate the signal peptide cleavage sites, and the boxed nucleotide sequence indicates the newly created *Eco*RI site. As a result of the mutation, the first three residues of the mature OmpA were altered from Ala-Pro-Lys to Gly-Ile-Gln; this change had no effect on the rate of OmpA production, OmpA secretion across the inner membrane, and OmpA assembly in the outer membrane. The mutation was constructed by oligonucleotide-directed mutagenesis. The DNA fragment containing the region coding for the *ompA* gene was then removed by *Pst*I-*Hin*dIII double digestion and inserted into pYM007 to generate pYT200. The wild-type signal peptide contained on a DNA fragment excised by digestion with *Eco*RI and *Pst*I was replaced with the corresponding fragment containing the nine kinds of mutant OmpA signal peptide genes that were previously isolated (9, 16, 17; Fig. 1). The resulting plasmids thus contain the *ompA* gene with the mutated signal peptides under the control of the strong *lpp* promoter and the inducible *lac* promoter-operator. To make the OmpA signal peptide deletion mutant, a DNA fragment containing the OmpA mature region was removed from pYT200 wild type by *Eco*RI-*Hin*dIII double digestion and insertion into pINIII-A3 to generate pYT300 (ΔSP in Fig. 1).

Three mutations in the mature region of the OmpA protein were constructed by oligonucleotide-directed mutagenesis using pYT200 as the template. In each case, arginine was substituted for a nonpolar residue located in a hydrophobic stretch of the protein as follows:

R144:	Phe-144 → Arg	R200:	Val-200 → Arg
	TTC → CGT		GTT → CGT
R257:	Val-257 → Arg		
	GTT → CGT		

Determination of processing half-life. For pulse-chase labeling experiments, 15-ml cultures were grown in M9 minimal medium supplemented with ampicillin (50 μg/ml), uracil (50 μg/ml), and leucine (50 μg/ml) to a Klett reading (blue filter) of 60. Then 6 ml of culture was transferred to a 10-ml beaker, and isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. After induction for 4 min, 100 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham Corp.) was added. After 15 s, nonradioactive methionine was added to a final concentration of 40 μg/ml, and a 0.9-ml sample was removed and added to a tube containing 100 μl of 100% (wt/vol) trichloroacetic acid. After chases for 15, 30, 60, 120, and 300 s, other 0.9-ml samples were removed and treated as described above. In the case of the R14 mutant, chase time points were 30 s, 1 min, 5 min, 10 min, and 30 min. The samples were then kept on ice for 1 h. Immunoprecipitation was carried out essentially as described previously (17), using rabbit anti-OmpA antisera. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out with use of 17.5% gels as described previously (17).

The intensities of the bands on the autoradiograms were determined by using a Hoefer Scientific Instruments GS300 scanning densitometer. Precursor half-lives were determined in the following manner. The percent precursor value was first obtained by determining the band intensities of the precursor and mature proteins combined and multiplying by 100. The band intensity for the precursor OmpA was multiplied by 0.833, since the precursor has six methionines that are labeled and the processed protein only has five. The natural log of the percent precursor was then plotted against time, and the slope of the line was used in the following equation: half-life = 0.693/(−slope of line).

Expression of mutant genes. For expression of mutant genes, cultures were grown as described above to a Klett reading (blue filter) of 60 in a sidearm flask. At various times following induction, cultures were rapidly cooled on ice and cells were collected by centrifugation (3,000 × *g*, 5 min) and washed with 10 mM Tris (pH 7.0). Then the cells were separated into soluble and insoluble membrane fractions by sonication and centrifugation (110,000 × *g*, 30 min). The insoluble pellet was resuspended in 50 μl of 10 mM Tris (pH 7.0) and incubated for 30 min at 50°C or 5 min at 100°C with SDS buffer. Then samples were applied to 17.5% SDS-polyacrylamide gels and stained with Coomassie brilliant blue. The intensity of each band on the Coomassie brilliant blue-stained gel was determined by a Hoefer Scientific Instruments GS300 scanning densitometer, and the concentration of each protein was estimated.

Protein localization was studied by separating cells into soluble, inner, and outer membrane fractions. Cells were obtained from 10-ml cultures by centrifugation and were then ruptured by sonication in an ice bath and separated into

soluble and membrane fractions by centrifugation (110,000 × *g*) for 30 min. The membrane pellet was resuspended in 50 μl of 10 mM sodium phosphate (pH 7.0), 50 μl of 1% sodium sarcosinate was added to solubilize the inner membranes (12), and inner and outer membranes were separated by centrifugation (135,000 × *g*). The samples were incubated for 30 min at 50°C or 5 min at 100°C and subjected to SDS-polyacrylamide gel electrophoresis. The periplasmic fraction was isolated as previously described (12).

RESULTS

Construction of OmpA with mutant signal peptides. We had previously constructed, by site-directed mutagenesis, a series of mutations within the OmpA signal peptide (9, 16, 17). In our prior work, we had studied the effect of OmpA signal peptide mutations on the processing of the heterologous proteins OmpA-nuclease and OmpA–β-lactamase. To examine the processing phenotypes of these same OmpA signal peptide mutations when they were directing secretion of homologous OmpA protein, we constructed the *ompA* gene carried by pYT200. This plasmid was constructed by insertion of an *Eco*RI site in pAM103 at the point of cleavage of precursor OmpA. This permits the ready fusion of the preexisting OmpA signal peptide mutations shown in Fig. 1 to the mature OmpA protein.

