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Recombinant forms of M13 procoat with an OmpA leader sequence or a large carboxy-terminal extension retain their independence of *sec*Y function

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The assembly of phage M13 procoat protein into the plasma membrane of *Escherichia coli* is independent of the *sec*Y protein. To test whether this is caused by the unusually small size of procoat, we fused DNA encoding 103 amino acids to the carboxy-terminal end of the procoat gene. The resulting fusion protein, which attains the same membrane-spanning conformation as mature coat protein, still does not require the *sec*Y function for membrane assembly. To determine whether the leader sequence governs interaction with the *sec*Y protein, we genetically exchanged the leader peptides between procoat and pro-OmpA, a protein which does require *sec*Y for its membrane assembly. Each of the resulting hybrid proteins assembles across the plasma membrane, though at a reduced rate. Membrane assembly of the fusion of procoat leader and OmpA required *sec*Y function, whereas assembly of the pro-OmpA leader/coat protein fusion was independent of *sec*Y. Properties of the entire procoat molecule, rather than its small size or a specific property of its leader peptide determines its mode of membrane assembly.

Key words: membrane assembly/M13 coat protein

Introduction

Biochemical and genetic studies have revealed several common features of bacterial protein export (Michaelis and Beckwith, 1982; Wickner and Lodish, 1985). One is the presence of amino-terminal leader (signal) sequences, which are removed shortly after the pre-proteins translocate across the plasma membrane (Zimmermann *et al.*, 1982). Detailed comparisons of leader sequences for bacterial and eukaryotic secreted proteins show strongly conserved features (von Heijne, 1983; Perlman and Halvorson, 1983). Leader peptides are 15–30 residues in length with a basic amino-terminal region, an apolar central domain and characteristic sequences near the cleavage site. Secondly, protein translocation into or across the plasma membrane requires the electrochemical potential (Date *et al.*, 1980a,b; Enequist *et al.*, 1981; Daniels *et al.*, 1981). A third characteristic of protein export is the requirement for the products of several *sec* genes (Michaelis and Beckwith, 1982). One of these, *sec*Y, codes for an integral protein of the bacterial plasma membrane (Akiyama and Ito, 1985). Despite intensive study, the precise role of *sec*Y and the other *sec* gene products has remained elusive.

These three features of bacterial protein export are not invariant and several exceptions have been described. For example, most proteins of the inner membrane do not have a cleaved leader sequence (Buchel *et al.*, 1980; Santos *et al.*, 1982; Poulis *et al.*, 1981; Wolfe *et al.*, 1983). Moreover, certain exported proteins do not require *sec*A or *sec*Y function (Oliver and Beckwith, 1981; Wolfe *et al.*, 1985; Liss and Oliver, 1986). Also, mutants have been described which affect the energetics of protein export (Zimmermann *et al.*, 1982).

In this study, we have investigated why M13 procoat does not require *sec*Y for its membrane assembly. This protein has a typical leader sequence of 23 amino acid residues. It is processed to coat protein by leader peptidase prior to its assembly onto extruding viruses. We have tested two postulates as to why procoat might be independent of *sec*Y function. One is that the assembly properties of procoat are predicated on its small size. The other is that the *sec*-encoded 'machinery' may resemble eukaryotic signal recognition particle (Walter and Blobel, 1981) in binding directly to the leader sequence with the corollary that procoat leader may have the unusual property of not being recognized by sec proteins. Neither of these simple postulates fits our observations; rather, it is the mature region of procoat which appears to specify *sec*-independent membrane assembly.

Results

To test whether procoat does not require *sec*Y functions because of its small size, we created a fusion of all but the last residue of procoat with a 103 amino acid polypeptide derived from the polar, carboxy-terminal domain of leader peptidase. This fusion protein of 177 residues (mol. wt 19 456) is processed by leader peptidase and assembles into the plasma membrane with its amino terminus facing the periplasm and its carboxy terminus remaining in the cytoplasm (Kuhn *et al.*, 1986a). We transformed the plasmid bearing this larger procoat/leader peptidase fusion into

