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GENETIC ANALYSIS OF PROTEIN TRANSLOCATION

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Abstract

Cells in all domains of life must translocate newly synthesized proteins both across membranes and into membranes. In eukaryotes, proteins are translocated into the lumen of the ER or the ER membrane. In prokaryotes, proteins are translocated into the cytoplasmic membrane or through the membrane into the periplasm for Gram-negative bacteria or the extracellular space for Gram-positive bacteria. Much of what we know about protein translocation was learned through genetic selections and screens utilizing *lacZ* gene fusions in *Escherichia coli*. This review covers the basic principles of protein translocation and how they were discovered and developed. In particular, we discuss how *lacZ* gene fusions and the phenotypes conferred were exploited to identify the genes involved in protein translocation and provide insights into their mechanisms of action. These approaches, which allowed the elucidation of processes that are conserved throughout the domains of life, illustrate the powerof seemingly simple experiments.

The term outer membrane (OM), as it is currently applied to Gram-negative bacteria, first appeared in the literature in 1964 [1]. The discovery that these bacteria have two membranes led to the recognition of a distinct, aqueous cellular compartment between the two membranes called the periplasm, a term credited to Peter Mitchell [2]. The peptidoglycan cell wall is a distinct structure located in the periplasm. By the end of the decade, the anatomy of the Gram-negative cell envelope had finally been clarified [3].

Fractionation methods were developed to separate the two membranes and to isolate the proteins present in the periplasm [4–7]. These showed that the OM, which was protein rich, contained a small number of very abundant proteins. In contrast, the inner membrane (IM) contained the enzymes, such as NADH dehydrogenase, that are typically found in the cellular organelles of eukaryotic cells. The periplasm was shown to contain degradative enzymes such as alkaline phosphatase (PhoA) and soluble sugar- or amino acid-binding proteins that functioned as components of the transport systems for these molecules [8]. Clearly each different cellular compartment had a distinct set of proteins. How were these proteins translocated from their site of synthesis in the cytoplasm to their final destination and how did the cell know the location to which they should be transported? It is the first question that this article is concerned with. In particular, it is concerned with the genetic approaches that were used to identify the cellular components required for this essential, protein translocation process.

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In a similar time frame, work on protein translocation in eukaryotic cells was focused on the endoplasmic reticulum. Palade had shown that proteins destined for secretion are made on ribosomes attached to the endoplasmic reticulum (ER) and this provided strong evidence that secreted proteins were translocated into the ER lumen as they were being translated [9]. Blobel and Sabatini (1971) suggested that the signal that directed ribosomes making secreted proteins to the ER was located at the amino terminus of these proteins [10]. Milstein et al. (1972) showed that the antibody light chain was made initially in precursor form [11]. The signal hypothesis was born when Blobel and Dobberstein (1975) developed an *in vitro* system and a protease protection assay to show that the precursor form of this protein is processed during the translocation reaction [12,13]. Inouye and Beckwith (1977) showed that PhoA from *Escherichia coli* is made *in vitro* in precursor form providing evidence that the process of protein translocation was conserved in all of the domains of life [14]. Accordingly, it was clear that bacterial genetics could be used to study this process.

Unfortunately, there was no obvious way to approach the protein translocation problem genetically. To illustrate the problem, consider the *lamB* gene, which specifies an OM protein that functions as the receptor for bacteriophage λ and as maltoporin [15–17]. Cells lacking LamB can grow on maltose, because the disaccharide is small enough to cross the OM through the general porins OmpF and OmpC [18]. However, transport of maltose polymers (dextrins) requires LamB absolutely [19]. The *lamB* gene thus provides a direct selection for the loss of the protein (resistance to phage λ, λ^R) and a direct selection for restoration of the protein in the OM (growth on dextrins as sole carbon source, Dex^+). Despite these very useful phenotypes, classical genetic approaches failed to find *lamB* mutations that block translocation of LamB. The basic problem here is that mutations that prevent targeting of LamB to the OM will confer the same phenotype as mutations that prevent synthesis of LamB (Dex^-, λ^R), and the latter are far more frequent than the former. Mutations that alter the signal sequence of LamB do confer λ^R, but when they were discovered and mapped, it became clear that they lie earlier in the gene than any of the hundreds of *lamB* mutations known at that time [20]. New approaches were required to overcome the failure of more classical methods.