Introduction of the *Eco*RI site in the *ompA* gene altered the first three mature amino acids from Ala-Pro-Lys to Gly-Ile-Gln. This change had no effect on precursor OmpA processing. The precursor OmpA processing half-life was 27 s when the protein was encoded by pAM103 and 28 s when it was encoded by pYT200 (data not shown). Alteration of the N terminus of the mature OmpA did not affect the level of protein expression (data not shown). Like chromosomally encoded OmpA, the mature OmpA protein synthesized is localized in the outer membrane of *E. coli* (see Fig. 7) and demonstrates the phenomenon of heat modifiability (see Fig. 3 and 5).

OmpA that is properly inserted in the outer membrane migrates on SDS-polyacrylamide gels with an apparent molecular size of 31 kDa if the sample is prepared for electrophoresis by heating at 50°C. Boiling of the sample causes the protein to migrate to a position consistent with its unfolded size (36 kDa). Presumably some conformation conferred by proper insertion in the outer membrane is retained at 50°C. Thus, the demonstration of heat modifiability is a measure of proper OmpA assembly (8).

The mature OmpA produced in this investigation migrated in SDS-polyacrylamide gels with chromosomally coded OmpA when samples were boiled but migrated slightly faster than the chromosomally encoded protein when samples were heated at 50°C (data not shown). This slight difference may be due to the charge alteration resulting from the replacement of the lysine at position 3 of mature OmpA with glutamine.

Effects of signal peptide mutations on OmpA secretion. Effects of signal peptide mutations on OmpA secretion were assessed by measuring the processing half-lives of mutant precursor OmpA species. The processing half-life was determined in pulse-chase experiments as described in Materials and Methods 4 min following induction of OmpA expression with IPTG. No toxic effects due to OmpA overproduction were evidenced within the first 30 min after induction (see Fig. 4). The processing half-life as determined by this method was highly reproducible. In four separate experiments conducted over a span of 1 year, the processing half-life for wild-type OmpA was found to be 28 ± 4 s.

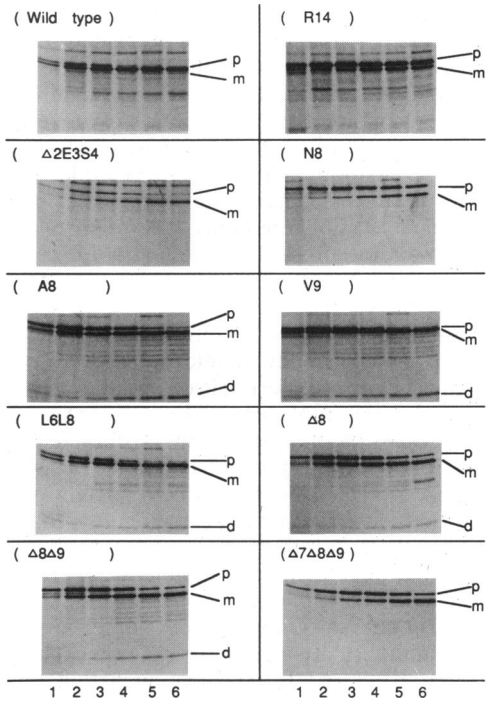


FIG. 2. Immunoprecipitates from pulse-chase experiments. Cultures were labeled with [^{35}S]methionine as described in Materials and Methods. The wild-type or mutant signal peptide used is indicated on the top of each panel. Experiments were carried out with a 15-s pulse (lanes 1) followed by chase times of 15, 30, 60, 120, and 300 s (lanes 2 to 6, respectively). In the case of R14, chase times were 30 s, 1 min, 5 min, 10 min, and 30 min (lanes 2 to 6, respectively). Bands: p, precursor; m, processed product; d, degradation product.

Results of the pulse-chase experiments used to determine the processing half-lives of the mutant OmpA-precursors are shown in Fig. 2. The calculated processing half-life for precursor OmpA is presented in Table 1. For comparison,

TABLE 1. Summary of processing half-lives

Signal peptide mutation	Processing half-life (s)[a]		
	OmpA	Nuclease A[b]	β-Lactamase[c]
Wild-type	28	46	90
V9	42	25	90
L6L8	26	39	ND
A8	65	144	90
Δ8	151	247	90
Δ8Δ9	134	330	90
Δ7Δ8Δ9	180	∞	∞
Δ2E3S4	160	∞	∞
N8	225	∞[d]	∞[d]
R14	3,780 (685)[e]	∞	∞

[a] ND, Not determined; ∞, no processing within 5 min was observed.
[b] As reported by Lehnhardt et al. (16, 17) and Goldstein et al. (9).
[c] As reported by Lehnhardt et al. (16, 17).
[d] As determined by Goldstein (8a).
[e] The number in parentheses indicates the OmpA processing half-life as calculated on the basis of the first three time points from Fig. 2 (see text for discussion).

the half-lives of the heterologous precursor proteins OmpA-nuclease and OmpA–β-lactamase are presented. From Table 1, three classes of signal peptide mutations can be identified.

