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**New insights into amino-terminal translocation as revealed by the use of YidC and Sec  
depletion strains**

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Different attributes of membrane protein substrates have been proposed and characterized as translocation-pathway determinants. However, several gaps in our understanding of the mechanism of targeting, insertion and assembly of inner membrane proteins exist. Specifically, the role played by hydrophilic N-terminal tails in pathway selection is unclear. In this study we have evaluated length and charge density as translocase determinants using model proteins. Strikingly, the 36 residue N-tail of 2Pf3-Lep translocates independent of YidC-Sec. This is the longest known substrate of this pathway. We confirmed this using a newly constructed YidC-Sec double-depletion strain. Increasing its N-tail length with uncharged spacer peptides led to YidC dependence and eventually YidC-Sec dependence, hence establishing that length has a linear effect on translocase dependence. Tails longer than 60 residues were not inserted, however an MBP-2Pf3-Lep fusion protein could be translocated. This suggests that longer N-tails can be translocated if it can engage SecA. In addition, we have examined how the positioning of charges on the translocated N-tail affects the insertion pathway. Additional charges can be translocated by the Lep TM when the charges are distributed across a longer N-tail. We tested charge density as a translocase determinant and confirmed that the addition of positive or negative charges led to a greater dependence on YidC-Sec when they were placed close to each other than away. Findings from this work make an important advance in our existing knowledge about the different insertion mechanisms of membrane proteins in *E. coli*.

## Introduction

The bio-machineries responsible for membrane protein biogenesis are universally conserved. Facilitated insertion and assembly of inner membrane proteins are catalysed by two known protein translocases in *E. coli*, Sec and YidC [1]. However, some proteins like KdpD [2], KscA [3], Pf3-Lep [4] have been proposed to insert by an unassisted pathway. The question of what features of a membrane protein substrate determines its insertion-pathway is incompletely answered. In this study, we have addressed important gaps in this area to improve our understanding of the fundamental mechanism of membrane protein insertion and assembly. Specifically, we have studied the features of the substrate N-terminal tails that dictate its translocase requirements, which heretofore have been difficult to address experimentally.

Sec is the membrane integration site for majority of the inner membrane proteins. The holotranslocon consists of the SecYEG channel, and the accessory elements SecDF, YajC and YidC [5]. Majority of its substrates are targeted to SecYEG as ribosome nascent-chain complexes (RNCs) by the SRP-FtsY pathway [6] and the substrate hydrophilic domain is translocated across the membrane in an unfolded state through a pore, while the TM segment exits the channel through a lateral gate to integrate into the membrane [7]. SecA is an associated ATPase that powers the movement of periplasmic proteins, and large hydrophilic domains of membrane proteins across the channel [8]. YidC is responsible for the insertion of a smaller subset of proteins like Pf3 coat and M13 procoat [9], subunit c of FoF1ATPase [10], MscL [11] and SciP [12]. In addition, it also assists in the folding, assembly and membrane partitioning of certain Sec-dependent proteins like MalF [13] and LacY [14, 15]. Recent studies showed that YidC possesses an aqueous cavity in the inner leaflet that likely can host a substrate N-tail and reduce its membrane crossing distance [16]. YidC and Sec can also work together to insert substrates, presumably at the YidC TM “greasy slide” and Sec lateral gate interface via the YidC-Sec pathway [17]. Substrates of this pathway include NuoK [18], subunit a of F0a F0F1ATPase [19, 20] and CyoA [21, 22].

It is unclear how substrates are destined for translocation by these pathways. Previous studies suggest that YidC has limited potential to function independently and recruits Sec for the translocation of more sophisticated proteins in terms of the size, charges and hydrophilicity [4, 23, 24]. Typical substrates of the YidC-only pathway have short translocated N-tails like Pf3 whereas Sec is required for the membrane insertion and translocation of large periplasmic domains of substrate proteins like leader peptidase [25],  $\beta$ -lactamase [26] and alkaline phosphatase [27]. Exception to this is ProW, which has been proposed to insert by a Sec-independent mechanism [28]. Cao *et al* showed that the Sec-independent protein Pf3-Lep requires Sec on increasing its N-tail length [29]. However, it is unclear what is the size-limit for N-translocation by YidC-Sec independent, YidC-only and YidC-Sec pathways. Pathway selection based on charges have been controversial and needs further investigation. It has been shown that negative charges on the N-tail and TM segment can act as YidC determinants and positive charges as Sec determinants [4, 18]. But the unfavourable distribution of positive charges have also been proposed to act as YidC-determinants [30].

To further our understanding of N-terminal tail translocation, we have examined length and charge features as pathway determinants by employing single-spanning model substrates. We have evaluated the critical length required for YidC-only and YidC-Sec mediated substrate insertion using a new strategy to regulate expression of two essential genes (*yidC* and *secE*) in the same cell. On increasing the N-tail length, we find that substrates switch from independent to YidC-only to YidC-Sec. Beyond 60-residue N-tail length, the substrates were not translocated. However, the large periplasmic protein maltose-binding protein (MBP) could be translocated in the N-terminal direction likely because it can engage SecA. We also find that longer N-tails could translocate additional charges, both positive and negative, if they are distributed away from each other. This led to the hypothesis that N-tail charge density plays a role in necessitating translocation dependency. Our results show that crowding of charges causes a switch in translocation pathway from YidC-Sec independent to dependent.

## Results

### N-tail length requirement for translocase dependence

To study if N-tail length is a determinant for the translocase requirement for insertion, we used Pf3-Lep, based on the YidC-Sec independent model protein used in [4]. Pf3-Lep has the 18-residue long Pf3 coat N-tail with two negative charges, followed by leader peptidase from positions 4-323. A positive charge added at position 79 renders its TM2 defective for insertion [29]. This prevents the translocation of the C-terminal P2 domain and allows us to monitor the translocation of the amino-terminus alone. To study the length requirement, the Pf3 tail segment was first doubled by adding another Pf3 N-tail ahead of the TM segment to make 2Pf3-Lep (Fig. 1a, Supp. Fig. 1a). To further increase the N-tail length, uncharged spacer residues used in [29] were inserted between residue 36 and 37 of 2Pf3-Lep (Fig. 1b).

The translocase requirements for these substrates were studied using the YidC-depletion strain JS7131 [9] and the SecE-depletion strain CM124 [31] that has either the *yidC* or the *secE* gene under the *araBAD* promoter, respectively. SecE depletion has been shown to affect SecYEG dependent substrates since SecE is required for the stabilization of SecY [32]. The depletion of the respective translocases was confirmed using western blot analysis (Supp. Fig. 3a), which showed a steep decline in the translocase levels under conditions where transcription of *secE* and *yidC* was blocked. To test YidC-dependence for membrane insertion, the substrates were expressed in JS7131 and labelled using [<sup>35</sup>S] methionine for 1 min under YidC expression (0.2% arabinose) and YidC depletion (0.2% glucose) growth conditions. N-tail translocation was studied using a protease-accessibility assay as described in [33]. Briefly, spheroplasts were generated using lysozyme to allow access to the inner membrane and then treated with Proteinase K for 30 min. When the N-tail of the substrate protein (full length indicated by *P* in Fig. 2) was translocated across the membrane, the N-tail was digested by the externally added protease (PK) and a smaller band corresponding to the protease-resistant fragment was

observed (indicated by *F* in Fig 2); whereas when the amino-terminal region of the substrate was not translocated, it is not protease-accessible, so a band whose size corresponds to the full-length protein was seen. Similarly, to determine Sec dependence of the various constructs, CM124 cells expressing the model proteins were labelled with [<sup>35</sup>S] methionine for 1 min under SecE expression (0.2% arabinose) and SecE depletion conditions (0.2% glucose), and the protease mapping assay was carried out as described above.