A BRIEF SUMMARY OF THE TRANSLOCATION PROCESS

To facilitate discussion of the approaches developed, a brief summary of what is currently know about the translocation process is warranted. Protein translocation from the cytoplasm in bacteria like *E. coli* is catalyzed by a heterotrimeric complex of IM proteins, SecYEG, and it is driven by the ATPase SecA and the proton motif force (for review see [21]). SecYE and SecA are sufficient for protein translocation *in vitro* [22]. SecG is not essential, but it does increase translocation *in vitro* in in some strains at low growth temperatures [23]. In the cell, seven different membrane proteins, SecYEG-SecDF-YajC-YidC, make up what is called the holo-translocon [24]. SecDF couple the proton motive force to protein translocation [25]. YidC functions in the insertion of IM proteins [26]. The function of YajC is not known, but it is not required for cell viability or protein translocation [27].

Two different pathways target protein to the translocon (Fig. 1). Many proteins like LamB and MalE are secreted post-translationally and are kept in a secretion-competent

conformation by the cytoplasmic chaperone SecB [28]. In contrast, many proteins destined for the IM like MalF are targeted to the translocon by Signal Recognition Particle (SRP) and are translocated cotranslationally [29]. Nearly all of the genes mentioned in this brief summary were discovered using the approaches described below.

GENE FUSIONS

The first hybrid gene was made by a spontaneous deletion that fused the T4 *rIIA* gene to the *rIIB* gene. This gene fusion was isolated by Benzer and Champe (1962) and exploited by them to show that nonsense mutations are chain terminating [30]. It was also used by Crick et al. (1961) to provide evidence that frameshift mutations alter the reading frame and that there is a fixed starting point for translation [31]. Jacob et al. (1965) identified a deletion that fused the intact *lacY* gene to the *purA* promoter [32] and Beckwith and colleagues (1966) learned more about operon structure from the study of various fusions that placed the *lac* genes under the control of the *trp* promoter [33]. However, the methods used to generate these useful tools were not easy to generalize.

Malcolm Casadaban appreciated the power of gene fusions and using nothing but toothpicks and logic, he developed a method that allowed one to fuse LacZ to any non-essential gene in *E. coli* [34]. At the time, Casadaban was a graduate student in Jon Beckwith's lab and Beckwith envisioned using this technology to study the protein translocation problem. From today's vantage point, it is hard to appreciate the intellectual leap this required. Beckwith saw LacZ as what would now be called an epitope tag, albeit a very large one, and he felt this tag could be used to follow amino-terminal fragments of cell envelope proteins on their journey through the cell envelope. Nowadays this logic is so commonly used it is hard to find a cell biology paper in either prokaryotes or eukaryotes that does not use it.

The maltose transport system in *E. coli* is specified by five genes located in two divergently transcribed operons [35] (Fig. 2a), and it seemed a useful system for studying protein targeting because there was at least one maltose transport protein located in each cellular compartment. As noted above, LamB is located in the OM (Fig. 3) [15,16]. Maltose binding protein is located in the periplasm. It accepts substrates that diffuse through LamB and delivers them to a paradigmatic ABC (ATP-Binding Cassette) transporter located in the IM [36]. This transporter is composed of two IM proteins, MalF and MalG, and an associated, cytoplasmic ATPase, MalK [37–39].

The value of gene fusion technology for the analysis of protein targeting was first demonstrated with *malF-lacZ* fusions [40]. The amino-terminal fragment of MalF present in the hybrid proteins described was able to direct β-galactosidase to the IM. Since these fusion strains required LacY for growth on lactose, it was concluded that LacZ sequences were located in the cytoplasm. We now know that in one of these fusions, LacZ is fused to MalF at a point between transmembrane helices 6 and 7, which would position LacZ in the cytoplasm verifying this prediction [41]. The significance of this is discussed in more detail below.

Gene fusions that attach sequences of non-cytoplasmic proteins to β-galactosidase can confer one or two distinctive phenotypes that are closely associated with protein targeting (Fig. 2bc). Accordingly, these phenotypes could be exploited to obtain mutations that were targeting-defective. Information gained from the analysis of these fusions and the mutations obtained has provided key insights into both the signals and the cellular machinery responsible for protein targeting.

SECRETION-RELATED PHENOTYPES OF LACZ FUSION STRAINS

Perhaps the most striking phenotype exhibited by *lamB-lacZ* or *malE-lacZ* strains is a pronounced sensitivity to inducer, i.e. induction, which causes high level expression of these hybrid genes is lethal [42,43]. A second useful phenotype concerns the LacZ activity exhibited by these strains. Both fusion strains are Lac^+, i.e. they will grow on lactose minimal media. However, the LacZ activity exhibited by the fusion strains is much lower than expected [44]. Before recounting how these phenotypes were exploited in the study of protein translocation, in the interest of clarity, a description of what we now know about the molecular basis for these phenotypes is provided.