Class I mutations exhibited essentially no significant processing defect when directing the secretion of any of the three proteins. This class of mutants consisted of the alanine replacement at position 9 of the signal peptide with valine (V9 mutant) and the isoleucine substitutions at positions 6 and 8 with leucine (L6L8 mutant). Secretion of OmpA or β-lactamase was not affected by the V9 or L6L8 mutation. These same mutations actually increased the rate of nuclease export (9).

Class II mutations exerted no effect whatsoever upon the secretion of β-lactamase but significantly slowed the rate of nuclease A and OmpA export. Three mutants made up this group; the isoleucine replacement by alanine at position 8 (A8), the deletion mutant at this same position (Δ8), and the double-deletion mutant at positions 8 and 9 (Δ8Δ9). In each case (A8, Δ8, and Δ8Δ9), this kinetic defect was more pronounced for secretion of the heterologous protein nuclease A than for the natural OmpA.

Class III mutations drastically inhibited the processing of the heterologous proteins nuclease A and β-lactamase but only retarded precursor OmpA processing. The proteins in this class of mutants are markedly different. The Δ2E3S4 mutation had an altered N terminus such that the charge (+2) of the normally basic region of the signal peptide had been reduced to −1. The polar asparagine residue had replaced the hydrophobic isoleucine at position 8 of the signal peptide in the N8 mutation. Three amino acids at positions 7, 8, and 9 had been deleted from the hydrophobic region Δ7Δ8Δ9 mutation. In the final mutant (R14), the glycine at position 14 had been replaced by the charged residue arginine. The apparent processing half-life presented in Table 1 significantly underestimates the rate of processing for this mutant. After a 30-min induction, secreted OmpA accounted for approximately 25% of the total cellular protein in both the wild type and the R14 mutant (Fig. 3). Processing of the R14 mutant precursor at the rate indicated in Table 1 (3,780 s) could not possibly have accounted for the actual quantity of OmpA secreted. A large proportion of synthesized R14 precursor (60% after 30 min of induction; Fig. 3) was in a translocation-incompetent conformation which formed an inclusion body in the cell cytoplasm (data not shown). The presence of this nontranslocatable precursor OmpA greatly increased the apparent processing half-life. A better estimate of the R14 processing half-life could be calculated as 685 s by using only the early time points from the pulse-chase experiment shown in Fig. 2. At this early time, the accumulation of nontranslocatable R14 mutant precursor OmpA was relatively minor.

Mutations within the mature OmpA protein. One explanation for the pronounced difference between secretion of OmpA and β-lactamase or nuclease A exhibited by class III mutations could be the existence in the former protein of some sequence, presumably hydrophobic, within the mature protein which was capable of some signal function. This internal signal sequence could have permitted export of OmpA in the absence of a functional amino-terminal signal peptide. Lack of such an internal signal sequence in nuclease A or β-lactamase would have accounted for the block of export observed in class III mutants.

To test this possibility, three mutations were constructed by oligonucleotide-directed site-specific mutagenesis within three predicted hydrophobic stretches of mature OmpA. Phenylalanine at position 144 of the precursor protein (amino acid 123 of the mature OmpA) was replaced by arginine (R144). Similarly, valine at position 200 and valine at position 257 were substituted with arginine (R200 and R257, respectively). We postulated that insertion of a charged arginine residue within the hydrophobic stretch should effectively eliminate any signal-like function. These three mature region mutations were individually fused to the wild-type OmpA signal peptide and to two class III mutations (Δ2E3S4 and R14). The processing half-life of each mutant precursor was then determined (Table 2). Each of these mature region mutations caused only a marginal inhibition of wild-type precursor OmpA processing. These mutations clearly did not further inhibit OmpA secretion directed by either the basic region (Δ2E3S4) (Table 2) or R14 (unpublished results) class III mutant signal peptides. In

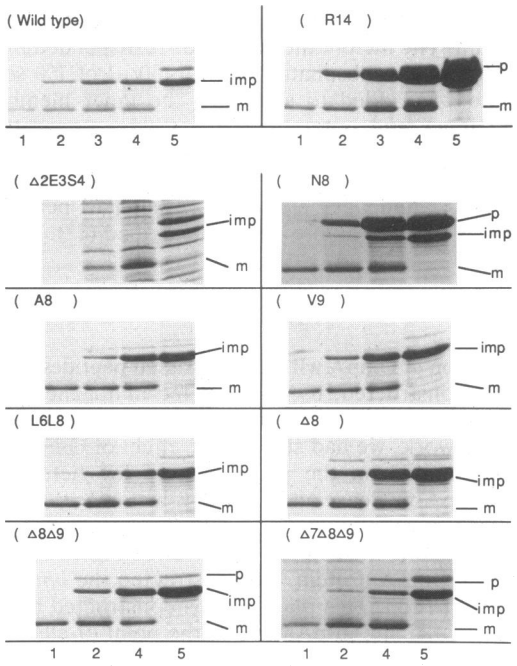


FIG. 3. Heat modifiability of OmpA translocated by wild-type and nine mutant signal peptides. When the Klett reading reached 60, IPTG was added to a final concentration of 1 mM. Induction times in lanes 1 to 5 were 0 min, 30 min, 1 h, 2 h, and 2 h, respectively. The membrane fractions were incubated in the solubilizing buffer (1% SDS–10% glycerol in 10 mM sodium phosphate buffer [pH 7.1]) for 30 min at 50°C (lanes 1 to 4) or 5 min at 100°C (lane 5). The samples were then subjected to SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. OmpA species are designated as mature (m), precursor (p), and immature processed (imp).