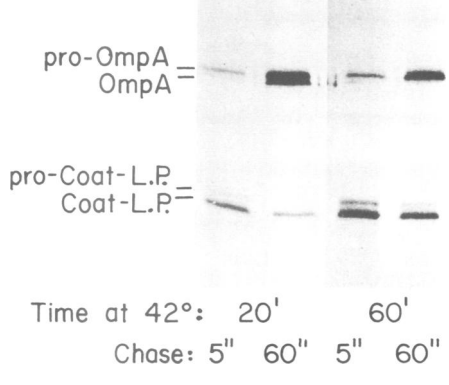

Fig. 1. The membrane assembly of a procoat-leader peptidase fusion does not require *sec*Y function. *E. coli* IQ292 (Shiba *et al.*, 1984) with plasmid pQN8ΔC12 (Kuhn *et al.*, 1986a) was grown in M9 minimal medium at 30°C to a density of 2 × 10^8 cells/ml, then shifted to 42°C. After the indicated times, portions were pulse-labeled for 30 s with [^{35}S]methionine and chased with an excess of non-radioactive methionine. After 5 and 60 s of chase, samples were mixed with trichloroacetic acid and then analyzed by immunoprecipitation with antisera to M13 coat protein and OmpA, SDS–PAGE and fluorography.

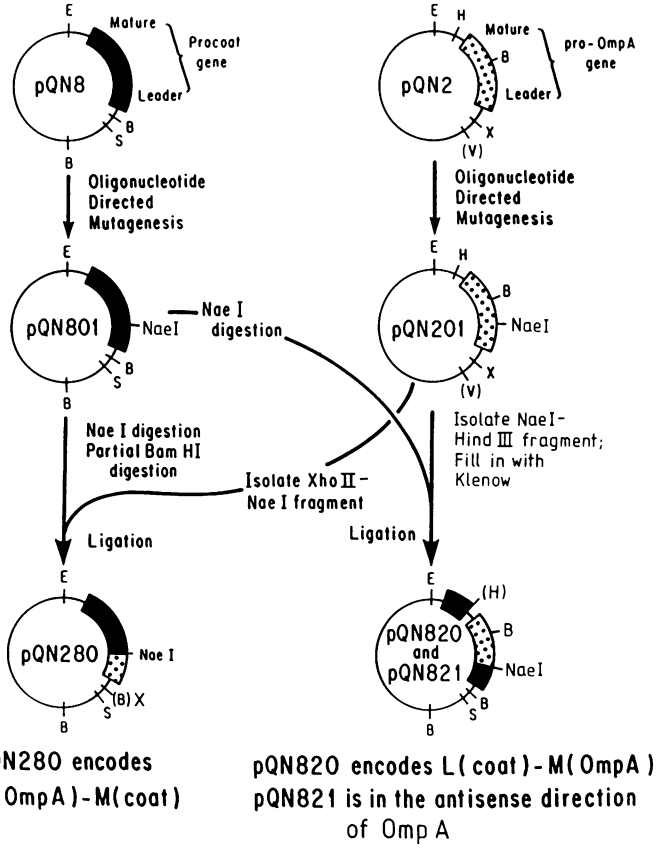

Fig. 2. Exchange of leader peptides of procoat and pro-OmpA. In order to create a unique restriction site at the junction between leader and mature regions of M13 procoat, and *Eco*RI−*Sal*I fragment of pQN8 (bearing the entire procoat gene) was cloned into M13 mp19. The synthetic oligonucleotide d(GGATCGTCACCCTCGCCGGCGAAAG)P was annealed and extended by the Klenow fragment of *E. coli* polymerase I in the presence of T4 ligase. Clones containing the newly introduced *Nae*I site were isolated and sequenced; they were termed pQN801. A similar procedure, using the oligonucleotide d(GCGCAGGCCGGCCCGAAAG)P was used to introduce a *Nae*I restriction site at the junction of the leader and mature regions of pro-OmpA. This plasmid was named pQN201. To prepare plasmid pQN280, the 139-bp *Nae*−*Xho*II fragment of pQN201, coding for the leader of pro-OmpA, was isolated by agarose gel electrophoresis and ligated with pQN801 that had been digested with *Nae*I and partially digested with *Bam*HI. The orientation of the insert was verified by analytical digestion with *Alu*I. To obtain pQN820, the 1084-bp *Nae*I−*Hin*dIII fragment of pQN201, encoding the mature region of OmpA, was isolated from an agarose gel, incubated with the large fragment of DNA polymerase I and deoxynucleoside triphosphates, and ligated with pQN801 which had previously been cleaved with *Nae*I and treated with bacterial alkaline phosphatase. The orientation of the cloned fragment was determined by analytical digestion with *Dde*I. Clones with the correct orientation (pQN820) and the antisense orientation (pQN821) could be identified. Abbreviated restriction sites are: B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, S = *Sal*I, V = *Eco*RV, X = *Xho*II.

