Polarity and Charge of the Periplasmic Loop Determine the YidC and Sec Translocase Requirement for the M13 Procoat Lep Protein*§

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**Background:** Procoat protein mutants require the Sec translocase in addition to YidC. 
**Results:** Subtle changes in the polarity of the translocated region alter the mode of insertion.

**Conclusion:** The translocase requirement increases as the energy transfer expense of the translocated region increases.

**Significance:** Selection of the translocase is determined by the energy barrier of translocation.

During membrane biogenesis, the M13 procoat protein is inserted into the lipid bilayer in a strictly YidC-dependent manner with both the hydrophobic signal sequence and the membrane anchor sequence promoting translocation of the periplasmic loop via a hairpin mechanism. Here, we find that the translocase requirements can be altered for P Clep in a predictable manner by changing the polarity and charge of the peptide region that is translocated across the membrane. When the polarity of the translocated peptide region is lowered and the charged residues in this region are removed, translocation of this loop region occurs largely by a YidC- and Sec-independent mechanism. When the polarity is increased to that of the wild-type procoat protein, the YidC insertase is essential for translocation. Further increasing the polarity, by adding charged residues, switches the insertion pathway to a YidC/Sec mechanism. Conversely, we find that increasing the hydrophobicity of the transmembrane segments of P Clep can decrease the translocase requirement for translocation of the peptide chain. This study provides a framework to understand why the YidC and Sec machineries exist in parallel and demonstrates that the YidC insertase has a limited capacity to translocate a peptide chain on its own.

In *Escherichia coli*, two main translocases have been identified to date, and they are involved in inserting inner membrane proteins (1–3). The Sec translocase is the major translocase of *E. coli* and is responsible for inserting the majority of the inner membrane proteins as well as translocating exported proteins across the inner membrane into the periplasmic space. The second is the YidC membrane insertase, which plays a role in inner membrane protein insertion and assembly.

The Sec translocase is a heterotrimeric protein composed of the SecYEG protein-conducting channel, the accessory SecDF-YajC trimeric complex, and the peripheral subunit SecA. SecA is mainly involved in the insertion of proteins with large periplasmic domains (4–6) and has been shown to be a motor ATPase involved in driving the protein through the translocation channel. It is believed to insert ~20–30 residues for every ATP hydrolysis cycle (7). The precise function of SecDF-YajC remains unclear, but it is important for the insertion of some membrane proteins (8). SecDF is believed to be involved post-translationally in translocation and helps prevent backward movement of the preprotein via interaction of the substrate-translocated domain with the large periplasmic P1 domain on SecD (9).

In the year 2000, a new inner membrane protein, YidC, was discovered to be involved with membrane protein insertion (10, 11). It is believed to function in concert with the Sec translocase to integrate proteins into the lipid bilayer and can also act, in certain cases, as a chaperone assisting membrane proteins to attain their correct membrane-embedded fold within the membrane (3). YidC can also function as an insertase, independent of the Sec translocase (12, 13). In this pathway, YidC promotes the membrane insertion of the M13 procoat and Pf3 coat protein, which were previously thought to insert into the membrane “spontaneously” (11, 14). At this time, YidC has been found to be required for the membrane insertion of several endogenous membrane proteins such as subunit C of F<sub>1</sub>F<sub>0</sub>-ATPase (13, 15–17), subunit II of cytochrome bo oxidase (18–20), TatC (21), and MscL (22).

All of the YidC-only pathway substrates identified have a small periplasmic domain. Because of this common feature, it has been suggested that YidC is only capable of acting as an insertase for proteins with small periplasmic domains, whereas larger periplasmic domains require the Sec pathway for translocation. Interestingly, several studies have shown that modification of the primary structure of different YidC-only substrates can alter their insertion pathway to a Sec-dependent mechanism. The first observation was that an extension of the periplasmic loop of the M13 procoat by 174 residues (from OmpA) switches the protein from a Sec-independent pathway

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**References:**

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2. This article contains **supplemental Table 1**.
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4. The abbreviations used are: OmpA, outer membrane protein A; P Clep, procoat lep; TM, transmembrane segment; PK, proteinase K.
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Materials—Sodium azide and lysozyme were purchased from Sigma. Proteinase K (PK) was purchased from Qiagen; isopropyl 1-thio-β-d-galactopyranoside was from Research Products International Corp.; PMSF was purchased from United States Biochemical (Affymetrix). Tran35S-label, a mixture of 85% [35S]methionine and 15% [35S]cysteine, 1000 Ci/mmol, was from PerkinElmer Life Sciences. Antiserum to leader peptidase (anti-Lep) and outer membrane protein A (anti-OmpA) were from our own laboratory collection.