To assess the efficiency of the formation of spheroplasts, degradation of outer membrane protein A (OmpA) was used as a positive control as it can be digested by Proteinase K from the periplasmic side of the membrane in properly prepared spheroplasts but cannot be accessed in intact cells. OmpA controls were performed for all the experiments reported in this study but are only shown once for representative purposes.

When the substrate N-tail was doubled to 2Pf3-Lep, the 36-residue long N-tail was found to be translocated efficiently under YidC or Sec depletion conditions and hence it was still YidC-Sec independent (Fig. 2a). Upon extending the N-tail with spacer pentapeptides to 41 (2Pf3+5-Lep) and 46 (2Pf3+10-Lep) residues, we observed a gradual increase in YidC dependence; but these substrates were still largely Sec-independent (Fig. 2b, 2c). Upon further increasing the N-tail length to 51 residues (2Pf3+15-Lep), the efficiency of insertion was reduced but it continued to be inserted by the YidC-only pathway and did not require Sec (Fig. 2d). At 56 residue N-tail length (2Pf3+20-Lep), the substrate required both YidC and Sec, albeit the insertion was not efficient (Fig. 2e). Further elongation of the N-tail to 61 residues (2Pf3+25-Lep) prevented its translocation completely (Fig. 2f). The substrate percentage translocated in each condition was measured by quantifying the bands using ImageJ (see “Materials and Methods”). As a control, we confirmed that OmpA (indicated by *M* in Fig. 2) was completely digested by the protease indicating good spheroplast formation (Fig. 2g). Additionally, OmpA export required Sec-only, so its protease-protected precursor form Pro-OmpA (indicated by *P* in Fig. 2) accumulated in Sec depletion condition but not in YidC.

### Sec-dependence on increasing N-tail length of the YidC-only substrate Pf3-23Lep

To test if increasing the N-tail length of a YidC-dependent substrate will cause a pathway-switch to require Sec, the model substrate Pf3-23Lep was employed. Pf3-23Lep contains the N-tail and TM segment of Pf3 coat protein fused to the 23<sup>rd</sup> residue of Lep and is a well-characterized YidC-only substrate [34]. Doubling the N-tail of Pf3-23Lep to 2Pf3-23Lep (Fig. 1b, Supp. Fig. 1b) did not cause a change in its translocation pathway; the substrate continued to insert by YidC-only mechanism (Fig. 3a). However, a gradual Sec dependence was observed in addition to the YidC dependence, when its N-tail length was increased to 41 (2Pf3+5-23Lep) and 46 (2Pf3+10-Lep) amino acids in length using the uncharged spacer residues used previously (Fig. 3b, 3c) [29]. Further increases in the N-tail length to 51 (2Pf3+15-23Lep) and 56 residues (2Pf3+20-23Lep) resulted in a strict dependence on both YidC and Sec, but the substrate was not translocated efficiently (Fig. 3d, 3e), as seen in the previous study. The longer N-tail of 61 residues length (2Pf3+25-23Lep) was not translocated (Fig. 3f).

### Translocation of the mature domain of MBP in the N-terminal direction

The model substrates tested above, which contained the duplicated Pf3 tails and spacers peptides, did not insert beyond a size of 60 residues length. This may have to do with the fact that there is something inherent about these sequences that prevent export, such as the lack of recognition sites for SecA/B machineries. Therefore, we examined whether a protein domain that is normally exported, i.e., in the C-terminal direction, could be exported in the amino-terminal direction. We also wanted to examine whether translocation of a long protein segment in the amino-terminal would require YidC. To test this, we fused the secretory protein MBP to the N-terminus of 2Pf3-Lep (MBP-2Pf3-Lep, Fig. 4a) and its translocation was studied as described above. The mature domain (indicated by *M* in Fig. 4) was translocated efficiently by Sec pathway without its cleavable signal sequence (Fig. 4b). However, YidC was dispensable as the translocated domain of the substrate was translocated and cleaved by the external protease to produce the protease-resistant fragment, (indicated by *F* in Fig. 4) even when YidC was

depleted. Based on the size of the protease-protected fragment of MBP-2Pf3-Lep, we predict that it consists of the Lep portion of MBP-2Pf3-Lep, which has 7 Met residues, as compared to the full length MBP-2Pf3-Lep that has 16 Met residues. The data suggests that the substrate C-terminal TM behaves as a reverse signal to open the SecYEG channel. We confirmed this by adding a pair of positive charges in the TM region (MBP-2Pf3-Lep L41R, L48R) that disabled MBP translocation (Supp. Fig. 2a). Next, we truncated the periplasmic region of this substrate by deleting all but the first 60 residues of the MBP mature domain (MBP-2Pf3-Lep  $\Delta$ 61-325, Fig. 4a). At this intermediate N-tail length of 96 residues, we observed that the substrate was inserted by Sec and YidC (Fig. 4c). PreMBP-2Pf3Lep that still has the N-terminal signal sequence of MBP attached, was also tested as a positive control. The precursor protein (indicated by *P* in Fig. 4d) accumulates under Sec depletion conditions whereas it doesn't require YidC.

### Testing charge density hypothesis

Previously it was shown by Zhu *et al.* that charges within the N-tail can act as translocase determinants [4]. When a negative or a positive charge was inserted in the Pf3-Lep N-tail, it caused a switch in translocase requirement from YidC-Sec independent to YidC-only or YidC-Sec dependent respectively. However, we observed that additional charges can be translocated when they are distributed across a longer N-tail in the 2Pf3-Lep construct, which has twice as many charges (Fig. 2a). This led us to propose and test the hypothesis that it is the charge density of the N-tail rather than just the presence of charges per se that determines its translocation pathway.

First, we introduced additional negative charges to the 2Pf3-Lep N-tail (Fig. 5a) and examined its translocase preference. Addition of one negative charge at position 15 on the first N-tail (2Pf3-Lep V15D), or 15' that is on the N-tail closer to the TM (2Pf3-Lep V15'D), did not influence the translocation pathway (Fig. 5b, 5c); the substrates continued to insert independent of YidC and Sec. When two negative charges were added at positions 15 and 15' (2Pf3-Lep V15D, V15'D), the substrate showed partial dependence on both YidC and Sec (Fig. 5d). To increase the

local density of charges in one of the two N-tails, the two negative charges were either added at positions 12 and 15 in the first N-tail (2Pf3-Lep L12E, V15D) or at the same positions in the second N-tail (2Pf3-Lep L12'E, V15'D). The charges introduced in the first N-tail resulted in a greater dependence on both YidC and Sec (Fig. 5e). However, when the charges were positioned closer to the TM, the N-tail was not efficiently translocated and strictly required YidC and Sec (Fig. 5f).