Escherichia coli IQ292, a strain with a temperature-sensitive mutation in the *sec*Y gene. The transformant was grown at 30°C, induced for the synthesis of the fusion protein and shifted to 42°C. After 20 or 60 min at the non-permissive temperature, portions of the culture were pulse-labeled for 30 s with [^{35}S]methionine, then chased for 5 or 60 s with an excess of non-radioactive methionine. Samples were analyzed by immunoprecipitation with antiserum to coat protein and OmpA (a major outer membrane protein), SDS−PAGE and fluorography. The fusion protein rapidly and efficiently chased to its mature mol. wt (Figure 1). In contrast, the processing of pro-OmpA was dramatically inhibited, as expected from previous studies (Shiba *et al.*, 1984). These data show that it is not simply the small size of procoat which governs its membrane assembly properties.

In order to determine whether the different interactions of procoat and pro-OmpA with *sec*-encoded proteins might be due to different properties of their leader sequences, we exchanged the leader peptides between these two proteins. As described in detail in Figure 2 and its legend, oligonucleotide-directed mutagenesis was used to introduce a *Nae*I restriction site into plasmid-borne genes for each of these proteins precisely at the junction between the leader and mature domains. This change converted the alanyl residue at the start of the mature part of each protein to a glycyl residue. As shown below, this had no measurable effect on the assembly properties of either protein. Using the unique *Nae*I sites thus generated in the two genes, the segments coding for the respective leader and mature regions were exchanged. The protein with the leader from procoat joined to the mature OmpA protein is termed L(coat)−M(OmpA); the other fusion protein is termed L(OmpA)−M(coat).

The plasmids containing these newly constructed genes were transformed into *E. coli* HJM114. Cells were pulse-labeled with [^{35}S]methionine, then chased with excess non-radioactive amino acid. At the indicated times, aliquots were mixed with cold trichloroacetic acid, then analyzed by immunoprecipitation, SDS−PAGE and fluorography (Figure 3). The mutation of alanine to glycine at the first residue of mature coat (Figure 3B) or OmpA (Figure 3E) had no detectable effect on the membrane assembly process. However, the maturation of L(OmpA)−M(coat) (Figure 3C) and L(coat)−M(OmpA) (Figure 3F) was considerably slower than that of the wild-type proteins (Figure 3A and D). This suggests a basically similar function of the two leader peptides, but also that there is an optimal match between the leader and mature regions for membrane assembly. In cells synthesizing L(coat)−M(OmpA), approximately half of the precursor form of OmpA matured rapidly, i.e. by the end of the 5-s chase period (see Figure 3F). We assume that this is the chromosomally encoded pro-OmpA made with its normal leader peptide (see below).

Protease mapping was used to test whether the processing observed in the experiment shown in Figure 3 truly reflects translocation rates. Cells were labeled with [^{35}S]methionine for 1 min, chased for 1 min with non-radioactive methionine, then chilled and treated with Tris, sucrose and EDTA to permeabilize their outer membrane. Protease digestion (Figure 4) showed that the precursors containing the exchanged leader peptides were not accessible to protease and thus not translocated, while the mature proteins had crossed the inner membrane and were accessible to proteolysis. This indicates that the leader peptidase is present in functional excess, as has been shown previously with the wild-type proteins (Date *et al.*, 1980b; Zimmermann and Wickner, 1983), and that the exchange of leader peptides simply retards translocation.