Strains, Plasmids, and Growth Conditions—JS7131, the E. coli YidC deletion strain, and MC1060 are from our collection. CM124, the SecE deletion strain, was obtained from Beth Traxler and is described in Ref. 26. Roos et al. (25) demonstrated that certain mutations in the procoat periplasmic loop result in the preprotein binding to the SecA protein, which then engages the SecYEG machinery. In contrast, with a single-spanning Pf3 TMLep model protein, the addition of a positively charged residue to the N-tail makes its insertion SecE-dependent but not SecA-dependent (28). Although these studies showed that mutations in the membrane proteins’ translocated region could change the translocase requirement, the reason for these results was not understood.

In this study, we present the polarity/charge hypothesis, which proposes that the membrane transfer expense of the translocated region determines the translocase requirement. We show that the insertion mechanism of Procoat lep (PClep) can be altered in a predictable way by changing the charge and polarity of the peptide region that is translocated. Although translocation of a peptide of low polarity can occur in a YidC/Sec-independent manner, YidC is required for translocation when the periplasmic loop is made more polar. Further increases in the polarity, by the substitution of positively charged residues or inserting negatively charged residues in PClep, change the mechanism such that both the YidC and Sec translocases are needed. However, substitution of apolar amino acids, with charged residues in the transmembrane segment of PClep to decrease its hydrophobicity, can also increase the requirements for a translocase.

EXPERIMENTAL PROCEDURES

PClep and PClep Mutants—The M13 coat protein is synthesized as a precursor called procoat with a cleavable signal sequence and a mature region consisting of a 20-amino acid periplasmic loop and a transmembrane segment (Fig. 1). The signal sequence of procoat is proteolytically removed by signal peptidase 1 (SP1, also known as leader peptidase) to generate the mature coat protein after membrane insertion. In our study, we used PClep in which the cyttoplasmic region is extended by 103 amino acids of the P2 domain of SP1 (30). This extension makes the protein easier to detect by using an antibody.

Increased Sec Requirement with Substituted Positive Charges to the Loop of Procoat—Wild-type PClep has a total of five charged amino acids in the periplasmic loop between the signal peptide and the membrane anchor. It contains four negatively charged and one positively charged residue, giving it a predicted net charge of −3 (Fig. 1). The YidC dependence of wild-type PClep was assayed using the YidC depletion strain, JS7131, which has yidC under the control of the araBAD promoter (11). Membrane insertion can be studied under YidC depletion conditions by growing the bacteria in growth media supplemented with glucose for 3 h, as described under “Experimental Procedures.” JS7131 cells expressing different protein constructs were labeled with [35S]methionine for 1 min under YidC expression (0.2% arabinose) and YidC depletion conditions (0.2% glucose). They were then analyzed by the protease acces-
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FIGURE 1. Membrane topology of procoat lep and mutants. Sequence of procoat is shown highlighting the amino acid residues that were modified in this study. The precursor form of procoat is made with a cleavable N-terminal signal peptide and a transmembrane segment in the mature region (42). The PClep used in this study has a P2 epitope fused at the C terminus of procoat corresponding to residues 220–323 of SP1. The residues in the signal peptide are denoted with a —, and residues in the mature domain are denoted with a + with respect to the cleavage site. SP1 cleaves PClep between the two Ala residues at the −1 and +1 positions. The gray circles indicate where substitutions were made. The ▽ indicates the site of insertion of additional amino acids for the −4 and −5 PClep mutants.

sibility assay (31). Briefly, JS7131 cells were converted to spheroplasts, by the addition of lysozyme and EDTA to allow the protease to have access to the inner membrane. A portion of the cells was treated with PK for 60 min to probe membrane insertion of PClep.

When the 35S-labeled PClep was expressed under YidC expression conditions, it was processed to the mature form (Fig. 2B, 2nd lane). PK added to the spheroplasts cleaves the translocated periplasmic loop causing a further shift on the gel (Fig. 2B, 1st lane, labeled f, which denotes the fragment with an approximate molecular mass of 15.1 kDa). Under YidC depletion conditions, procoat accumulated in the cytoplasm (Fig. 2B, lane 4) and was protected from PK cleavage (Fig. 2B, lane 3) (Fig. 2B, labeled p with a molecular mass of 19.46 kDa; corresponding to PClep). When YidC was present, PClep was inserted across the membrane where SP1 cleaved the signal peptide, giving rise to a lower molecular mass protein (Fig. 2B, labeled c, corresponding to coat lep with a molecular mass of 17.07 kDa).