Similarly, to evaluate the effect of adding positive charges, an Arg residue was substituted in the first and second N-tail of 2Pf3-Lep at the same positions (Fig. 6a) as with the previous study. 2Pf3-Lep V15R was largely independent of YidC and Sec (Fig. 6b), but 2Pf3-Lep V15'R that bears the substitution closer to the TM segment was partially dependent on both YidC and Sec (Fig. 6c). On adding two Arg residues at positions 15 and 15' (2Pf3-Lep V15R, V15'R), the substrate became strictly dependent on YidC and Sec (Fig. 6d). When the two positive charges were placed close to each other in the first N-tail (2Pf3-Lep L12R, V15R) the substrate was poorly inserted and required both YidC and Sec (Fig. 6e). When the charges were substituted in the second N-tail (2Pf3-Lep L12'R, V15'R), it was not inserted (Fig. 6f).

### **2Pf3-Lep is YidC-Sec independent in double-depletion conditions**

*In vitro* membrane insertion studies have shown that some Sec-dependent substrates display promiscuity and can be also translocated by the YidC-only pathway [35]. This promiscuity may explain why 2Pf3-Lep can insert under YidC depletion conditions, where it may go by the Sec pathway, and vice-versa. Therefore, it is critical to evaluate its insertion under YidC-Sec double depletion conditions to rule out the possibility of a promiscuous insertion pathway. For this, we utilized CRISPR interference (CRISPRi) [36] to repress expression of *secE* in the YidC depletion strain JS7131 and the translocation of 2Pf3-Lep was assayed using the protease-mapping assay as described above. The results showed that 2Pf3-Lep was fully inserted when both YidC and Sec were depleted at the same time (Fig. 7a).

To further characterize the YidC/SecE depletion strain, we tested the insertion of YidC-Sec dependent protein subunit a of the FoF1 ATPase with a C-terminal P2 epitope of Lep that served as a cytoplasmic tag for immunoprecipitation (F0a-P2). The substrate was largely blocked under YidC or Sec depletion conditions (Fig. 7b). However, it remained uninserted when both YidC and Sec were depleted simultaneously. We also evaluated the insertion of the YidC-only model protein wild-type Procoat-Lep (PC-Lep) and a YidC-Sec dependent mutant protein ARGRR-Procoat-Lep (ARGRR-PC-Lep) [23]. The YidC-only substrate PC-Lep (indicated by *P* in Fig. 7) was converted to the mature coat protein (indicated by *C* in Fig. 7) by the action of signal peptidase upon insertion under YidC expression conditions (Fig. 7c). In contrast, PC-Lep was largely uninserted when YidC was depleted and only slightly affected by Sec depletion (Fig. 7c). However, the YidC-Sec substrate ARGRR PC-Lep mutant was largely blocked in any translocase depletion condition, as expected (Fig. 7d). Next, we evaluated the export of the Sec-only substrate OmpA and found it was affected as seen by ProOmpA accumulation under Sec-depletion and YidC-Sec depletion conditions (Fig. 7e). However, the export was not completely inhibited, which could be because OmpA has a higher affinity for the Sec apparatus. To further characterize the double-depletion strain, immunoblotting analysis was performed (Supp. Fig. 3b). We observed a dramatic decrease in the translocase levels to under 5% of the levels measured in their wild type conditions (i.e., over 20 fold decrease), thus demonstrating the depletion of both YidC and SecE under the conditions tested. We also verified that 2Pf3-Lep was fully inserted (data not shown) under a more stringent depletion condition (over 40 fold depletion) of both YidC and SecE. Based on these results, we favor the idea that 2Pf3-Lep can translocate by a Sec/YidC independent mechanism.

## Discussion

Typically, substrates that are inserted by the YidC-only pathway have short translocated segments, like Pf3 coat (18 residues), Procoat (20 residues), SciP (11 residues), DjlC (1 residue), Flk (1 residue), FoF1 ATPase subunit c (8 residues), MscL (29 residues), and the N-region of

CyoA (26 residues) [9-12, 37, 38]. Longer translocated regions typically require the Sec system [26, 27]. YidC-Sec independent insertion is rare and observed in proteins with short translocated regions like KdpD (10 residues), Pf3-Lep (18 residues) model protein, and KscA (30 residues) [2-4]. In this study, we report that a 36-residue tail can be translocated independent of Sec and YidC. This result was confirmed for the first time using a new strategy to deplete both YidC and SecE in the same *E. coli* strain to confirm that the substrate is not promiscuously being inserted by Sec when we deplete YidC, and vice-versa. Under the YidC/Sec depletion conditions, where the insertion of 2Pf3-Lep was unaffected, we confirmed that YidC was depleted over 30 fold and SecY over 20 fold. The combined data suggests the possibility that unassisted translocation of polar domains across the membrane is feasible in nature and it is conceivable that the hydrophobicity of the TM segment of the protein fuels this process. The hydrophobicity of TM1 of 2Pf3-Lep is very high, much higher than the 2Pf3-23Lep construct that inserts by the YidC-only pathway. However, the presence of residual levels of translocases under depletion conditions and the involvement of any other unknown translocation mechanism needs to be considered as well and tested in future studies.

On evaluating size of the translocated region as a pathway-determinant, we found that increasing the length of the YidC-Sec independent substrate N-tail with uncharged residues led to YidC dependence and eventually Sec dependence as well. Similarly, increasing the length of a YidC-dependent substrate N-tail caused a switch in its translocase requirement to YidC-Sec mode of insertion. This suggests that these substrates insert into the membrane at the YidC-Sec interface. In both cases, the N-tails were poorly inserted beyond a certain length and substrate N-tails of about 60-residue length was found to be the upper limit of translocation capacity for these model proteins. Thus, we observe a correlation between length of the translocating region and the number of translocating devices employed by the substrate. This agrees with previous studies suggesting a limited role for YidC to function independently and often require the assistance of Sec for more sophisticated proteins in terms of the energy barrier of translocation [23].

We should point out that this length study here is a reinvestigation of amino-terminal translocation. Previously, we reported that short tails up to 38 residues are efficiently translocated in a SecA and SecY-independent manner whereas longer tails are poorly inserted [29]. This agrees with the results reported in this paper. In Cao and Dalbey (1994), we could obtain translocation of a long N-tail only when we added a leader sequence to the N-terminus of the protein. However, the N tail translocation of  $\beta$ -lactamase and alkaline phosphatase were reported in later studies to occur even in the absence of an amino-terminal signal peptide [39, 40]. Therefore, we wanted to reinvestigate the properties of the mature domain that can be translocated in the N-terminal direction. Specifically, we wanted to look at whether this requires YidC, which was discovered in 2000 [9], and also examine Sec-dependency of insertion using the SecE depletion strain that was constructed in 1996 [31].