In order to determine whether the pro-OmpA leader peptide would cause *sec*Y-dependence of procoat membrane assembly, plasmids encoding wild-type procoat, procoat with alanine (+1) changed to glycine, and L(OmpA)−M(coat) were each transformed into *E. coli* IQ292, a strain with a thermo-sensitive mutation in *sec*Y. Cells were grown at 30°C, then shifted to 42°C. After 20, 60 or 100 min of growth at the non-permissive temperature, aliquots were pulse-labeled with [^{35}S]methionine for 30 s, then chased for 5 s or 1 min with non-radioactive methionine. After only 20 min at 42°C, pro-OmpA chased only slowly to OmpA, and this was dramatically delayed when the cells had grown for 60 or 100 min at the non-permissive temperature (Figure 5). As reported previously (Wolfe *et al.*,

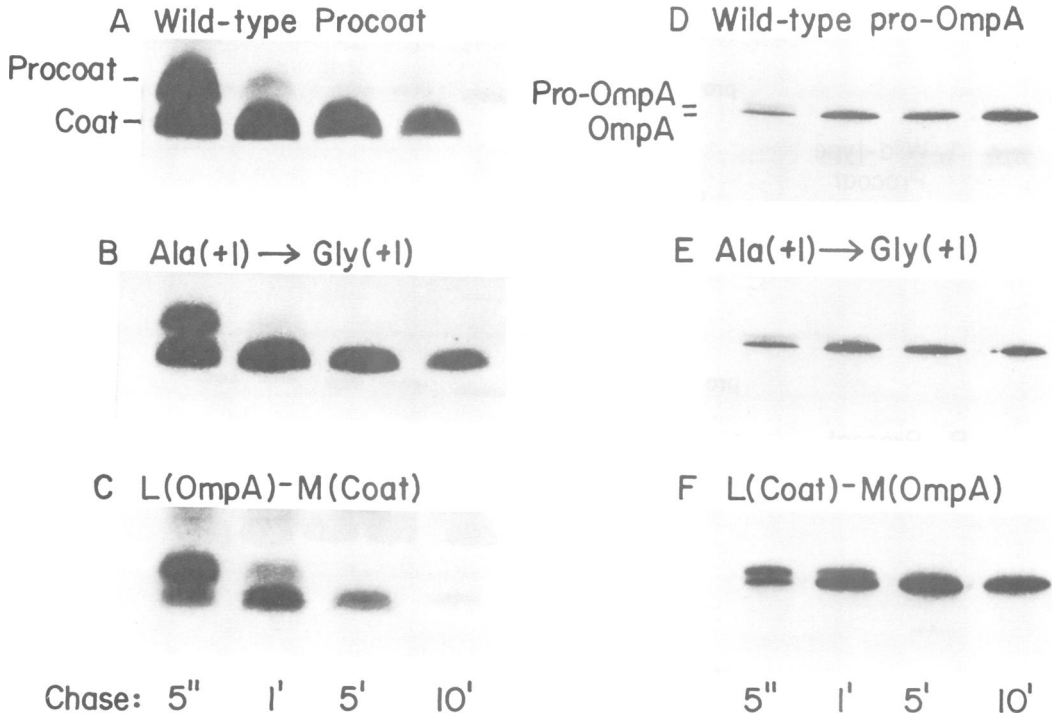

Fig. 3. The effect of leader peptide exchange on the kinetics of assembly. *E. coli* HJM114 was grown at 37°C in M9 minimal medium with 0.2% arabinose to early log phase. Cells were pulse-labeled with [^{35}S]methionine for 30 s, then chased with non-radioactive methionine. At the indicated times, samples were precipitated with trichloroacetic acid, then analyzed by immunoprecipitation with antiserum to M13 coat protein (**A**–**C**) or OmpA (**D**–**F**). Cells bore the following plasmids; (**A**) pQN8 encoding wild-type procoat. (**B**) pQN801 encoding procoat with Ala (+1) changed to Gly, (**C**) pQN280 encoding L(OmpA)–M(coat), (**D**) pQN2 coding for wild-type OmpA, (**E**) pQN201 coding for pro-OmpA with Ala (+1) changed to Gly, (**F**) pQN820 coding for L(coat)–M(OmpA).