The Sec dependence of the wild-type PClep and respective mutants was studied in the SecE depletion strain, CM124. Cells bearing the respective mutants on a plasmid were back-diluted in M9 minimal media supplemented with 0.4% glucose (SecE depletion conditions) or 0.4% glucose + 0.2% arabinose (SecE expression conditions) for 8–9 h. The cells were then transferred to fresh M9 medium and grown for 30 min before induc-
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![Image of amino acid sequence and protease accessibility]

**FIGURE 2. Increased Sec requirement with positively charged residues in the loop of PClep.** A, schematic of the amino acid sequence of PClep mutants with substituted arginine residues within the polar region that is translocated to the periplasmic space. B, YidC, SecE, and SecA requirement for membrane insertion of wild type. ARGNN (C), ANGRR (D), and ARGRR PClep were tested (E). Representative data of OmpA indicate the effectiveness of the protease as well as inhibition in OmpA export under SecE depletion conditions or by azide to inhibit SecA function (F). E. coli JS7131 (YidC depletion strain) expressing the various PClep proteins were grown for 3 h under YidC expression (0.2% arabinose) or YidC depletion conditions (0.2% glucose), labeled with [35S]methionine for 1 min, and analyzed by protease accessibility, as described under “Experimental Procedures.” The Sec dependence of membrane insertion was tested using CM124, the SecE depletion strain. CM124 transformed with the respective pLZ1 plasmid was grown under SecE expression (0.2% arabinose and 0.4% glucose) or SecE depletion conditions (0.4% glucose) to Sec dependence with more than half of the protein being accumulated in the cytoplasm under SecE depletion conditions or when SecA was inhibited. Substitution of aspartic acid residues at both positions in the protein (L/TM-D PClep) caused the protein to be SecA- and SecE-dependent for insertion, in addition to being completely YidC-dependent (Fig. 3G).

Next, we examined whether the substitution of a neutral hydrophilic residue would have the same effect as a charged residue when added to the same positions as the charged residues in the signal peptide or TM sequence. The mutants L-Q, TM-Q, and L/TM-Q showed no Sec requirement, while remaining completely dependent on YidC for insertion (Fig. 3, H–J). Taken together, these studies show that Sec requirement was enhanced only when a charged residue was added and not with a neutral polar residue.

Requirement for YidC and Sec Increases with Increased Polar-...
To test whether we can reduce the YidC requirement for membrane insertion by decreasing the polarity of the periplasmic loop, we studied the ANGNN PClep mutant with the negatively charged residues at positions \(\pm 1\), \(\pm 3\), and \(\pm 5\) of wild-type PClep substituted with neutral residues (30). Fig. 4D shows that ANGNN PClep inserted into the membrane in a less YidC-dependent manner with a small portion of the protein inserting into the membrane even under YidC depletion conditions compared with a complete block for the wild-type PClep. Decreasing the polarity further, by substituting alanine instead of the uncharged but still polar asparagine residues at the \(\pm 2\), \(\pm 4\), and \(\pm 5\) positions (AAGAA PClep), led to a similar insertion efficiency as the ANGNN mutant under YidC-depleted conditions (Fig. 4E).

### FIGURE 3. Charged residues in the hydrophobic region of the signal peptide or the transmembrane segment of PClep make insertion more Sec-dependent.

A, schematic of the amino acid sequence of PClep mutants with arginine, aspartic acid, or glutamine residue substitutions in the signal peptide and/or in the transmembrane segment. The YidC, SecE, and SecA requirements of membrane insertion of L-R (B), L-D (C), TM-R (D), TM-D (E), L/TM-R (F), L/TM-D (G), L-Q (H), TM-Q (I), and L/TM-Q PClep (J) were tested. Membrane insertion of the PClep mutants was examined under YidC depletion, SecE depletion, and SecA inhibition conditions as described in Fig. 2. Cells were labeled with \(^{35}\text{S}\)methionine and analyzed by protease accessibility (see “Experimental Procedures”).