It has long been clear that induction of *lamB-lacZ* or *malE-lacZ* fusions causes a “jamming” of the secretion machinery (Fig. 4). Signal peptidase is located in the periplasm, so if a protein cannot be translocated, the signal cannot be removed. The lack of translocator activity in the dying fusion strains was evidence by the accumulation of the precursor form of other periplasmic and outer membrane proteins [45]. The cell is trying to translocate these hybrid proteins in a posttranslational manner. In its native, cytoplasmic environment, LacZ is folding and the translocator cannot deal effectively with folded proteins. When uninduced, the translocator has enough time to handle the low levels of hybrid protein produced (Fig. 4a). Under these conditions, a significant fraction of the hybrid protein is translocated into the periplasm where it is inactivated (see below). The low level of LacZ activity observed under noninducing conditions is due to a fraction of the hybrid protein that is not translocated and remains in the cytoplasm. The translocators cannot deal effectively with the large amounts of hybrid protein that are made under inducing conditions and this leads to cell death (Fig. 4b).

Why translocator jamming leads to cell death is not clear. Induction of the *lamB-lacZ* fusion leads to the degradation of the jammed translocator by the protease FtsH [46]. However, this does not happen with *malE-lacZ* fusion strains; here death seems dependent on reactive oxygen species [47]. In the *malE-lacZ* fusion strain, the *malE* gene is disrupted and the expression of *malF* and *malG* is prevented by polarity (Fig. 2c). Accordingly, to see induction, *malEFG* must be provided in trans. The *lamB* gene is not required for growth on maltose so haploid *lamB-lacZ* fusion strains can be induced. If this strain is made diploid, the extra copies of the divergent promoters that control expression of the *malEFG* and the *malK lamB* genes titrate the positive activator MalT and reduce expression and the toxicity of the *lamB-lacZ* hybrid gene [48]. It would not be surprising if lethal jamming of the diploid *lamB-lacZ* fusion would phenocopy lethal jamming of the diploid *malE-LacZ* fusion.

Manoil and Beckwith devised a genetic approach to analyze the topology of IM proteins using the periplasmic enzyme PhoA [49]. This protein contains disulfide bonds and their formation is catalyzed by the periplasmic enzyme DsbA [50]. Consequently, even though the precursor form of PhoA is active *in vitro* [14], *in vivo* PhoA is only active in the periplasm. So if PhoA is fused to a periplasmic loop of an IM protein it will exhibit high activity. If fused to an internal loop it will exhibit very low activity.

LacZ behaves in the opposite fashion from PhoA [41]. If fused to a periplasmic loop it is inactive. Active LacZ requires fusion to an internal loop. Recall that IM proteins like MalF are targeted to the translocon by SRP and are translocated cotranslationally. Accordingly, LacZ sequences never exist in the cytoplasm and therefore, jamming does not occur. Indeed, if the signal sequence of LamB is made more hydrophobic so that it becomes SRP dependent then the altered LamB-LacZ hybrid protein is directed efficiently to the periplasm [51]. LacZ has a number of cysteine residues but no disulfide bonds. In the periplasm, DsbA oxidizes LacZ and forms disulfide-bonded aggregates. With LamB-LacZ these periplasmic aggregates accumulate to toxic levels. With MalF-LacZ expression levels are much lower and toxicity is not seen unless the hybrid protein is purposely overproduced. With either fusion, removal of DsbA allows LacZ to fold normally into a non-toxic, active enzyme [48].

SIGNAL SEQUENCE MUTATIONS

The inducer-sensitive (Mal^s) phenotype exhibited by both the *lamB-lacZ* and *malE-lacZ* fusion strains is clearly related to the cell's attempted translocation of these hybrid proteins from the cytoplasm. The most common survivors of a selection for resistance to inducer, which in these cases is a selection for growth on maltose (Mal^+), are simply mutants that for one reason or another fail to make the toxic hybrid protein. As noted above, the problem with classical approaches for targeting defective mutations was always that the phenotype of mutants that lack the protein and mutants that target the protein incorrectly are identical. However, gene fusion provides a simple way to distinguish these two classes of mutation. Mutants that don't make the hybrid protein will be $LacZ^-$, mutants that make the hybrid protein, but fail to target it from the cytoplasm will be $LacZ^+$. Using this logic, mutations that alter the signal sequence of LamB and MalE were identified [20,43,52,53]. Generally speaking, these mutations either reduced the size or introduced charge into the hydrophobic core of the signal sequence. When these signal sequence mutations were recombined back into the wild-type *lamB* or *malE* gene, they blocked translocation and caused the precursor form of the protein to accumulate in the cytoplasm. Thus, they provided the first direct evidence for Gunther Blobel's signal hypothesis [12,13].