TABLE 2. OmpA processing half-lives

Signal peptide mutation	Mature region mutation	Processing half-life (s)
Wild-type	Wild type	28
	R144	38
	R200	58
	R257	43
Δ2E3S4	Wild type	160
	R144	151
	R200	95
	R257	108

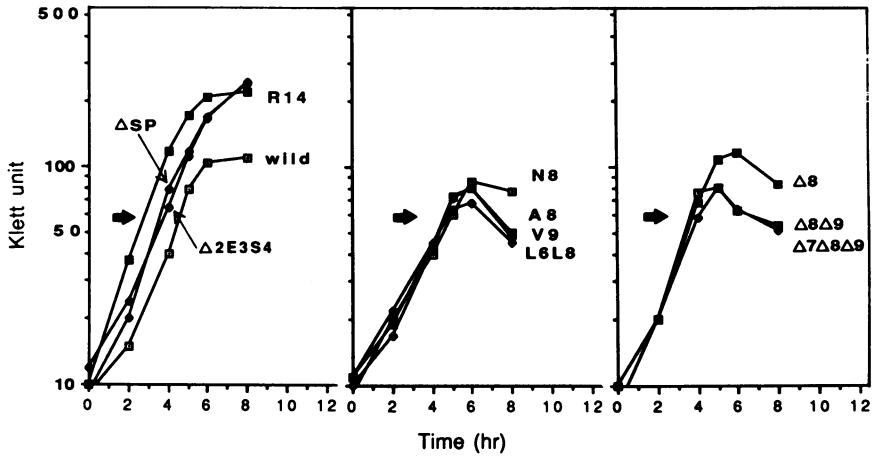


FIG. 4. Effect of induction of the wild-type and mutant precursor OmpA on cell viability. When the Klett reading reached 60, IPTG was added to a final concentration of 1 mM. An arrow indicates the point of induction.

addition, we have determined that mature OmpA lacking a signal peptide was incapable of being secreted by *E. coli* cells (see below). Clearly there was no evidence to support the existence of an internal signal sequence within mature OmpA.

Effects of OmpA signal peptide mutations on OmpA synthesis. Figure 3 shows the production of OmpA after induction of gene expression. Because of the toxic effects of overexpression (discussed below), only lane 2 (30 min after induction) should be used to compare the effects of different signal peptide mutations on OmpA production and assembly. At this time point, all cultures were growing at the same rate and toxic effects (Fig. 4) are not yet evident. Quantitation by densitometry indicated that the A8 and V9 signal peptide mutations had either no or only a slight stimulatory effect on the total amount of OmpA produced compared with production directed by the wild-type signal peptide. Total OmpA production was calculated as the sum of all OmpA species (mature OmpA plus precursor OmpA plus imp-OmpA [defined below]) synthesized in the 30 min following induction. These OmpA species are designated in Fig. 3 as m, p, and imp, respectively. The L6L8, Δ8, and Δ8Δ9 mutations resulted in an approximately twofold increase in OmpA synthesis. The N8 and R14 mutations stimulated production by three- to fivefold. With these latter mutations, a large proportion of the produced OmpA was unprocessed precursor (Fig. 3). The R14 mutant precursor accumulated as a cytoplasmic inclusion body (data not shown). In contrast, the basic region mutant Δ2E3S4 and hydrophobic deletion mutant Δ7Δ8Δ9 slightly decreased the level of OmpA production.

The observation that mutant signal peptides can stimulate OmpA protein synthesis suggested that a functional wild-type signal peptide somehow down-regulated OmpA production. Inactivation of the signal peptide through mutation or its complete removal would be expected to relieve this down-regulation. To test this possibility, a mutant protein that lacked the entire signal peptide (ΔSP) was constructed. Expression of this protein lacking a signal peptide resulted in synthesis of a cytoplasmic OmpA that formed an inclusion body at levels identical to that of the wild-type protein, which was normally exported (0.5 h in Fig. 5 and 6). The inability of the ΔSP mutant protein to assume the heat-modifiable conformation of OmpA (Fig. 5) indicated that this protein was not exported. In Fig. 6, both exported OmpA (wild type) and cytoplasmic OmpA (ΔSP) accounted for approximately 25% of the total cellular protein. In comparison, the R14 signal peptide mutation caused threefold greater OmpA production than did the wild type after 30 min of induction. Prolonged overexpression of wild-type precursor OmpA led to an inhibition of cell growth (Fig. 4). Comparison of protein production at the latter time points shown in Fig. 5 and 6 is therefore not valid. We concluded that the observed increase in protein synthesis caused by signal peptide mutations was not due to the release of some negative regulatory control exerted by a wild-type signal peptide.