| YidC + | YidC - | SecE + | SecE - | SecA + | SecA - |
|---|---|---|---|---|---|
| PK | PK | PK | PK |
| L-R | L-D | TM-R | TM-D |
| L/TM-R | L/TM-D | L-Q | L/TM-Q |

To further test whether the YidC requirement is correlated with the translocation of a loop that is highly polar, we decreased the polarity of the loop even more by changing the lysine at the +1 position and the glutamic acid residue at +20 position to alanines, in addition to the AAGAA mutations. Fig. 4F shows that AAGAA/AA PClep mutant inserted into the membrane almost completely independent of YidC. All the uncharged mutants studied in this section, namely the AAGAA, ANGNN, and the AAGAA/AA mutants, inserted independent of the Sec translocase.

### Increasing the Hydrophobicity of the TM Segment Can Decrease the YidC Requirement of Insertion

The TM4L PClep mutant has a more hydrophobic TM segment as compared with WT PClep due to the substitution of the residues at positions...
+24 to +27 with leucines (from tyrosine, alanine, tryptophan, and alanine, respectively) (Figs. 1 and 5A). This mutant, TM4L PClep, inserted into the membrane in a largely YidC-independent manner with roughly half of the mutant being membrane-inserted in the JS7131 strain. The insertion of this protein was completely unaffected by SecE depletion or SecA inhibition (Fig. 5B). Possibly, the increased hydrophobicity of the mutant allowed a direct interaction with the hydrophobic core of the membrane, driven by its own hydrophobicity.

Next, we tested whether combining the TM4L PClep mutant with the ANGNN mutation in the loop of PClep would result in a lower dependence on YidC on insertion. Fig. 5C shows the TM4L/ANGNN PClep protein inserts also largely independent of YidC as well as SecE and SecA. A completely YidC- and Sec-independent mechanism of insertion is seen with the TM4L/AAGAA mutant, which contains alanine residues at the +2, +3, and +5 position instead of the more polar asparagine residues (Fig. 5D). Possibly, the increased hydrophobicity of the mutant allowed a direct interaction with the hydrophobic core of the membrane, driven by its own hydrophobicity.

With the increased hydrophobic driving force of the TM4L PClep construct, it may be possible that the loop containing the ARGRR could be translocated with less dependence on the Sec translocase. Indeed, TM4L/ARGRR PClep inserted across the membrane in a largely SecYEG-independent manner, although the protein still required YidC for insertion (Fig. 5E). Therefore, the energy barrier for translocation due to the positively charged arginines was still high, even with the increased hydrophobic TM segment of PClep. To test whether the TM4L segment could drive translocation of the −5 loop with less dependence on the Sec machinery, we tested the TM4L/−5 PClep mutant under SecE depletion conditions or when the SecA function was inhibited. Fig. 5F shows this construct inserted almost completely independent of the Sec machinery, suggesting that the negatively charged periplasmic loop is easily translocated when the hydrophobic driving force is increased by the 4-leucine substitution and can largely do this without requiring SecA and SecYEG.

**DISCUSSION**

Our hypothesis is that the polarity and charge of the periplasmic loop determines the Sec and YidC requirements for M13 procoat translocation. The translocase requirement of a protein is thermodynamically determined because the higher the polarity of the loop, the higher the translocation barrier for moving the peptide chain through the lipid phase. What we found is compelling. By increasing the polarity of the loop region, either by incorporating negatively or positively charged residues, the Sec requirement for insertion of PClep was increased linearly. In contrast, decreasing the polarity of the translocated loop region from that of the wild-type protein decreased the degree to which YidC is needed for insertion.

Starting with PClep, which is YidC-dependent, we found that when two negatively charged residues were added, membrane insertion was completely Sec/YidC-dependent (−5 PClep), whereas adding only one negatively charged residue did not change the insertion mechanism (−4 PClep). Thus, YidC on its own can translocate the PClep periplasmic loop with one additional negatively charged residue but cannot with two added charged residues. This latter −5 PClep mutant required SecYEG and also SecA. Interestingly, previous studies with the MscL protein (33), which spans the membrane twice, found that adding negatively charged residues to the periplasmic region made the protein SecYEG-dependent for membrane insertion.
insertion but did not affect its SecA independence for insertion. However, in this study, we found a remarkable correlation between the level of SecE dependence with the extent of SecA dependence for the Sec-dependent PClep mutants.