MUTATIONS THAT ALTER THE TRANSLOCATION MACHINERY

Two general approaches were taken initially to identify the genes that specify components of the translocation machinery. One exploited the signal sequence mutations that were obtained using gene fusions as described above. Basically, this approach sought dominant, gain-of-function mutations that enabled translocation of an internalized protein. The mutations obtained using this approach were termed *prl* (PRotein Localization). The other approach exploited the low level of LacZ activity exhibited by the LacZ fusion strains, in particular

the *malE-lacZ* fusion, to identify recessive, loss-of-function mutations that internalized a translocated protein, increasing LacZ activity. The mutations isolated using this approach were termed *sec* (SECretion). Satisfyingly, these two distinct approaches led to the identification of the same gene multiple times, providing strong evidence that these gene products functioned directly in protein translocation. Today, these genes are still known by both the *prl* and the *sec* mnemonic. In what follows, we will often refer to a gene using both names. When doing so, the name that appears first reflects the mutation in question. For example, *prlA* (*secY*) mutations are dominant suppressors of signal sequence mutations and *secY* (*prlA*) mutations are recessive loss of function mutations that compromise the protein translocation process.

Suppressors of signal sequence mutations

The signal sequence mutations likely block protein translocation because they prevent recognition of the mutant precursor protein by the translocation machinery. If this was true, then genetic logic suggests that it may be possible to identify mutations that alter the translocation machinery and restore recognition of the mutant signal. This approach, termed interactive suppressors, conformational suppressors, or allele-specific suppressors, was first used in prokaryotes by Jarvik and Botstein in their study of phage P22 proteins [54]. It is difficult to prove direct interaction genetically and the best that can be done is to demonstrate strict allele specificity. You can imagine a mutant key that won't fit a mutant lock, but a "suppressor" lock mutation that fits one mutant key should not fit any other mutant key. There are many examples where this approach has been applied successfully. For example, this technique was used to show that λ cl activates transcription by direct interaction with the σ subunit of RNA polymerase [55] and the direct interaction between the amino acids affected by these two mutations in the wild-type proteins has been confirmed by structural analysis [56].

The lamB signal sequence mutations were especially useful for this type of suppressor analysis for the following reason. Suppressors can be selected by demanding growth on maltodextrins. It turns out that there are mutations that allow growth on maltodextrins (Dex $^{+}$) by bypassing the requirement for LamB altogether. Such mutations can increase expression of a normally cryptic porin [57], increase the pore size of a typical porin like OmpC [58], or they can disrupt the OM permeability barrier [59]. However, a suppressor that restores translocation of a mutant LamB protein with a defective signal sequence will not only be Dex^{+} but it will also become sensitive to phage λ, whereas the bypass suppressors mentioned above will remain phage resistant.

Suppressors of MalE signal sequence mutations can be obtained by demanding growth on maltose. Mutations that bypass the maltose transport system are known [60], so restoration of MalE translocation must be confirmed biochemically.

The first suppressors of signal sequence mutations *(lamB)* identified mapped to a gene in the largest ribosomal gene cluster in *E. coli* and were termed *prlA* [61]. Subsequent work showed that the suppressor mutations mapped to a previously unidentified gene at the end of this operon [62]. Following localized mutagenesis, Ito et al. identified a temperature-sensitive lethal mutation that mapped to this region [63]. When shifted to the non-permissive

temperature, pulse-chase experiments show that this mutant, like the other *sec* mutants described below, accumulated the precursor form of MalE, demonstrating a block to translocation. The Nomura lab had identified an open reading frame at the end of this operon and called it Y [64]. Accordingly, Ito et al. named the gene *secY* [63].

The first gene identified using the LacZ fusion approach described in the next section was *secA* [44]. Suppressors of signal sequence mutations that mapped to *secA*, termed *prlD* mutations, were identified by Fikes and Bassford [65]. A more extensive hunt for suppressors of *lamB* signal sequence mutations revealed additional *prlA* and *prlD* suppressors but also suppressors in a new gene termed *prlG* [66]. The paper describing this work appeared back-to-back with a paper describing a new *sec* gene, *secE* that was obtained as described below [67]. By screening PCR mutagenized *secG* libraries, suppressors of MalE signal sequence mutations have also been obtained and these are termed *prlH* [68].