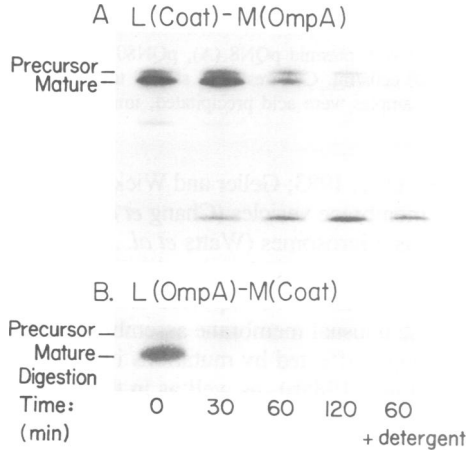

Fig. 4. Mapping the topology of precursor and mature fusion proteins. *E. coli* HJM114 with (**A**) plasmid pQN820 or (**B**) plasmid pQN280 was grown at 37°C in M9 medium containing 0.2% arabinose. Cultures in mid-log phase were pulse-labeled with [^{35}S]methionine for 1 min and chased with non-radioactive methionine for 1 min. Cells were chilled and osmotically shocked by mixing with an equal volume of ice-cold 60 mM Tris-HCl (pH 8.0), 40% sucrose and 20 mM EDTA. Proteinase K was added to a final concentration of 1 mg/ml, and incubation continued at 0°C for the indicated times. A portion of the culture was lysed by addition of Triton X100 to a final concentration of 2% prior to digestion with proteinase K. The protease was inhibited by the addition of 1 mg/ml phenylmethylsulfonyl fluoride. Samples were mixed with trichloroacetic acid, then immunoprecipitated with antiserum to (**A**) OmpA or (**B**) coat protein prior to SDS–PAGE and fluorography.

1985), the conversion of procoat did not show such a delay (Figure 5A), nor did the mutant with glycine instead of alanine at position +1 (Figure 5B). Even after 60 min at the non-permissive temperature, >90% of procoat was processed within a chase period of 1 min (see Figure 5A) and this was complete within 5 min chase (data not shown). Surprisingly, the precursor comprised of the leader from pro-OmpA and the mature part of coat protein was also *sec*Y-independent in its membrane assembly (Figure 5C).

In a complementary experiment, IQ292 cells were transformed with the plasmid pQN820, encoding L(coat)–M(OmpA), or the control plasmid pQN821, which contains the OmpA gene in reverse orientation. As a control, both types of cells were incubated for 1 min with radioactive methionine and the amount of radioactive OmpA present on the gels was estimated by the method of Suissa (1983). In cells containing plasmid pQN820, 2.05 times as much OmpA was detected compared to cells containing plasmid pQN821. This indicates that the wild-type OmpA derived from the chromosomal gene was synthesized in about the same amount as the plasmid-derived hybrid polypeptide. The experiment with cells containing plasmid pQN821, which synthesize only the chromosomal OmpA, is shown in Figure 6A. In the parallel experiment with plasmid pQN820, the excess, plasmid-derived L(coat)–M(OmpA) polypeptide was found to have the same assembly phenotype (Figure 6B) as the normal pro-OmpA, i.e. a block at the non-permissive temperature. Our results with these hybrid proteins thus indicate that *sec*Y dependence, or independence, of membrane assembly is not simply mediated by structural features of certain leader peptides.

Discussion

Previous *in vivo* studies of coat protein biogenesis (Wickner, 1983) have shown that procoat is synthesized on free polysomes and then rapidly binds to the plasma membrane. Its insertion across the membrane is independent of ongoing protein synthesis