YidC has difficulty transporting the periplasmic loop with a small number of positively charged residues added to the loop showing that positive charges have a bigger requirement for Sec assistance than negative charges. This is seen most clearly by comparing the wild-type PClep containing AEGDD with the ARGRR PClep mutant, where the positively charged mutant has arginines at the same position as the wild-type negative charges (H11001, H11001, and H11001); otherwise, the periplasmic sequence is identical. The triple arginine mutant was completely YidC/SecE- and SecA-dependent, whereas the wild-type protein was Sec-independent. Interestingly, adding one arginine to the sequence with the other neutral residues caused the protein (ARGNN PClep) to insert into the membrane in a slightly SecE- and SecA-dependent manner while maintaining its dependence on YidC. Substituting another arginine led to a further increased Sec dependence of insertion (as in PClep ANGRR).

The ARGRR PClep mutant, like PClep, was completely Sec-dependent, indicating that three positively charged residues lead to a Sec dependence similar to 5 negatively charged residues. Our results with the positively charged PClep mutants are different from recent results obtained with a single span Pf3-Lep model protein (28). With this Pf3-Lep model protein, the addition of a single positively charged residue to the periplasmic region switched the insertion pathway from Sec-YidC-independent to Sec-YidC-dependent, whereas the Pf3-Lep model protein inserts with only one hydrophobic domain (34).

We found that by decreasing the polarity of the wild-type PClep periplasmic loop by changing the negatively charged residues at H11001, H11001, and H11001 to either asparagines (ANGNN PClep) or alanines (AAGAA PClep) (Fig. 4, D and E), we were able to decrease the YidC dependence of insertion to ~40–50% YidC-independent insertion. Further decreasing the polarity of the loop (AAGAA/AA PClep), by substituting all the charged residues with alanines, led to an even further decrease in YidC dependence providing support for our hypothesis that the polarity of the loop of PClep is a major determinant for pathway selection.

Increasing the hydrophobicity of the TM regions should provide more thermodynamic force to drive the translocation of the PClep periplasmic loop. The ΔG_{app} for the wild-type membrane anchor of PClep is ~20.2 kJ/mol as compared with ~32.4 kJ/mol for the TM4L mutant (35). Indeed, we observed membrane insertion was over 50% independent of YidC with the TM4L mutant (Fig. 5; Table 1). Moreover, translocation of the less hydrophilic periplasmic loop of the AAGAA PClep mutant, in combination with the TM4L, was completely YidC-independent as the hydrophobic force was increased with the presence of the more hydrophobic TM segment (TM4L + AAGAA). Intriguingly, the very hydrophobic TM segment was insufficient to drive insertion of a loop independent of a translocase when it contained a number of positively charged residues as seen with the TM4L/+ARGRR PClep mutant. We assume that the translocation barrier was too high to go by the

**FIGURE 5.** Increasing the hydrophobicity of the transmembrane segment of PClep reduces the YidC and Sec requirements for membrane insertion. A, schematic of amino acid sequence of PClep mutants with leucine mutations in the transmembrane segment to increase the hydrophobic driving force of membrane insertion. The italicized amino acids indicate insertions. The translocase requirements are as follows: TM4L (B), TM4L + ANGNN (C), TM4L + AAGAA (D), TM4L + ARGRR (E), and TM4L/+5 PClep (F). YidC depletion, SecE depletion, and SecA inhibition and protease accessibility studies were performed as described in Fig. 2.
YidC-only pathway, and therefore, this positively charged mutant remained slightly Sec-dependent. This is to be compared with the ARGRR PClep mutant (without the 4 leucines substituted in TM1) that was almost completely dependent on the SecYEG machinery, in addition to YidC, for insertion. Similar results were observed with the TM4L/−5 mutant with a number of negatively charged residues in the translocated region. Remarkably, the more hydrophobic TM segment reduces the Sec translocase requirement for translocation of both negatively and positively charged residues (Table 1).