It is clear that none of the *prl* suppressors restores recognition of the mutant signal sequence used for their selection. These suppressors do not exhibit allele-specificity. Indeed all of the *prl* mutations except for *prlD* will suppress a complete signal sequence deletion [69,70,68]. As noted below, the *prlD* mutations act by a distinct mechanism. The *prlAGH* suppressors are really bypass suppressors; they bypass the requirement for a signal sequence [71].

Besides identifying genes, the *prl* mutations also provided insight into mechanism. Studies with double mutants revealed several pairs of *prlA* and *prlG* mutations that exhibited allele-specific synthetic lethality. Moreover, these pairs of alleles were also topologically specific, i.e. a mutation in a transmembrane helix of one protein was synthetically lethal with a mutation in the other protein that was also in a transmembrane helix. This suggested that these mutations identified domains of interaction between the two proteins. Lethality occurred because together these mutations prevented an essential interaction between the two proteins [72].

One of the synthetically lethal *prlA* and *prlG* mutant pairs alters an amino acid in an external loop of both proteins. In order to demonstrate that these amino acids were in close proximity *in vivo*, the amino acids were changed to cysteine. Expression of the cysteine mutant pair resulted in a DsbA-dependent lethal phenotype. The lethality is dominant to either wild-type *secY* (*prlA*) or *secE* (*prlG*) provided in trans and protein translocation is largely unperturbed in the dying cells demonstrating that it is the disulfide-bonded complex itself that is causing toxicity [73].

The predictions about interactive domains from the synthetic lethal phenotypes were confirmed when the structure of the SecYEG complex was determined with one glaring exception [74]. The residues that could be crosslinked by disulfide bond *in vivo* were more than 20A apart. The structure determined is the “closed” form of the complex and, in this structure, the external loop where the cysteine residue in SecY introduced by the *prlA* mutation is located is folded back into the channel forming a plug (Fig. 5). The disulfide bonded complex explained how the plug moves to open the channel during the translocation reaction. In fact, the disulfide bonded complex is locked open explaining its dominant lethality.

The closed and open forms of the SecYEG complex exist in equilibrium and signal sequence recognition pushes the equilibrium towards the open form [74]. The *prl* mutations either stabilize the open form or destabilize the closed form thus reducing the requirement for a signal sequence [75]. Nonetheless, to be translocated, a protein must be delivered to the translocator. This happens because these precursors lacking a signal sequence are still recognized by SecB and SecB delivers them to SecA and thus to SecYEG. Suppression of signal sequence deletions requires SecB absolutely [69,70].

The *secA* (*prlD*) mutations work by a different mechanism(s). Most of the *prlD* alleles also confer resistance to azide [76]. In fact, the first suppressor of signal sequence mutations was actually isolated by Joshua Lederberg [77]. SecA is an ATPase and it is likely that the prlD alleles affect the nucleotide binding and hydrolysis cycles [78].

Mutations that increase the LacZ activity gene fusion strains

As noted above, *lamB-lacZ* and *malE-lacZ* fusion strains exhibit two unusual phenotypes. One of these is inducer sensitivity and this was exploited as described above to identify signal sequence mutations. The other phenotype is the low level of LacZ activity. Inducer sensitivity is caused by translocator jamming [46]; low LacZ activity is caused by the targeting of LacZ to the periplasm [48] (Fig. 4).

***malE-lacZ phoA-lacZ* and *lamB-lacZ fusions*.—**Oliver and Beckwith noted that mutations that alter the *malE* signal sequence in the *malE-lacZ* fusion increase the uninduced LacZ activity dramatically [44]. They reasoned therefore, that mutations that decrease the activity of the protein translocation machinery should also increase the LacZ activity of MalE-LacZ because a higher percentage of the hybrid protein would remain in the cytoplasm where LacZ is fully active. Using lactose tetrazolium agar, they searched for mutant Lac^+ papillae growing out of a lawn of the *malE-lacZ* fusion strain, which appears Lac^- on this media. Assuming that the genes they sought were essential, they screened for mutations that compromise the translocation machinery at low temperatures (30°) but kill the cell at high growth temperatures (TS lethal at 42°). In this way they could avoid signal sequence mutations and mutations that simply increase expression of the hybrid protein. They found a new gene, which they named *secA* [79]. At the non-permissive temperature, the precursor forms of periplasmic and OM proteins could be detected in pulse-chase experiments.