Interestingly here, we found that longer N-tails can be exported by the Sec system if the substrate has a downstream reverse signal. The mature domain of MBP fused to the N-terminus of the model substrate 2Pf3-Lep was translocated even when not preceded by its N-terminal signal sequence. One hypothesis is, Sec A/B engages with the MBP domain and directs it to the SecYEG channel, which is then opened by the C-terminal TM segment of the model protein, enabling the translocation of the large periplasmic domain. In keeping with this, we found that MBP-2Pf3-Lep is SecA dependent (Supp. Fig. 2b). Previously, the mature alkaline phosphatase has been shown to be exported without an attached signal peptide if a signal sequence is added in trans [41]. Later studies showed that regions of the translocated segment need also to contain SecA targeting signals that allow this region to bind to SecA, enabling SecA assisted translocation [42].

In addition to length, we have also tested charge density of the N-tail region as a translocase determinant. It was previously shown that the addition of charges to N-tail region of the model substrate Pf3-Lep resulted in a switch in its translocation pathway [4]. However, we observed that doubling this protein's N-tail length, hence also the number of charged residues it carries,

did not change its translocase requirements. This was surprising and suggested that additional charges on the N-tail could be translocated without altering the mechanism if they are distributed across a longer N-tail. This was confirmed by strategically placing charged residues either close to each other or away and studying the changes in its translocation pathway. While we found that positive charges had a greater effect on the translocation mechanism than negative charges, all mutants showed equal dependence on both YidC and Sec. This is contrary to the previous study that proposed negative charges as YidC determinant and positive charges as YidC-Sec determinants [4]. We hypothesize that this can be explained as an effect of charge crowding over the short N-tail of Pf3-Lep substrate that was tested. Although the negatively charged substrates tested in the previous study are YidC-dependent, our data suggests that increasing the number of negative charges appear to make it more Sec-dependent as well. Based on this, we propose that YidC has limited potential to insert charged substrates independently and requires Sec for more complex substrates.

In conclusion, we observed that both N-tail length and charge density can specify the insertion pathway of substrates. Shorter tails are translocated independently, whereas longer ones first recruit YidC and beyond a certain threshold, need both YidC and Sec. For these substrates, we hypothesize that insertion occurs at the YidC-Sec interface, between the greasy-slide and the lateral gate. Longer periplasmic domains are exported when followed by a reverse signal by the Sec apparatus. Additionally, crowding of charges in the translocated region, increases the energy barrier of translocation and thus requires the assistance of translocases for its membrane clearance.

## Materials and methods

**Materials.** Isopropyl 1-thio- $\beta$ -D-galactopyranoside was purchased from Research Products International Corp. Tran<sup>35</sup>S-label (mixture of 85% [<sup>35</sup>S] methionine and 15% [<sup>35</sup>S] cysteine at 1000 Ci/mmol concentration) was purchased from PerkinElmer Life Sciences. Anhydrotetracycline hydrochloride was purchased from ACROS Organics. Lysozyme was

purchased from Sigma, Proteinase K (PK) from Qiagen and PMSF from United States Biochemical (Affymetrix). Antisera to leader peptidase (anti-Lep) and outer membrane protein A (anti-OmpA) were from our lab collection. Restriction endonucleases, T4 DNA ligase, Q5 polymerase, Monarch PCR and DNA Cleanup kits and NEBuilder HiFi DNA Assembly Master Mix were purchased from New England Biolabs. PCR primers were synthesized by Integrated DNA Technologies (IDT).

**Plasmids and Site-directed mutagenesis.** To express the 2Pf3-Lep and 2Pf3-23Lep derivatives and mutants in JS7131, CM124 and YidC-Sec double depletion strain NB167, the genes were cloned into the pLZ1 vector [43] under the control of T7/lacUV5 promoter. The techniques described previously [44] were used for DNA manipulations. Site-directed mutations were made by QuikChange or Fusion PCR method. N-tail length of the substrates was increased by stepwise insertion of neutral spacer residues used in [29] (TQVLNAPTSGGQSLNPGTSAQGNLS). DNA sequencing of the entire gene verified all mutations. The N-terminal addition of MBP and Pre-MBP to 2Pf3Lep was done by sub-cloning the respective genes amplified from the plasmid pMAL-c2X (Addgene #75286), a gift from Paul Riggs.

**Bacterial Strains.** The *E.coli* YidC depletion strain JS7131 is from our collection [9]. The SecE depletion strain CM124 was obtained from Beth Traxler [31]. These strains have either the *yidC* or the *secE* genes expressed under the control of the *araBAD* promoter while their endogenous *yidC* or *secE* genes are inactivated. The YidC-Sec double depletion strain NB167 is a derivative of JS7131 with the *secE* gene repressed using CRISPRi [36] by inducing the expression of a catalytically defective Cas9 (dCas9). This strain was constructed by synthesizing dCas9 (IDT), originally from *Streptococcus pyogenes*, with codons optimized for expression in *E. coli*. This synthetic construct was introduced into a modified pAH63 vector [45] containing a tetracycline inducible promoter to yield pTR-dCas9. The *tetR* – *dcas9* region from this plasmid was PCR-amplified using P1 and P2 primers (Supp. Table S1.) and cloned into the Tn7-based vector

pMS26 by USER cloning as described [46], yielding plasmid pTn7-TR-dCas9. This plasmid was then transformed into JS7131 and ampicillin resistant transformants were streaked on LB agar at 42°C for plasmid curing, as described [46]. Colony PCR (primers P3 and P4) was performed on individual, ampicillin sensitive colonies to confirm successful chromosomal integration of *tetR-dCas9* into *attTn7*. To target dCas9 to *secE* a guide RNA array was constructed by first identifying three distinct 20-bp pair proto-spacer target sequences adjacent to a NGG PAM site within the *secE* promoter region. These 20-bp sequences were each introduced into pgRNA [36] by inverse PCR. Each individual guide RNA was then assembled as an array into pDLC29, a low copy ColE1-like plasmid compatible with other vectors used in this study [47] (primers P11-P16 and P17-P18) using the NEBuilder HiFi DNA Assembly Master Mix. The resulting plasmid, psgRNA-*secE*123 was transformed into JS7131 strain harboring *tetR-dCas9* at the *attTn7* site to yield NB167.

**Growth Conditions.** The YidC depletion strain JS7131 was cultured at 37 °C for 3.5 h in LB media with 0.2% arabinose (YidC expression conditions) or 0.2% glucose (YidC depletion conditions). The SecE depletion strain CM124 was cultured in M9 media with 0.2% arabinose plus 0.4% glucose (SecE expression conditions) or 0.4% glucose (SecE depletion conditions) for 8 h at 37 °C. YidC-Sec double depletion strain NB167 was cultured at 37 °C for 4 h in LB media with 0.2% arabinose (YidC-Sec expression conditions) or 0.2% arabinose plus 0.02 µg/ml ATc (SecE depletion conditions) or 0.2% glucose (YidC depletion conditions) or 0.02% glucose plus 0.02 µg/ml ATc (YidC-Sec depletion conditions). For all conditions tested, the cells were exchanged into fresh M9 media and shaken for 30 min at 37 °C before inducing the plasmid-encoded substrate protein. SecA dependence was evaluated by treating the samples with 3 mM sodium azide 5 min prior to induction.