As mentioned above, our results indicated that positively charged residues added to the periplasmic region have a larger effect on the requirements for a translocase than negatively charged residues. Therefore, not only polarity, but also the charge of the peptide chain, needs to be taken into consideration because of the membrane potential. The membrane potential (+ side periplasmic) favors the transfer of negatively charged residues across the membrane but hinders the translocation of positively charged residues (23, 36). Table 1 summarizes the predicted standard free energy needed (not considering the contribution of the membrane potential) for transfer of the periplasmic region across the membrane for the PClep mutants. The values for the membrane transfer expense were determined using the GES scale for each amino acid and include the contribution of the peptide bond (37). As expected, as the GES values decrease from the wild-type PClep to the ANGNP PClep, AAGAA PClep, to the AAGAA/AA PClep mutant, membrane insertion becomes less dependent on YidC. Intriguingly, as the number of the positively charged residues is increased (going from ANGNN ⇒ ANGR ⇒ ARGRR), both the Sec dependence and the GES values of the periplasmic domain increase. The insertion of two negatively charged residues leads to a Sec-dependent insertion (−5 PClep), although one added negatively charged residue (−4 PClep) did not change the YidC-only insertion mechanism. Because the protonotive force (+ side on the periplasm) supports the membrane transfer of negatively charged residues but restricts the transfer of positively charged residues, this might account for the Sec independence of −4 PClep that has a +278 GES value, whereas the ANGRR that has a +251 GES value is slightly Sec-dependent. The periplasmic region poses a bigger barrier for the ANGRR over the −4 PClep mutant because the transfer of positive charge increases the standard free energy due to the membrane potential, whereas a negative charge decreases the standard free energy.

The mutants that show a low energy barrier for translocation of their periplasmic domain and possess an increased hydrophobic TM segment require neither YidC nor Sec translocase. Although insertion could be facilitated by another not yet identified component, it is conceivable that in this case the TM regions directly insert into the lipid bilayer, and even the periplasmic domain is translocated through the lipid bilayer directly. This unassisted mechanism has also been observed for proteins that insert into the thylakoid membrane (38). Moreover, a recent in vitro study has shown that the Foc subunit inserts into liposomes with substantial efficiency (39). Also, Koch and co-workers (40) have shown that a minor amount of MtlA was inserted into liposomes in the presence of SRP and FtsY but without YidC and Sec translocase. The addition of YidC, SecYEG, or both improved the insertion efficiency. In agreement with these studies, our results indicate that small periplasmic domains with low polarity and charge can insert without assistance. However, when YidC is present, the hydrophobic TM segment of PClep will readily interact with the TM regions TM1, TM3, TM4, and TM5 of YidC, which contain the substrate contact sites (41). Most likely, during the translocation process, the periplasmic domain is passively dragged along with the TM region into the bilayer by strong interactions between the TM segments of YidC and the substrate. Polar and charged residues within the periplasmic region need further assistance of the Sec translocase that likely shields these residues during their translocation process (Fig. 6).

In conclusion, we found that if the energy barrier of translocation is low, insertion can occur largely by an unassisted mechanism requiring neither YidC nor the Sec translocase. If the barrier is increased due to a more hydrophilic loop and below a certain polarity/charge threshold, then YidC can catalyze the translocation step by itself. However, if the barrier exceeds a certain threshold due to a higher polarity and charge of the periplasmic loop, then both YidC and Sec are required for insertion (Fig. 6). Exactly how YidC and the Sec machinery work together to drive insertion is still not known. We favor the

### TABLE 1

The membrane transfer expense for translocation of the periplasmic region of the PClep constructs calculated using the GES scale for each amino acid (37)

| PClep mutant | YidC requirement | Sec requirement | Standard free energy (kJ/mol) GES |
|--------------|------------------|-----------------|-----------------------------------|
| WT (AEGDD)   | + + +            | −               | +238                              |
| ANGNP        | + +             | −               | +188                              |
| AAGAA        | + +             | −               | +108                              |
| AAGAA/AA/A   | −               | −               | +23                               |
| ARGRR        | + + +           | +               | +219                              |
| ARGRR        | + + +           | +               | +251                              |
| −4 (AEEGD)   | + + +           | −               | +282                              |
| −5 (AEEGD)   | + + +           | −               | +317                              |
| TM4L         | +               | −               | +238                              |
| TM4L + ANGNN | +               | −               | +188                              |
| TM4L + AAGAA| −               | −               | +108                              |
| TM4L + ARGRR| + + +           | +               | +282                              |
| TM4L/−5 (AEEGD) | + + +         | +               | +317                              |

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idea that PClep inserts at a YidC/SecYEG interface such that both translocases can facilitate translocation simultaneously. In addition, the work presented here shows that subtle changes throughout the procoat protein, i.e. to the leader sequence, the TM segment, or periplasmic loop of the M13 procot can change the insertion pathway from YidC-only to YidC/Sec or to YidC/Sec-independent.

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