Kumamoto and Beckwith performed an extensive hunt for Lac^+ derivatives of the *malE-lacZ* fusion strain and found an additional gene that they termed *secB* [80]. Unlike *secA, secB* is not essential, but they showed that strains lacking this protein show pleiotropic defects in protein translocation. Similar Lac^+ selections with *phoA-lacZ* and *lamB-lacZ* fusions yield mutations at another locus termed *secD* [81]. Subsequent analysis showed that the *secD* gene is in an operon with another gene termed *secF*. Both of these proteins are membrane proteins and strains lacking either are very cold sensitive.

It is remarkable that the Lac^+ selections with the *malE-lacZ* and the *phoA-lacZ* and the *lamB-lacZ* fusion strains found different, non-overlapping genes. Also, remarkable is the fact that mutations in *secY* (*prlA*) did not answer any of them. As noted above, the *secY*

mutations were found following localized mutagenesis of the *prlA* region of the chromosome. Why this is so has never been clarified.

secA-lacZ fusions.—Oliver and Beckwith discovered that SecA production is increased up to 10-fold when protein translocation is compromised [82]. We now know that the *secA* gene is located in an operon downstream of a gene called *secM*. This gene specifies a periplasmic protein that is rapidly degraded; its only real function is in regulation. If protein translocation is compromised, a sequence near the carboxy terminus of SecM gets stuck in the ribosome exit tunnel, and this changes the conformation of the downstream mRNA exposing the Shine-Dalgarno sequence of *secA*. If the translocation machinery is not compromised and is working properly it actually pulls SecM out of the ribosome, the mRNA structure is not altered and SecA synthesis is kept at a low level (for review see [83]).

Riggs et al. exploited this novel translational regulation of *secA* to design a new screen for mutations that alter the protein translocation machinery [84]. Starting with a strain that carried a *secA-lacZ* fusion in trans to $secA^+$, they looked for mutations that increased LacZ production. Mutations in *secY* (*prlA*), *secA* (*prlD*), *secDF* [84] and *secE* (*prlG*) [67] answered this screen. It is not surprising that *secB* and *secG* mutations were not obtained because it was known that these mutations do not increase SecA production. The *secA-lacZ* screen proved ultimately to be the most generally useful screen for mutants with protein translocation defects.

Many of the *sec* alleles confer cold-sensitive growth defects. The first *secE* alleles were cold sensitive, but they did not alter the protein sequence. Rather, they reduced synthesis by reducing translation [85]. This likely reflects the fact that protein translocation is an inherently cold-sensitive process.

malF-lacZ fusions.—In eukaryotic cells, Signal Recognition Particle (SRP) recognizes signal sequences as they emerge from the ribosome and delivers the ribosome and the nascent chain to the endoplasmic reticulum where translation resumes and the protein is simultaneously translocated from the cytoplasm [86]. DNA sequence analysis revealed that the *E. coli* chromosome contains homologues of the protein subunit that binds signal sequences, a protein called Ffh (Fifty-Four Homologue) [87–89], as well as a homologue of 7S RNA, an RNA called Ffs (Four point Five S RNA) [90,91]. Ffh [92] and Ffs are essential, but that might reflect a role in translation for Ffs [93,94]. Using depletion strains some defects in protein translocation could be detected, but the observed defects were clearly not as severe as the defect observed in the *sec* mutants [90–92]. Moreover, extensive genetic and biochemical analysis had failed to identify a role for the diminutive prokaryotic SRP. We now know that this is because the genetics and biochemistry had been done using periplasmic and outer membrane proteins as model systems, but as noted above, in bacteria, SRP is primarily used for inner membrane proteins [29].

Using the logic described above with *malE-lacZ* fusion strains, the Beckwith lab had designed a selection using a *malF-lacZ* fusion for mutants defective in the assembly of inner membrane proteins. The *malF-lacZ* fusion employed exhibited low LacZ activity because LacZ was fused to a periplasmic loop. However, Bardwell et al. discovered that mutations

that increased LacZ activity only yielded mutations that disrupted disulfide bond formation [50]. We now know that this is because the *malF-lacZ* fusion directed LacZ to the periplasm where DsbA inactivated the enzyme by forming disulfide-bonded aggregates. In the absence of DsbA, LacZ folds normally into an active enzyme [48]. Nearly a decade later, in 2000, Tian et al. modified the *malF-lacZ* selection to get the mutations that were originally sought [95]. Basically, instead of selecting mutants with strongly increased LacZ activity, they isolated mutants that only partially restored LacZ activity. These mutants had defects in SRP.