**Protease Accessibility Studies.** Protein substrates were expressed by induction using 1 mM IPTG (final concentration) for 5 min at 37 °C. Cells were labelled with [<sup>35</sup>S] methionine for 1 min and converted to spheroplasts. Briefly, after labelling, the cells were collected by centrifugation

and resuspended in spheroplast buffer (33mM Tris-HCl, pH 8.0, 40% (m/v) sucrose). The resuspended cells were then treated with 1 mM EDTA (pH 8.0) and 10 µg/ml Lysozyme on ice for 30 min. To an aliquot of this, Proteinase K (0.75 mg/ml) was added. After 30 min digestion on ice, the reaction was quenched by the addition of 5mM PMSF for 5 min. Another aliquot was not treated with PK. An equal volume of ice-cold 20% (m/v) TCA was added to the solution and incubated on ice for 1 h. The total protein was then spun down at 13000 rpm for 10 min and washed with the sample volume of ice-cold acetone. The protein pellet was solubilized in Tris-SDS buffer (10mM Tris-HCl, pH 8.0, 2% (m/v) SDS) overnight at room temperature. The samples were then immunoprecipitated with antiserum to leader peptidase, which precipitates the Lep derivative substrates, or precipitated with antiserum to OmpA. The samples were analyzed by SDS-PAGE and phosphorimaging.

**Signal Peptidase Processing Assay.** Substrates were expressed using 1mM IPTG (final concentration) for 5 minutes at 37°C and labelled with [<sup>35</sup>S] methionine for 1 min. The total protein was precipitated on ice for 1 h with an equal volume of ice-cold 20% (m/v) TCA. The samples were spun down at 13000 rpm for 10 min and washed with an equal volume of ice-cold acetone. The pellet was solubilized in Tris-SDS buffer (10mM Tris-HCl, pH 8.0, 2% (m/v) SDS) overnight at room temperature. The samples were then immunoprecipitated with antiserum to leader peptidase, which precipitates the Lep derivative substrates, or precipitated with antiserum to OmpA. The samples were analyzed by SDS-PAGE and phosphorimaging.

**Western blot.** *E. coli* strains JS7131, CM124 and NB167 were grown for various times (JS7131 and NB167 for 3.5 h, and CM124 for 7 h) in the presence of arabinose or glucose (with or without ATc for NB167 in each condition) at 37 °C. The cells were then collected by centrifugation and washed with ice-cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.4)). After normalizing the cells to A<sub>600</sub> of 1.2, the cell samples were pelleted, washed, and dissolved in 40 µl of SDS gel loading buffer, and 5 µl of the protein samples were loaded on a 15% (SecY visualization) and a 10% (YidC visualization) SDS-

polyacrylamide gel. YidC was analyzed by a Western blot using the total protein. To analyse SecY expression, membrane vesicles were prepared from cells grown in arabinose and glucose conditions, adjusted to  $A_{600}$  of 1.2, respectively and the proteins resolved on the 15% SDS-polyacrylamide gel. Antisera to YidC and SecY (each diluted 1:5000) and secondary antibody (goat-to-rabbit IgG horseradish peroxidase, 1:10,000) were used for the Western blot.

**Quantification of Membrane Insertion Data.** The percent of substrate translocation efficiency was determined by quantifying the intensities of the protease-protected bands in each condition using ImageJ, a tool developed by National Institutes of Health as described in [34]. The intensities of the cleaved and uncleaved substrate bands were divided by the predicted number of methionine residues they contain and substituted in the Equation below. For example, 2Pf3-Lep uncleaved substrate contains 9 methionine residues, where the cleaved product has 7. Hence, the equation to be used becomes:

$$\% \text{ translocation} = \left[ \frac{\frac{9}{7} \text{cleaved } 2Pf3Lep}{\left\{ \frac{9}{7} \text{cleaved } 2Pf3Lep + \text{uncleaved } 2Pf3Lep \right\}} \right] * 100$$

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**FIGURE 1. Construction of model substrates.** A schematic of the fusion proteins with doubled N-tails used in this study, 2Pf3-Lep and 2Pf3-23Lep (a). 2Pf3-Lep has two consecutive 18-residue long Pf3 coat N-tails, followed by Lep from positions 4-323. 2Pf3-23Lep contains the two consecutive Pf3 N-tails followed by the TM segment of Pf3 fused to the 23<sup>rd</sup> residue of Lep. Pf3 portions are shown in Purple and Lep portions are shown in Green. An arginine is present at position 79 of Lep following TM2 in both proteins. The amino acid sequence of the uncharged pentapeptides (b) added between the N-tail and the TM segments of the model proteins used to

construct the substrates 2Pf3+5-Lep, 2Pf3+10-Lep, 2Pf3+15-Lep, 2Pf3+20-Lep and 2Pf3+25-Lep with 2Pf3-Lep; 2Pf3+5-23Lep, 2Pf3+10-23Lep, 2Pf3+15-23Lep, 2Pf3+20-23Lep and 2Pf3+25-23Lep with 2Pf3-23Lep

**FIGURE 2. N-tail length requirement for translocase dependence.** *E. coli* JS7131 cells bearing different plasmids were grown for 3.5 h under YidC expression (0.2% arabinose) or YidC depletion conditions (0.2% glucose). The substrates 2Pf3-Lep (a), 2Pf3+5-Lep (b), 2Pf3+10-Lep (c), 2Pf3+15-Lep (d), 2Pf3+20-Lep (e) and 2Pf3+25-Lep (f) were expressed from pLZ1 plasmid using 1mM IPTG for 5 min and labelled with [<sup>35</sup>S] methionine for 1 min. Translocation of the substrate N-tail was analysed using the protease-accessibility assay, where the cells were converted into spheroplasts, and a portion was treated with Proteinase K (PK) (see “Material and Methods”). *E. coli* CM124 bearing the same plasmids (a, b, c, d, e, f) were grown for 8 h under SecE expression (0.2% arabinose+0.2% glucose) or SecE depletion conditions (0.4% glucose) and analysed using the protease-accessibility assay. A representational OmpA immunoprecipitation data is included for both studies (g). The percent translocation of the substrate was quantified as described in “Methods and Materials”. *P* denotes the full-length protein, whereas *F* denotes the protease-resistant fragment. In panel (g) *M* denotes the mature OmpA whereas *P* denotes the precursor protein.

**FIGURE 3. Increasing N-tail length directs YidC-only N-tails to YidC-Sec pathway.** *E. coli* JS7131 cells bearing different plasmids were grown under YidC expression or depletion conditions. The substrates 2Pf3-23Lep (a), 2Pf3+5-23Lep (b), 2Pf3+10-23Lep (c), 2Pf3+15-23Lep (d), 2Pf3+20-23Lep (e) and 2Pf3+25-23Lep (f) were expressed from pLZ1 plasmid using 1mM IPTG for 5 min and labelled with [<sup>35</sup>S] methionine for 1 min. Translocation of the substrate N-tail was analysed using the protease-accessibility assay, where the cells were converted into spheroplasts, and a portion was treated with Proteinase K (PK) (see “Material and Methods”). *E. coli* CM124 expressing the same substrates (a, b, c, d, e, f) were grown under SecE expression or depletion conditions and analysed using the protease-accessibility assay. Representational

OmpA data is included at the bottom for both studies (g). The percent translocation of the substrate was quantified as described in “Methods and Materials”. *P* denotes the full-length protein, whereas *F* denotes the protease-resistant fragment. In panel (g) *M* denotes the mature OmpA whereas *P* denotes the precursor protein.