Effects of signal peptide mutations on OmpA assembly. Although many of the signal peptide mutations affected the amount of total OmpA produced, the quantity of mature OmpA assembled in the outer membrane remained unaltered (designated m in Fig. 3, lane 2). This mature OmpA was heat modifiable (Fig. 3) and localized exclusively to the outer membrane (Fig. 7). Instead, several of the signal peptide mutations resulted in production of a processed but non-heat-modifiable form of OmpA (designated imp in Fig. 3) which was aberrantly folded and localized to both the inner and outer membranes (Fig. 7). This OmpA, produced as a

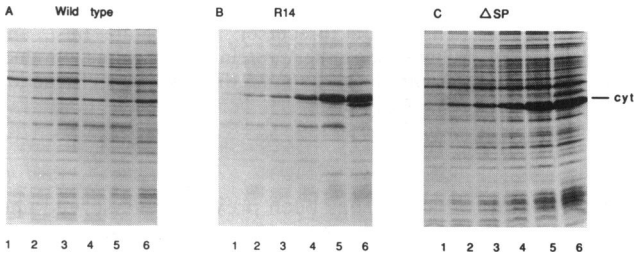


FIG. 5. Whole cell homogenate of cultures expressing wild-type OmpA and R14 and ΔSP mutant OmpA. When the Klett reading reached 60, IPTG was added to a final concentration of 1 mM. Induction times were 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), and 4 h (lanes 5 and 6). Whole cells were incubated in the solubilizing buffer (1% SDS–10% glycerol in 10 mM sodium phosphate buffer [pH 7.1]) for 30 min at 50°C (lanes 1 to 5) or 5 min at 100°C (lane 6). The samples were then subjected to SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue.

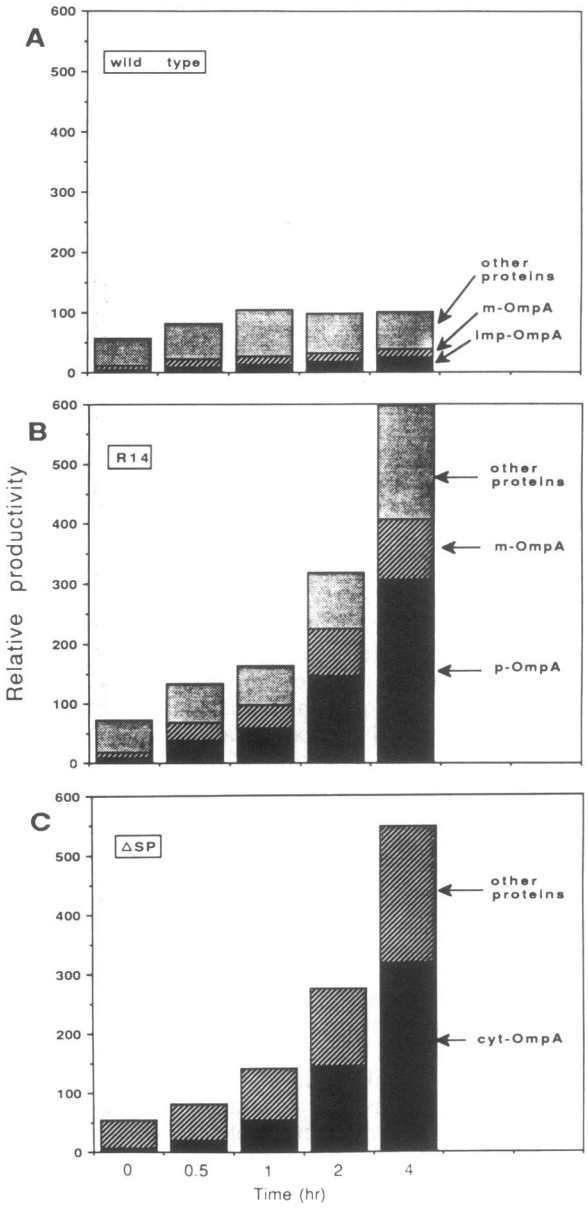


FIG. 6. Relative productivity of wild-type OmpA and of R14 and ΔSP mutant OmpA. The stained gels shown in Fig. 4 were subjected to densitometry as indicated in Materials and Methods. The relative productivity of each protein (precursor [p-], mature [m-], and cytoplasmic [cyt-] OmpA and other proteins) is indicated. Productivity was calculated with the assumption that wild-type production after 4 h of induction was 100%.

result of overproduction, has been previously observed and was termed immature, processed OmpA (imp-OmpA) (8). It has been demonstrated that this protein had not been properly assembled in the outer membrane. Signal peptide mutations that resulted in only a moderate processing defect (L6L8, Δ8, and Δ8Δ9) or had essentially no effect on processing (wild type, A8, and V9) (Table 1) produced large quantities of imp-OmpA (Fig. 3, lanes 2). More severe signal peptide defects decreased the amount of imp-OmpA produced (N8 and Δ7Δ8Δ9) or eliminated it entirely (Δ2E3S4 and R14) (Fig. 3, lanes 2).

