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The Filamentous Bacteriophage Assembly Proteins Require the Bacterial SecA Protein for Correct Localization to the Membrane

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The noncapsid assembly proteins pI and pI* of the filamentous bacteriophage f1 are inserted into the inner membrane of *Escherichia coli* via an internal signal sequence. Inhibition of the activity of SecA with low concentrations of sodium azide results in rapid accumulation of pI and pI* proteins in the cytoplasm. However, both proteins are inserted into the membrane under the same conditions when synthesized in bacteria containing a *secA* azide resistance mutation. The other noncapsid assembly protein, pIV, is an outer membrane protein synthesized with a cleavable signal sequence. Wild-type bacteria accumulate the precursor to pIV when protein synthesis is in the presence of low concentrations of sodium azide. These results suggest that the f1 bacteriophage assembly proteins require SecA and consequently the bacterial Sec system to reach their proper membrane location.

The filamentous bacteriophages f1, fd, and M13 consist of a covalently closed circular DNA genome contained in a protein tube formed by five capsid proteins (14). The capsid proteins, which appear to reside in the inner bacterial membrane, form around the phage DNA as it is extruded through the membrane assembly site (12). The major capsid protein, pVIII, is synthesized with a cleavable amino-terminal signal sequence but is inserted into the membrane independently of the *Escherichia coli* Sec proteins (10, 19). A minor capsid protein, pIII, also contains a cleavable amino-terminal signal sequence, but it does use the Sec system for translocation into the membrane (5, 13). The mechanism of insertion and the topology of the remaining minor capsid proteins (pVI, pVII, and pIX) are presently unknown.

In addition to the capsid proteins, the products of phage genes I and IV are required for the formation of phage particles and are referred to as the viral assembly proteins (17). The gene IV protein, pIV, is synthesized as a precursor and appears to reside primarily in the outer membrane (2). The transport of this protein to the outer membrane results in the induction of the four genes of the *E. coli* stress-induced phage shock (*psp*) operon (3, 4). Gene I encodes two proteins, the 348-residue pI and the smaller, 108-residue pI*, which is the result of an internal translation initiation event at codon 241 of gene I (6). Both proteins span the inner membrane via a 20-residue internal hydrophobic region so that the carboxyl-terminal 75 amino acids are exposed in the periplasm (6). Experiments with fusion proteins containing various regions of the pI protein suggest that the internal signal sequence is composed of a 13-residue sequence, beginning at Met-241, with the potential to form a positively charged amphiphilic helix, followed by the membrane-spanning region (9). Recent deletion analyses of specific fusion proteins suggest that the pI protein may require only the 20-residue hydrophobic region preceded by a single arginine residue for membrane insertion (7). The nature of the exact signal sequence for the pI* protein is still not known. Production of the pI and pI* proteins together, in the absence of the other phage proteins, leads to inhibition of cell growth, presumably due to a loss of membrane potential (8).

The nature of the pI-pI* protein internal signal sequence, the effect that the production of the assembly proteins has on cell viability and expression of the *psp* operon, and the different insertion pathways of the phage capsid proteins prompted us to examine whether the assembly proteins use the general Sec system to reach their membrane locations. SecA plays an integral part in the Sec translocation system (15, 18), and its ATPase and protein translocation activity have been shown by Oliver et al. (16) to be extremely sensitive to low concentrations of sodium azide. They showed that approximately 1 mM sodium azide results in an immediate block in protein export while bacteria containing azide resistance *secA* mutations are unaffected. The ability of the pI and pI* proteins to insert into the membrane was analyzed in wild-type MC4100 [F$^-$ *araD139* (*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi-1*] and its isogenic *secA* azide-resistant mutant DO309 (16) in the presence or absence of azide. These strains contained pJIH110, which encodes gene I under the control of the left promoter of lambda and the temperature-sensitive *c*I857 repressor (8). These bacteria also contained pJIH13 with the lambda gene N also under the control of the lambda left promoter. The presence of the N protein was required to read through the transcription terminator in gene I (8). Since the pI and pI* proteins do not appear in precursor form, their membrane localization was examined in the presence and absence of azide by a protease accessibility assay (6). The use of *sec* mutants to analyze the membrane insertion of pI-pI* proteins was not possible because of the combined lethal effect of inactive Sec proteins and production of pI-pI* proteins.