**FIGURE 4. Translocation of MBP in the N-terminal direction.** A schematic of MBP-2Pf3-Lep, MBP-2Pf3-Lep  $\Delta$ 61-375 and PreMBP-2Pf3-Lep substrates (a). Maltose-binding protein (shown in Orange) was fused to the N-terminus of 2Pf3-Lep either with its SP (PreMBP-2Pf3-Lep) or without (MBP-2Pf3-Lep). All but the first 60 residues of MBP were deleted to make the truncated MBP-2Pf3-Lep  $\Delta$ 61-375 substrate. *E. coli* JS7131 cells bearing pLZ1 plasmids expressing MBP-2Pf3-Lep (b), MBP-2Pf3-Lep  $\Delta$ 61-375 (c) or PreMBP-2Pf3-Lep (d) were grown under YidC expression or depletion conditions. The substrates were expressed using 1mM IPTG for 5 min and labelled with [ $^{35}$ S] methionine for 1 min. Translocation of the periplasmic domain was analysed using the protease-accessibility assay, where the cells were converted into spheroplasts, and a portion was treated with Proteinase K (PK) (see “Material and Methods”). *E. coli* CM124 bearing the same substrate plasmids (b, c, d) were grown under SecE expression or depletion conditions and analysed using the protease-accessibility assay. The percent translocation of the substrate was quantified as described in “Methods and Materials”. *M* denotes the mature domain region of MBP fused to 2Pf3-Lep, whereas *F* denotes the protease-resistant fragment. *P* denotes the full-length precursor protein in panel (d).

**FIGURE 5. Testing negative charge distribution on N-terminal tail as a pathway-determinant.** The positions of the negative charges introduced on the N-tail of 2Pf3-Lep (a). *E. coli* JS7131 cells bearing different plasmids were grown under YidC expression or depletion conditions. The substrates were expressed using 1mM IPTG for 5 min and labelled with [ $^{35}$ S] methionine for 1 min. Substrate N-tail translocation was analysed using the protease-accessibility assay, where the cells were converted into spheroplasts, and a portion was treated with Proteinase K (PK) (see “Material and Methods”). The plasmids encoded the proteins 2Pf3-

Lep V15D (b), V15'D (c), V15D/V15'D (d), L12E/V15D (e) and L12'E/V15'D (f) (mutations positioned in the second Pf3 N-tail ahead of the TM segment are indicated by (')). *E. coli* CM124 expressing the same substrate proteins (b, c, d, e, f) were grown under SecE expression or depletion conditions and analysed using the protease-accessibility assay. Representational OmpA data is included at the bottom for both studies (g). The percent translocation of the substrate was quantified as described in "Methods and Materials". *P* denotes the full-length protein, whereas *F* denotes the protease-resistant fragment. In panel (g) *M* denotes the mature OmpA whereas *P* denotes the precursor protein.

**FIGURE 6. Testing positive charge distribution on N-terminal tail as pathway-determinant.** The positions of the positive charges introduced on the N-tail of 2Pf3-Lep (a). *E. coli* JS7131 cells bearing different plasmids were grown under YidC expression or depletion conditions. The plasmids encoded the proteins 2Pf3-Lep V15R (b), V15'R (c), V15R/V15'R (d), L12'R/V15'R (e) and L12R/V15R (f). The substrates were expressed using 1mM IPTG for 5 min and labelled with [<sup>35</sup>S] methionine for 1 min. Translocation of the substrate N-tail was analysed using the protease-accessibility assay, where the cells were converted into spheroplasts, and a portion was treated with Proteinase K (PK) (see "Material and Methods"). *E. coli* CM124 expressing the same substrate proteins (b, c, d, e, f) were grown under SecE expression or depletion conditions and analysed using the protease-accessibility assay. Representational OmpA data is included at the bottom for both studies (g). The percent translocation of the substrate was quantified as described in "Methods and Materials". *P* denotes the full-length protein, whereas *F* denotes the protease-resistant fragment. In panel (g) *M* denotes the mature OmpA whereas *P* denotes the precursor protein.

**FIGURE 7. Confirming non-promiscuous insertion of 2Pf3-Lep using YidC-Sec double-depletion.** *E. coli* NB167 cells expressing 2Pf3-Lep (a) from pLZ1 plasmid, and Foa-P2 (b) from pMS119 plasmid were grown under YidC-Sec expression or YidC/Sec/YidC-Sec depletion conditions. The substrate was expressed using 1mM IPTG for 5 min and labelled with [<sup>35</sup>S]

methionine for 1 min. Translocation of the substrate regions was analysed using the protease-accessibility assay, where the cells were converted into spheroplasts, and a portion was treated with Proteinase K (PK) (see “Material and Methods”). NB167 cells bearing the pLZ1 plasmid expressing either the YidC-only substrate PC-Lep (c) or the YidC-Sec substrate ARGRR PC-Lep mutant (d) were grown under YidC-Sec expression or YidC/Sec/YidC-Sec depletion conditions and the substrate membrane insertion was analysed using the signal peptidase processing assay, where the substrate labelled with [<sup>35</sup>S] methionine for 1 min and precipitated with TCA were analysed for signal peptidase processing (see “Material and Methods”). OmpA signal processing assay data for the same conditions are also included (e). *P* denotes the full-length protein, whereas *F* denotes the protease-resistant fragment. *C* denotes the signal peptide processed mature Coat protein in panel (c, d). In panel (e), *M* denotes the mature OmpA whereas *P* denotes the precursor protein.

**Supplementary figure 1. Amino acid sequence of the model protein constructs.** (a) The 2Pf3-Lep model protein contains two consecutive Pf3 N-tails followed by the Lep<sup>TM</sup> segment. (b) In 2Pf3-23Lep model protein the Lep<sup>TM</sup> segment is replaced by a Pf3<sup>TM</sup> segment instead. Pf3 portions are shown in Purple whereas Lep portions are shown in Green. The <sup>TM</sup> segment of each construct is followed by residues 23–323 of Lep, where an arginine is present at position 79 of Lep following <sup>TM</sup>2.

**Supplementary figure 2. The translocation of MBP-2Pf3-Lep is blocked when Arg residues are added to its <sup>TM</sup> and analysis of its SecA-dependence.** (a) *E. coli* JS7131 cells expressing MBP-2Pf3-Lep L41R, L48R from pLZ1 plasmid was tested under YidC expression condition and the N-tail translocation was analysed using the protease-accessibility assay. (b) SecA-dependence of MBP-2Pf3 was analysed by treating *E. coli* JS7131 cells expressing MBP-2Pf3-Lep with or without 3mM sodium azide 5 mins prior to substrate induction under YidC expression conditions and the N-tail translocation was evaluated using the protease-accessibility assay. OmpA export was analysed under the same conditions as a control.