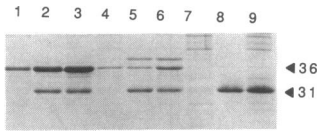


FIG. 7. Localization of the OmpA protein. OmpA production in cells harboring the wild-type (lanes 1 to 3), Δ7Δ8Δ9 mutant (lanes 4 to 6), and Δ2E3S4 mutant (lanes 7 to 9) OmpA was induced for 2 h by adding IPTG (2 mM) when the Klett reading reached 60. The inner membrane and the outer membrane were separated as described in Materials and Methods. These fractions were solubilized at 50°C. The inner membrane fractions were applied to lanes 1, 4, and 7; the outer membrane fractions were applied to lanes 2, 5, and 8; the whole membrane fractions were applied to lanes 3, 6, and 9. After SDS-gel electrophoresis, the gel was stained by Coomassie brilliant blue. Molecular sizes are indicated in kilodaltons on the right. The band at 31 kDa represents the heat-modifiable OmpA, and the 36-kDa band is non-heat-modifiable OmpA.

This inverse correlation between processing defect and imp-OmpA production was dramatically demonstrated in the experiment depicted in Fig. 8. We observed that introduction of an arginine residue at position 257 of mature OmpA prevented normal assembly of OmpA when export was directed by a wild-type signal peptide. Consequently, all of the produced OmpA was non-heat-modifiable imp-OmpA (Fig. 8A). Although improperly assembled, this imp-OmpA was still localized exclusively to the outer membrane (Fig. 8B). If export of the same R257 mutant protein was directed by the defective basic region signal peptide mutation (Δ2E3S4), all of the produced OmpA was heat modifiable, indicative of proper assembly in the outer membrane (Fig. 8A). The basic region signal peptide mutation significantly increased the processing half-life of the R257 precursor protein (Table 2) and suppressed the assembly defect of this mutant.

Toxicity of OmpA overproduction. Cultures that produced imp-OmpA as a result of overexpression of wild-type or mutant signal peptide precursor OmpA (wild type, N8, A8, V9, L6L8, Δ8, Δ8Δ9, and Δ7Δ8Δ9; Fig. 3) experienced a severe inhibition of growth following induction (Fig. 4). Cultures that produced severely processing defective mutant precursors (R14, Δ2E3S4, and ΔSP) did not accumulate

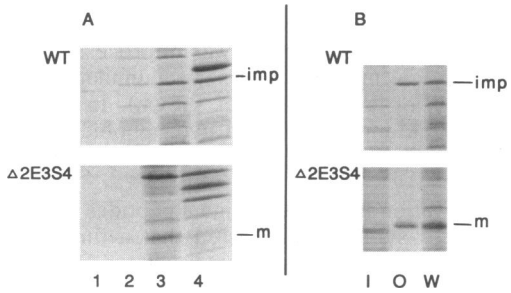


FIG. 8. Evidence that a mutant signal peptide suppresses the outer membrane assembly defect of an OmpA mature region arginine replacement mutation. (A) Production of the OmpA arginine replacement mutant (R257) before (lane 1) and 30 min (lane 2) and 2 h (lanes 3 and 4) after induction. (B) Localization to the inner membrane (I), outer membrane (O), or unfractionated envelope (W). Export directed by the wild-type (WT) or mutant (Δ2E3S4) signal peptide leads to immature, processed (imp) or mature (m) OmpA production, respectively. Samples in lanes 1, 2, 3, I, O, and W were heated at 50°C for 30 min; the sample in lane 4 was boiled for 5 min.

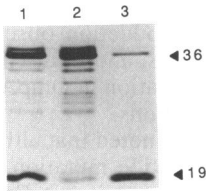


FIG. 9. Localization of the OmpA degradation products. Cells producing the wild-type OmpA were pulse-labeled with [^{35}S]methionine for 15 s and chased for an additional 5 min. Lanes: 1, whole cell; 2, fraction after removal of the periplasmic fraction; 3, periplasmic fraction. Cell fractionation was carried out as described in Materials and Methods. All of the fractions were solubilized in the solubilizing solution, incubated at 100°C for 5 min, and treated with anti-OmpA serum. The immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis. Numbers at the right indicate molecular sizes in kilodaltons.

imp-OmpA (Fig. 3) and experienced no toxic effect (Fig. 4). Accumulation of high levels of mature OmpA was not responsible for the toxic effect. Mature OmpA accounted for as much as 20% of total cellular protein when the R14 signal peptide mutant directed export (Fig. 6). Likewise, the very high levels of precursor OmpA and cytoplasmic OmpA produced by the R14 and ΔSP signal peptide mutations, respectively (Fig. 6), preclude the possibility that these OmpA species had a toxic effect. It is clear that imp-OmpA accumulation was responsible for the toxicity associated with OmpA overproduction.

Imp-OmpA degradation products. In pulse-chase experiments, we were able to observe the production of eight labeled proteins in the 19- to 35-kDa range which were immunoprecipitated by anti-OmpA antiserum (Fig. 9, lane 1; Fig. 2, labeled d). The intensity of radioactive label in these proteins increased with time of chase, with the 19-kDa protein predominating. Signal peptide mutations that caused production of high levels of imp-OmpA (wild type, A8, V9, L6L8, Δ8, and Δ8Δ9) produced these proteins within the 5-min span of a pulse-chase experiment (Fig. 9 and 2). Signal peptide mutations that resulted in the synthesis of only small quantities of imp-OmpA in a 30-min experiment (N8 and Δ7Δ8Δ9; Fig. 3) produced no observable lower-molecular-weight proteins in a 5-min pulse-chase experiment (Fig. 2). Signal peptide mutations that did not result in imp-OmpA production (R14 and Δ2E3S4; Fig. 3) did not produce these smaller proteins (Fig. 2). There was clearly a strong correlation between the production of imp-OmpA and these lower-molecular-weight proteins.