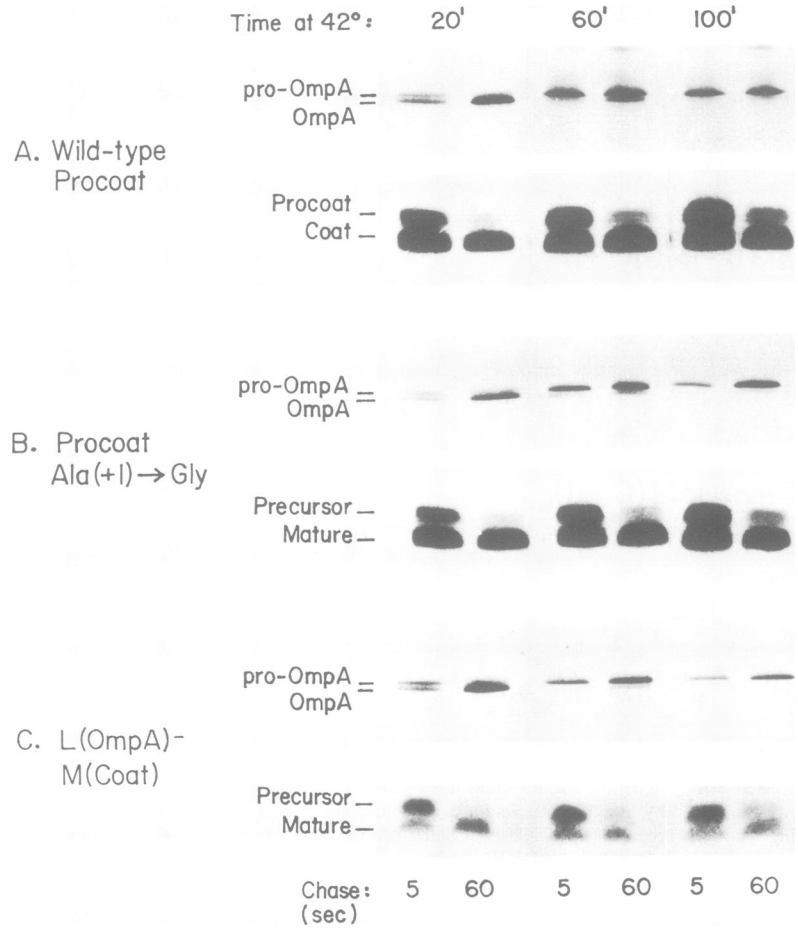

Fig. 5. The membrane assembly of L(OmpA)−M(coat) does not require *sec*Y function. *E. coli* IQ292 with plasmid pQN8 (**A**), pQN801 (**B**) or pQN280 (**C**) were grown at 30°C in M9 medium containing 0.2% glycerol and 0.2% arabinose to a density of 10^8 cells/ml. Cultures were shifted to 42°C. At the indicated times, aliquots were pulse-labeled with [^{35}S]methionine for 30 s. After 5 or 60 s of chase, samples were acid precipitated, immunoprecipitated with antiserum to coat protein and to OmpA, and analyzed by SDS−PAGE and fluorography.

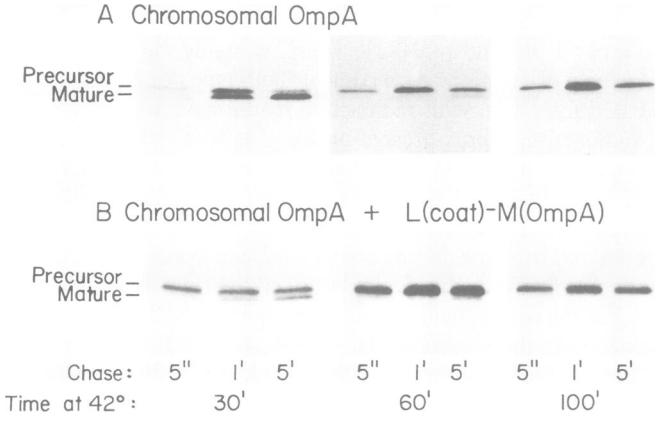

Fig. 6. The membrane assembly of L(coat)−M(OmpA) still requires *sec*Y function. *E. coli* IQ292 with plasmid pQN821 (**A**) or PQN820 (**B**) was grown at 30°C to mid-log phase, then shifted to 42°C. At the indicated time, aliquots were pulse-labeled for 1 min, then chased with an excess of non-radioactive methionine. After 5 s, 1 min or 5 min, portions were acid-precipitated, immunoprecipitated wtih antiserum to OmpA, and analyzed by SDS−PAGE and fluorography.

or the action of *sec*-encoded proteins, but requires the membrane electrochemical potential. It has been demonstrated that procoat also assembles *in vitro* into liposomes (Watts *et al.*, 1981; Ohno-Iwashita and Wickner, 1983; Geller and Wickner, 1985), inverted bacterial inner membrane vesicles (Chang *et al.*, 1979), and even into dog pancreas microsomes (Watts *et al.*, 1983). In the latter system, procoat does not require signal recognition particle or docking protein, as could be expected from the studies with liposomes. These unusual membrane assembly characteristics of procoat are strongly affected by mutations in the leader peptide (Kuhn and Wickner, 1985b), as well as in the membrane spanning and cytoplasmic portions of the mature protein (Kuhn *et al.*, 1986a,b).