**Supplementary figure 3. Detection of YidC and Sec depletion using western blot. (a)**

Western blot analysis of YidC and SecY protein expression under YidC/Sec expression and depletion conditions respectively, in strains JS7131 and CM124. (b) The YidC and SecY levels were evaluated in the double-depletion strain NB164 using western blot. The percent YidC/Sec remaining under depletion conditions were quantified for each case and reported as fold decrease relative to the corresponding wild-type levels. For details, see “Material and Methods” section.

**Supplementary Table 1. Oligonucleotide primers used in strain construction.****References**

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**Highlights**

- An increase in substrate N-terminal length or charge density mandates a switch in its translocase requirement from independent to YidC-only and YidC-Sec.
- The mature domain of MBP can be translocated in the amino-direction if followed by a C-terminal TM segment by the Sec channel.
- The YidC-Sec independent substrate 2Pf3-Lep is not promiscuously inserted by either YidC or Sec in the absence of the other, as confirmed by a double-depletion strain.

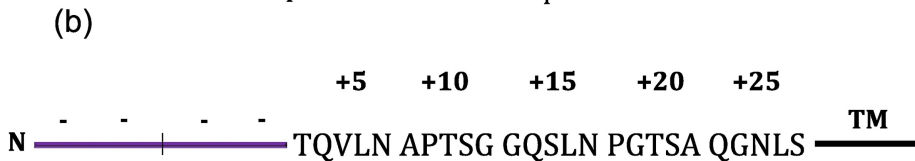
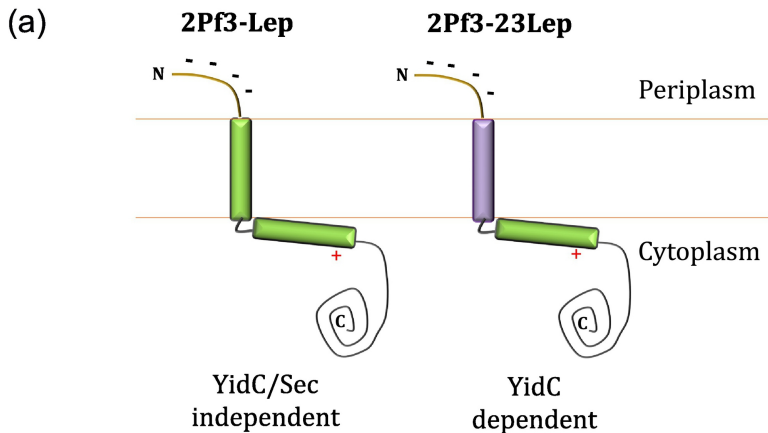