CONCLUSIONS

LacZ gene fusions enabled the power of bacteria genetics to be applied to the study of protein translocation and this stimulated both progress and interest in the new field of bacterial cell biology. It was an exciting time in part because of the realization that eukaryotic and prokaryotic researchers had much to learn from each other. We'd like to close with a sentence from the announcement for Blobel's Nobel Prize: The signal hypothesis describes a process that **"is a universal one: it operates similarly in plant, yeast and animal cells, including those of humans"** [96]. Actually, it is even more universal than that!

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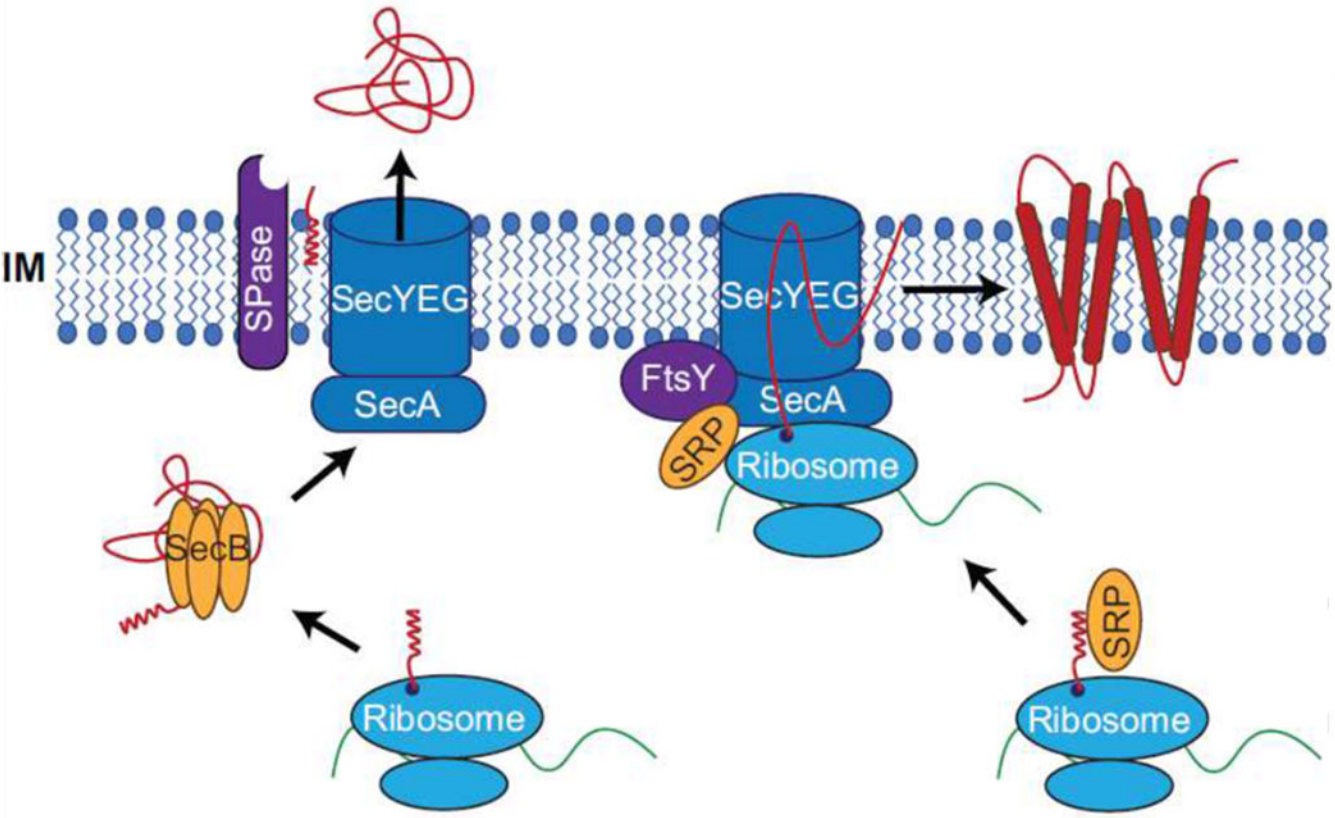

Fig. 1. Sec mediated protein translocation.