It has been demonstrated that OmpA has a periplasmic domain composed of the C-terminal sequence of residues 178 to 325 (7). The size of this domain was similar to that of the 19-kDa protein. To determine whether this 19-kDa protein could be an OmpA degradation product derived from the C terminus of OmpA, the region encoding amino acids 263 to 325 was replaced with a short oligonucleotide containing an open reading frame of 21 codons plus a termination codon. When production of the truncated OmpA was examined, a band similar in intensity to the 19-kDa band was shifted to 16 kDa (data not shown). This result confirms that the 19-kDa protein was a degradation product derived from the C terminus of OmpA. Furthermore, this degradation product was localized in the periplasmic fraction from labeled cells (Fig. 9, lane 3). The other seven degradation products of this truncated OmpA remained associated with the membrane fraction (Fig. 9, lane 2). Very little immunoprecipitable material was found in the cytoplasmic fraction (data not shown). Taken together, these results strongly suggested that the degradation products were derived from imp-OmpA.

DISCUSSION

Class I and II mutations were designed to test the functional importance of the overall hydrophobicity and secondary structure of the OmpA signal peptide (9). The OmpA signal peptide has an approximately equal probability of forming an α helix or β sheet. An increase in β-sheet structure would favor membrane insertion if a transition from β sheet to α helix is critical for signal peptide loop formation (1, 3). When examined within this framework, the processing defects of class I and II mutants, when directing OmpA or nuclease A secretion, agree quite well with the predicted phenotypes. Mutations that decrease the predicted β-sheet structure and hydrophobicity (A8, Δ8, and Δ8Δ9) resulted in defective processing of precursor nuclease and precursor OmpA. Only a slight decline in β-sheet secondary conformation and no change in hydrophobicity were predicted for the L6L8 mutant. As expected, this signal peptide alteration did not induce any defect in OmpA processing and actually improved nuclease A maturation. The V9 mutation increased both the probability of β-sheet conformation and hydrophobicity, thereby stimulating precursor nuclease processing (9), but had no effect on precursor OmpA export. The observed phenotypes described here appear to fit well the model that the signal peptide secondary structure and hydrophobicity are essential because they are required either for direct membrane interaction or for recognition by proteins involved in the secretion pathway.

The processing defects caused by class I and II signal peptide mutations were dependent on the mature region of the precursor protein. The class I mutations (V9 and L6L8) were beneficial or had essentially no effect on the export of either OmpA or nuclease A. Class II mutations (A8, Δ8, and Δ8Δ9) caused relatively moderate kinetic processing defects for both of these precursor proteins. Remarkably, these same signal peptide mutations (class I and II) had absolutely no influence on the processing half-life of precursor lactamase. In effect, β-lactamase processing was independent of the structural modifications induced by class I and II signal peptide mutations.

Class III mutations vary considerably. The Δ2E3S4 mutation alters drastically the charge at the N terminus of the precursor from +2 to −1. Mutations in other precursor proteins (lipoprotein [24], staphylokinase [11], maltose-binding protein [19], and PhoE [5]) which change the signal peptide basic region net charge from positive to negative result in severe processing defects both in vivo and in vitro. This region of the signal peptide is widely believed to be important for precursor interaction with the acidic phospholipids of the cytoplasmic membrane (5, 13). A synthetic mutant PhoE signal peptide with a net charge of −2 at the N terminus was found to bind normally to phospholipid vesicles but was unable to cause a lipid phase transition (14). In contrast, Δ7Δ8Δ9 and N8 mutations are probably defective at the level of translocation. The results of a series of experiments comparing the ability of synthetic wild-type and these mutant OmpA signal peptides to interact with phospholipid vesicles indicate that the mutant signal peptides are unable to insert into the vesicle membrane (10a). It is therefore likely that these mutations are blocked at translocation in vivo. Processing of the R14 mutant precursor

OmpA was decidedly slower than that of the wild type (Fig. 2). However, as a result of a three- to fivefold increase in the level of precursor synthesis, roughly equivalent quantities of this mutant precursor OmpA and wild-type precursor OmpA were exported in a 30-min period following induction of protein expression (Fig. 3). Class III mutations (Δ2E3S4, N8, Δ7Δ8Δ9, and R14) cause a complete block in the in vivo secretion of the heterologous proteins nuclease A and β-lactamase (Table 1), while remarkably, these same mutations are capable of supporting the export of the homologous OmpA protein (Fig. 2), albeit at markedly reduced rates (Table 1). The mature portion of the precursor-OmpA can therefore in some manner suppress these processing defects.