Figure 1

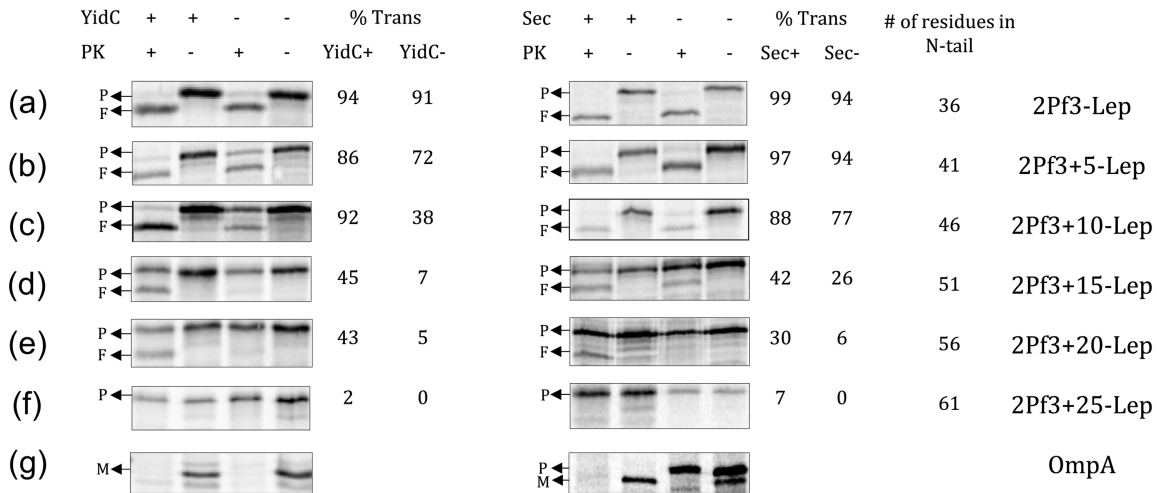


Figure 2

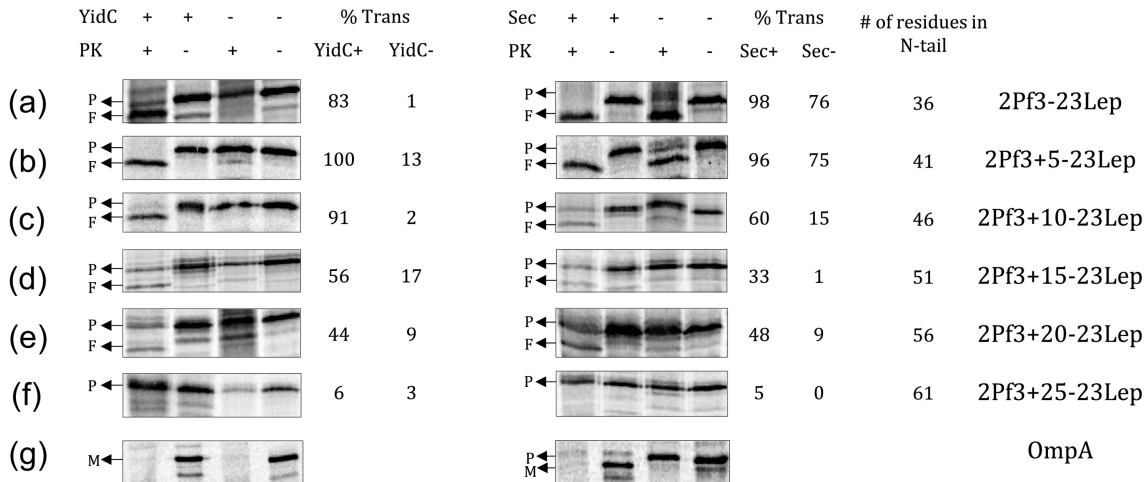


Figure 3

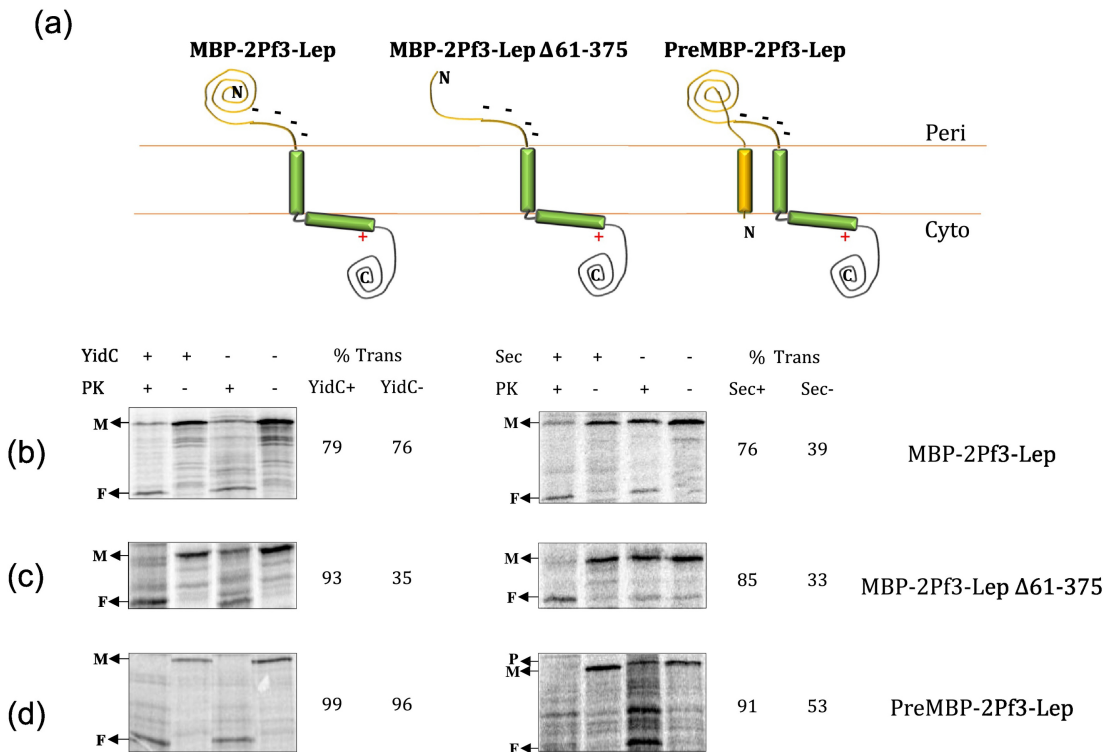


Figure 4

(a)

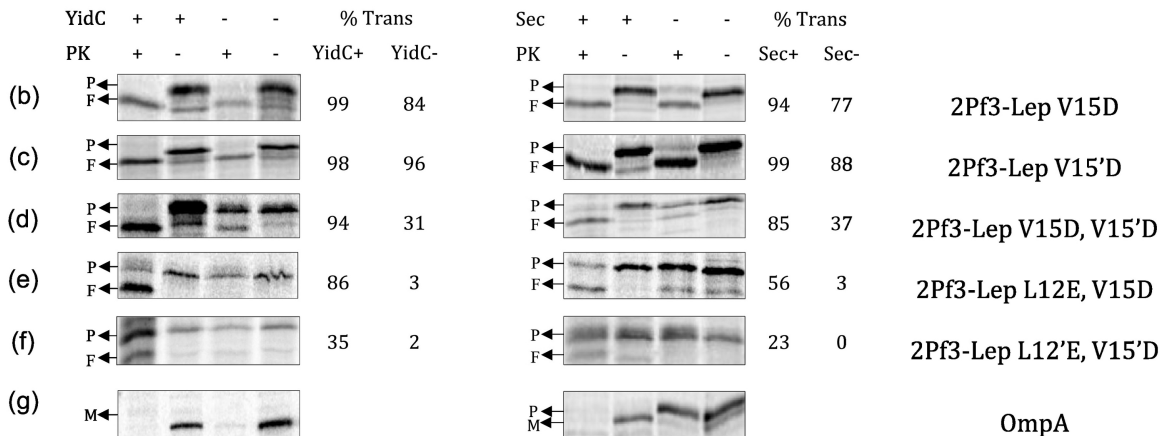


Figure 5

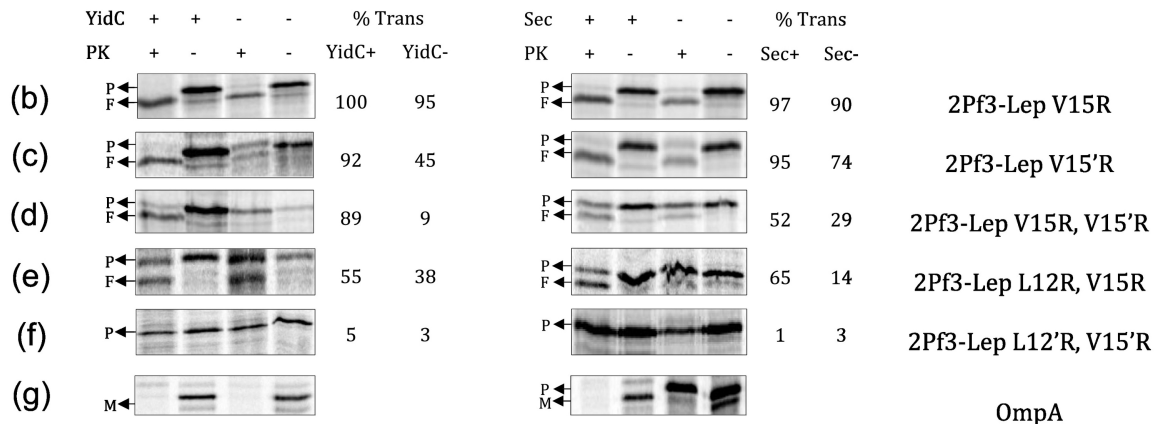
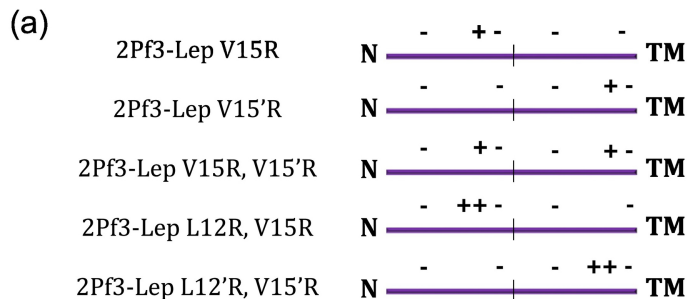
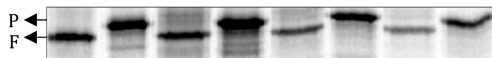


Figure 6

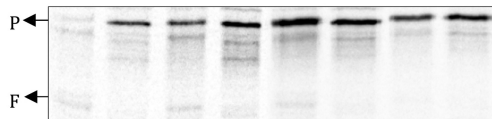
|      |   |   |   |   |   |   |   |   |
|------|---|---|---|---|---|---|---|---|
| YidC | + | + | - | - | + | + | - | - |
| Sec  | + | + | + | + | - | - | - | - |
| PK   | + | - | + | - | + | - | + | - |

(a)



2Pf3-Lep

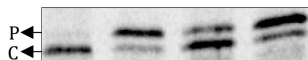
(b)



Foa-P2

|      |   |   |   |   |
|------|---|---|---|---|
| YidC | + | - | + | - |
| Sec  | + | + | - | - |

(c)



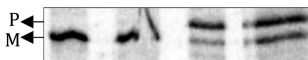
PC-Lep

(d)



ARGRR PC-Lep

(e)



OmpA

Figure 7