The bacteria were grown in Luria-Bertani media supplemented with 0.4% maltose at 32°C, a temperature at which only a low level of pI protein is synthesized. When the bacteria reached a concentration of 2×10^8 cells per ml, they were shifted to 39°C for 5 min in the presence or absence of 0.5 mM sodium azide. At this temperature, the pI protein is

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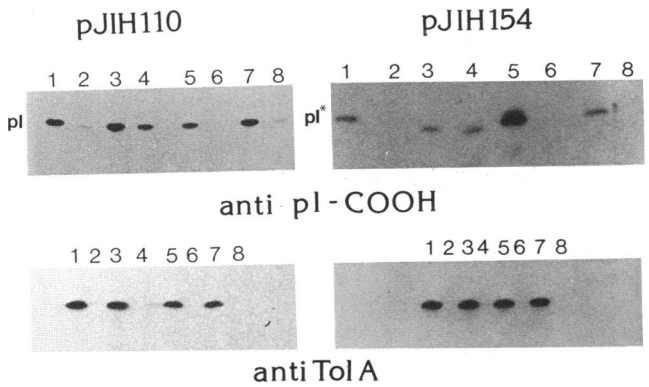

FIG. 1. Sodium azide inhibits membrane insertion of the pI and pI* proteins. Bacteria containing the appropriate plasmids were grown at 32°C and shifted to 39°C in the presence or absence of 0.5 mM sodium azide. After 5 min, the bacteria were harvested, and one-half were subjected to proteolysis by trypsin as described in the text. The proteins were then examined by Western blot analysis using both ^{125}I-protein A and either antibody to the carboxyl-terminal portion of pI protein (anti-pI-COOH) or antibody to TolA. Shown are the radioautograms from analyses of wild-type MC4100 (lanes 1 to 4) and *secA* mutant DO309 (lanes 5 to 8) bacteria containing the indicated plasmids. Cells in lanes 1 and 5 were not treated with either azide or trypsin. Cells in lanes 2 and 6 were treated with trypsin but no azide. Cells in lanes 3 and 7 were treated with azide but no trypsin, and cells in lanes 4 and 8 were treated with both azide and trypsin.

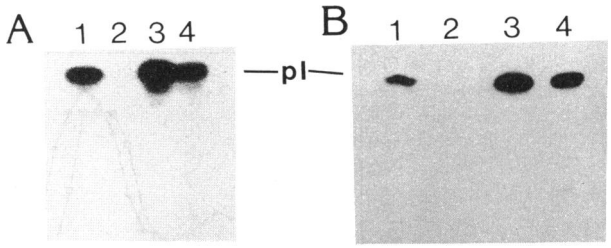

FIG. 2. Sodium azide sensitivity of the pI protein for insertion into the membrane. (A) K91 bacteria containing pJIH110 and pJIH13 were grown and subjected to the same experimental conditions described in the legend to Fig. 1. The radioautograms show Western blot analysis using anti-pI-COOH antibody. Lane 1, cells not treated with azide or trypsin; lane 2, cells treated with trypsin but no azide; lane 3, cells treated with 0.5 mM azide but no trypsin; lane 4, cells treated with both azide and trypsin. (B) Same experiment as in panel A except that the azide concentration was 2 mM.