OmpA is an integral outer membrane protein (7). In contrast, both nuclease A and β-lactamase are soluble proteins. It is possible that the hydrophobic nature of the OmpA protein leads to the prolonged residence of precursor OmpA class III signal peptide mutations on the inner face of the cytoplasmic membrane. This would increase the probability of the fruitful insertion into the membrane of a defective signal peptide and result in translocation. In contrast, the analogous mutant precursor nuclease and β-lactamase proteins may readily dissociate from the membrane, or be degraded (16), following delivery there.

Numerous observations of an effect of signal peptide mutations on the level of secretory protein synthesis have been reported (2, 9, 10, 23, 24). Usually the observed effect was a decrease in protein synthesis. Here we presented evidence that a variety of OmpA signal peptide mutations (L6L8, Δ8, Δ8Δ9, N8, and R14) increase the level of precursor protein synthesis. The OmpA R14 signal peptide mutation was the most extreme example within this group, being synthesized at three to five times the level of wild-type precursor OmpA. Overproduction of the R14 mutant protein led to accumulation in an inclusion body of very high levels (50% of total protein) of this defective precursor. The predicted mRNA secondary structure was not altered by the point mutation that generated the R14 lesion. The OmpA protein completely lacking a signal peptide (ΔSP) formed a cytoplasmic inclusion body following induction, but the rate of synthesis was indistinguishible from that of wild-type precursor OmpA under equivalent growth conditions (Fig. 5 and 6, 0.5 h). The R14 mutation therefore would appear to positively modulate protein synthesis rather than bypass some inhibitory mechanism inherent in a functional signal peptide.

The assembly of OmpA has been studied extensively by Henning and co-workers (15). OmpA is believed to be inserted in the outer membrane by eight transmembrane β strands at the N-terminal domain of the protein, with the C-terminal domain exposed to the periplasm (7). Overproduction of OmpA results in the formation of a processed but immature OmpA (imp-OmpA) species that cannot assume the heat-modifiable conformation characteristic of properly assembled outer membrane OmpA (8). These investigators find that overproduced imp-OmpA is attached to the inner face of the outer membrane. With the exception of the R257 mature region mutant (see below), we observed imp-OmpA to be distributed between inner and outer membranes (Fig. 7).

We have demonstrated here that signal peptide mutations affected imp-OmpA production under conditions of protein overproduction (Fig. 3). One possible cause for imp-OmpA accumulation is saturation during overexpression of an OmpA assembly site. Overproduction would then be responsible for the imp-OmpA accumulation observed when a wild-type signal peptide directed export. The further increase in imp-OmpA production observed when defective signal peptides (L6L8, Δ8, and Δ8Δ9) directed export would then be due to the stimulation in OmpA synthesis caused by the signal peptide mutations.

However, it should be noted that although severely defective, the R14 signal peptide mutation permitted significant amounts of mature, heat-modifiable OmpA to be assembled in the outer membrane. This amount of mature OmpA was roughly equivalent to the sum of mature OmpA and imp-OmpA produced when a wild-type signal peptide was directing OmpA export (compare R14 and wild type in Fig. 3, lane 2, and Fig. 6, 0.5 h). Even at very high levels of overproduction, the R14 signal peptide lesion did not lead to the appearance of imp-OmpA (Fig. 3 and 5). This result indicates that suppression of the OmpA assembly defect (imp-OmpA formation) caused by certain signal peptide mutations (R14 and Δ2E3S4) may in some manner be a reflection of the slowed processing rate of these mutant precursors.

For unknown reasons, insertion of arginine at position 257 of the mature OmpA protein prevented assumption of the heat-modifiable conformation of OmpA indicative of proper outer membrane assembly. It should be noted that the mutant R257 OmpA was exclusively localized to the outer membrane (Fig. 8) and not distributed between inner and outer membranes as was imp-OmpA (Fig. 7). Arginine insertion at position 144 or 200 of the mature protein did not prevent OmpA outer membrane assembly or folding to the heat-modifiable form (data not shown). When secretion of this mature region R257 mutant OmpA was directed by the Δ2E3S4 mutant signal peptide, all of the synthesized protein was found to be heat modifiable (Fig. 8). The level of R257 mutant OmpA production was essentially the same whether secretion was directed by the wild-type or basic region mutant signal peptide (Fig. 8). Therefore, only the difference in processing rate could have accounted for this dramatic shift in OmpA assembly.

The correlation between imp-OmpA accumulation and cell toxicity (Fig. 4) argues strongly that imp-OmpA mediated this toxic response. Mutations that prevented imp-OmpA accumulation in induced cultures (R14, Δ2E3S4, and ΔSP) were nontoxic. Induction of wild-type and all other signal peptide mutant precursor OmpA proteins resulted in the production of imp-OmpA and the concurrent cessation of culture growth (Fig. 4). Imp-OmpA appears to be degraded to a 19-kDa soluble periplasmic fragment corresponding to the OmpA C terminus. It follows that the observed membrane-bound degradation products must be derived from the N-terminal region (Fig. 9). Improper assembly of the protein may increase the susceptibility of imp-OmpA to proteolysis. It remains to be established whether the intact imp-OmpA or one of the degradation products is the direct cause of cell toxicity. A better understanding of the mechanism of imp-OmpA toxicity could shed light on the normal process of OmpA assembly in the outer membrane.

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