In the studies reported in this communication, two possible reasons for the *sec*-independent assembly of procoat were tested. One was that the unusually small size of procoat (73 amino acids) is responsible for this phenotype. This was prompted by analogies to eukaryotic systems, where it has been estimated that 70−80 amino acids are protected by the large subunit of the ribosome and through interaction with signal recognition particle (Walter and Blobel, 1981). Recent studies with small eukaryotic precursors like prepromelittin, which contains only 70 residues (Suchanek *et al.*, 1978) have shown that their interaction with microsomes is indeed independent of signal recognition particle (Zimmermann and Mollay, 1986; R.Zimmermann, personal communication). It appeared possible that the small size of procoat might have analogous consequences, precluding its interaction with the *sec*-encoded 'secretion apparatus'. Our results are at variance with this notion. It could be shown that a fusion

protein of 177 residues, containing 72 residues of procoat fused to 103 polar amino acids from the carboxyl end of leader peptidase, assembles into a trans-membrane conformation independent of *sec*Y protein.

A second possibility was that a specific structural property of the procoat leader sequence caused its independence of *sec*Y. By exchanging the leader peptides of procoat and pro-OmpA, hybrid precursors were constructed and their membrane assembly properties were tested. We found that the L(OmpA)−M(coat) fusion was still *sec*Y-independent, while L(coat)−M(OmpA) required a functional *sec*Y protein. This demonstrates that, in this respect, the hybrid precursors retain the characteristics of the respective mature protein. However, the membrane assembly of these hybrid proteins was clearly retarded indicating that both the leader peptide and the mature region contribute to the overall rate of this process.

These results, then, suggest that *sec*-independence is not simply a function of the leader peptide. A remaining possibility is that *sec*-encoded proteins alter some physical property of the membrane to permit insertion of a class of proteins. The availability of *in vitro* systems, where the bacterial membrane assembly is *sec*Y-dependent, may allow this question to be addressed directly.

Materials and methods

Bacterial strains

E. coli K12 strain HJM114 (Wickner and Killick, 1977) and IQ292, which has a temperature-sensitive defect in *sec*Y (Shiba *et al.*, 1984) were from our laboratory collection and from Dr T.Ito (Kyoto), respectively. Cells were grown in M9 minimal medium (Adams, 1959) containing thiamine (2 μg/ml) and 20 μg/ml of each amino acid except methionine. Fructose (0.5%) and glycerol (0.2%) were the carbon sources for growth of HJM114 and IQ292, respectively. Cultures were grown overnight at 37°C (HJM114) or 30°C (IQ292).

Plasmids

The plasmid pQN8 (formerly termed pQN805; Kuhn and Wickner, 1985a) and its derivative pQN8ΔC12 (Kuhn *et al.*, 1986a) have been described. pQN2 carried the 1200-bp *Eco*RV−*Hin*dIII fragment of pUC8 OmpA (Geller *et al.*, 1986), coding for wild-type pro-OmpA. The construction of the other plasmids is described in Figure 2.

Labeling techniques

Overnight cultures were diluted 1:100 into M9 medium with the same carbon source and grown to a density of 10^8 cells/ml. To induce the transcription of plasmid-borne genes, 0.2% arabinose was added and growth continued to 2×10^8 cells/ml. Aliquots (0.1 ml) were labeled with 10 μCi of [^{35}S]methionine (>1000 Ci/mmol, Amersham) and chased with non-radioactive methionine (200 μg). Samples were mixed with an equal volume of chilled 40% trichloroacetic acid. Precipitates were collected and processed for immunoprecipitation (Kuhn and Wickner, 1985a), SDS−PAGE (Ito *et al.*, 1980) and fluorography.

DNA techniques

Restriction enzymes and the Klenow fragment of DNA polymerase I were purchased from Boehringer. DNA kinase was from Bethesda Research Labs, bacterial alkaline phosphatase from International Biotechnologies and T4 ligase from New England Biolabs. For transformations, the method of Cohen *et al.* (1973) was used. All enzyme reactions and DNA preparations were performed as described by Maniatis *et al.* (1982). Oligonucleotide-directed mutagenesis was by the method of Zoller and Smith (1983). Cloning from the plasmids to M13 mp19, and from the virus back to the plasmid, was done after digestion with *Eco*RI and *Sal*I (Kuhn *et al.*, 1986b). After transformation into *E. coli* HJM114, colonies were screened by hybridization with ^{32}P-labeled oligonucleotide. The mutants were verified by analytical restriction digestion wtih *Hpa*II and *Nae*I.

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