expressed at a high rate. After harvesting by centrifugation, the bacteria were resuspended in 12% sucrose–30 mM Tris-HCl (pH 7.8)–1 mM EDTA and incubated on ice for 15 min in the presence or absence of 0.025 mg of trypsin per ml. The resulting cells were then solubilized in sample buffer and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis using antibody against the carboxyl-terminal portion of the pI protein and ^{125}I-protein A (Fig. 1, left panels) as described by Guy-Caffey et al. (6). The presence of azide rendered the newly synthesized pI protein resistant to the action of trypsin in the MC4100 wild-type strain (compare lanes 2 and 4 in top panel) but not in the DO309 *secA* azide-resistant strain (compare lanes 6 and 8 in top panel), suggesting that SecA is required for the insertion of the pI protein into the membrane. As a control for the effectiveness of the trypsin digestion, the same samples were subjected to Western blot analysis using anti-TolA antibody. TolA is a constitutively synthesized inner membrane protein which is sensitive to the presence of periplasmic trypsin (11). The bottom left panel of Fig. 1 shows that the membrane-associated TolA is accessible to the action of trypsin in the presence or absence of azide as expected.

The role of SecA in the membrane insertion of the pI* protein was analyzed in the same manner with bacteria containing pJIH154 instead of pJIH110. pJIH154 differs from pJIH110 by the presence of an amber mutation in the region coding for the amino-terminal portion of the pI protein and thus effectively synthesizes only the pI* protein when induced (6, 8). The top right panel of Fig. 1 shows that azide prevents the insertion of the pI* protein into the membrane in wild-type bacteria but not in *secA* mutant cells, suggesting that the pI* protein also requires an active Sec system for translocation into the membrane.

The same trypsin accessibility experiments were repeated with another *E. coli* strain, K91 (HfrC *thi phoA tonA22 garB10 ompF relA pit-10*), with azide concentrations of 0.5 mM (Fig. 2A) and 2 mM (Fig. 2B). This was the strain used to characterize the topology of pI-pI* proteins in the inner membrane (6). Again, the presence of azide prevented the insertion of pI protein into the membrane and also caused the accumulation of cellular pre-maltose-binding protein (preMBP) (data not shown). Maltose-binding protein (MBP) is a periplasmic protein whose secretion has been shown to be Sec dependent (1, 15). Cellular fractionation of the azide-treated bacteria into the periplasmic, cytoplasmic, and envelope components showed that the pI protein as well as preMBP was contained in the envelope fraction. To further examine this, the envelope was separated into inner and outer membranes as described by Guy-Caffey et al. (6) and probed for the presence of the pI protein and preMBP by Western blot analysis. In the absence of azide, pI and pI* proteins were associated only with the inner membrane and there was no membrane-associated MBP or preMBP (Fig. 3A). The fractions from the azide-treated bacteria showed accumulation of pI and pI* proteins and preMBP present at a position in the gradient (density greater than that of the outer membrane) where inclusion bodies usually sediment (Fig. 3B). Examination of these fractions by negative staining in the electron microscope revealed structures resembling inclusion bodies (data not shown). These structures rapidly accumulated in the presence of azide, even at concentrations as low as 0.5 mM. Thus, pI and pI* proteins rapidly accumulate in the cytoplasm in the absence of SecA function.

The effect of azide on the translocation of pIV across the membrane was tested in K1188 (F^+ *his bio thi gal* Sm^r) containing chromosomally encoded lambda N and *c*I857 genes and the plasmid pPMR72 (2). pPMR72 contains gene IV under the control of the left promoter of lambda. When this culture is shifted from 32 to 37°C, the production of pIV rapidly increases (Fig. 4, lanes 1 to 3). If the azide concentration is made 2 mM at the time of the temperature shift, precursor pIV accumulates, indicating that translocation of pIV is SecA dependent (Fig. 4, compare lanes 4 and 5). When the concentration of azide is lowered to 0.5 mM, the precursor of pIV appears more slowly (data not shown).

The data presented in this paper are consistent with the interpretation that the filamentous phage assembly proteins require active SecA protein and presumably the other membrane-associated Sec proteins to be properly inserted into

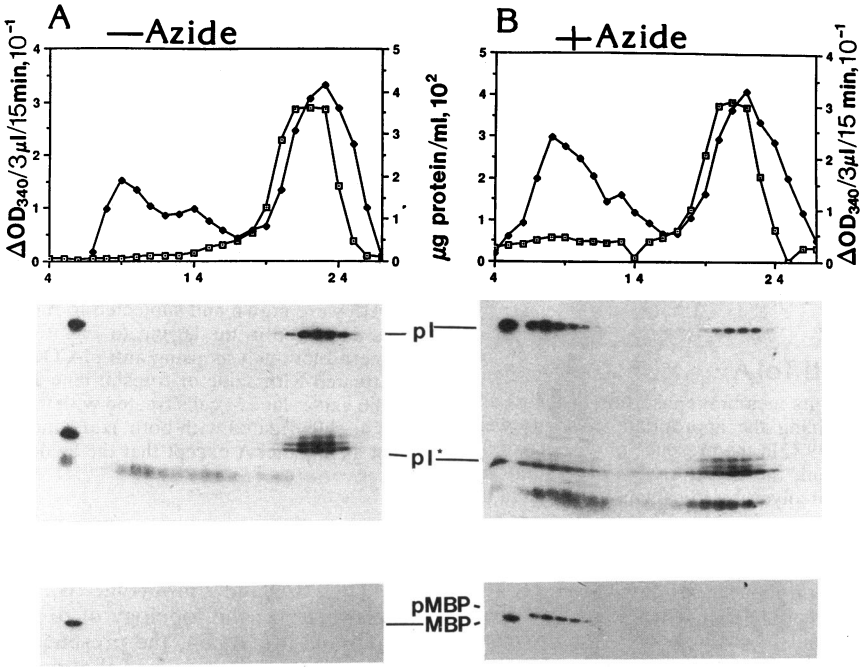

FIG. 3. Membrane fractionation of K91 bacteria containing pJIH110 and pJIH13. The bacteria were grown at 32°C and shifted to 39°C for 10 min in the absence (A) or presence (B) of 2 mM sodium azide. The membranes were separated by sucrose density gradient centrifugation. The top panels show the protein concentrations (■) or NADH oxidase activities (⊡) of fractions 4 to 27. ΔOD_{340}, change in optical density at 340 nm. The bottom of the gradient is to the left. The NADH activity marks the position of the inner membrane (fractions 14 to 24), while the outer membrane is found in fractions 4 to 12, as determined by the presence of outer membrane porins seen by Coomassie blue staining (data not shown). The middle panels show a Western blot analysis of an SDS-polyacrylamide gradient gel (10 to 16% acrylamide) of fractions 6 to 27 probed with antibody to the carboxyl region of pI. The bottom panels show a Western blot of the samples probed with antibody to MBP. The samples in the extreme left lanes are bacterial extracts in the absence of azide, Western blotted as markers for pI and pI* proteins, MBP, and preMBP (pMBP).

their membrane sites. We can only speculate why two systems are used to translocate the phage-specific proteins into the membrane. A large amount of the major coat protein, pVIII, must be rapidly synthesized and inserted into the membrane to allow assembly of the phage. Since this might overly tax the Sec secretion system, the pVIII major coat protein may require a separate insertion system. Since much less of the pIII capsid protein and the assembly proteins is needed, their insertion can be accommodated by the normal Sec system.

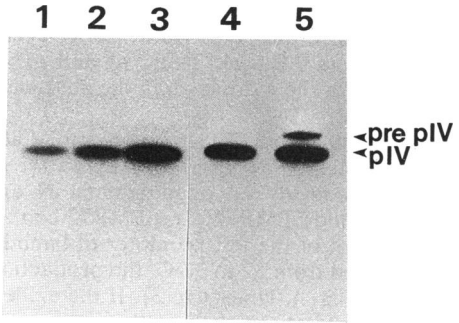

FIG. 4. Sodium azide sensitivity of pIV for insertion into the membrane. K1188 bacteria containing pPMR72 were grown at 32°C and then shifted to 37°C. At times after the temperature shift, the bacteria were collected and the proteins were subjected to Western blot analysis using anti-pIV antibody and ^{125}I-protein A. Lanes 1 to 3 show the labeled pIV at 0, 5, and 10 min, respectively, after the temperature shift. The remaining lanes show pIV or precursor (pre) pIV 10 min after the temperature shift in the absence (lane 4) or presence (lane 5) of 2 mM sodium azide.

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