The SecYEG complex is responsible for the translocation of unfolded proteins through the IM [22]. Two recognition pathways allow the either the translocation of the protein into the periplasm or the insertion of the protein into the IM. Post-translationally secreted polypeptides are recognized by SecB after translation and SecB maintains their unfolded state [28] while bringing them to the SecYEG complex through an interaction with SecA [97]. The polypeptide is then translocated through SecYEG. After secretion, the signal sequence is cleaved by signal peptidase 1 (SPase) releasing the polypeptide into the periplasm [98] where it can fold or can be protected by chaperones until OM insertion. Lipoproteins are lipidated following translocation and their signal sequences are cleaved by signal peptidase II [99,100]. In contrast, the highly hydrophobic signal sequence of co-translationally secreted proteins is recognized by SRP and the translating ribosome is brought to Sec through an interaction between SRP and FtsY, the SRP receptor [29,88]. Sec inserts the transmembrane helices of the IM proteins into the membrane as the protein is translated. Accessory Sec proteins are omitted from the diagram for clarity.

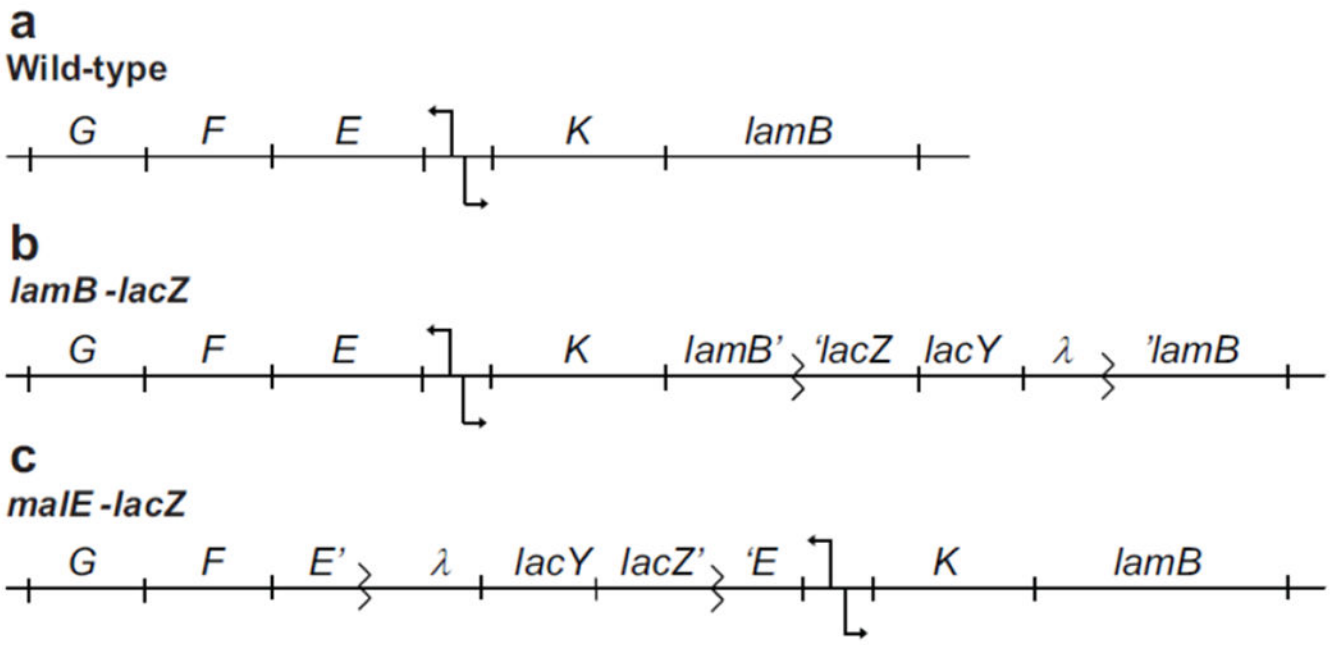

Fig. 2. Structure of maltose operons and gene fusions.

(a) The genes responsible for maltose import are found in divergent operons [35]. The transcription of these operons is activated by maltose bound MalT [101]. While one operon contains *malE, malF*, and *malG*, the other contains *malK* and *lamB*. (b) In the *lamB-lacZ* fusion, *lacZ, lacY*, and λ DNA is inserted into the genome in the *lamB* gene. This fuses the 5' end of the *lamB* gene to the *lacZ* gene [42]. **(c)** In the *malE-lacZ* fusion, the insertion is located in the *malE* gene. This fuses the 5' end of the *malE* gene to the *lacZ* gene [43].

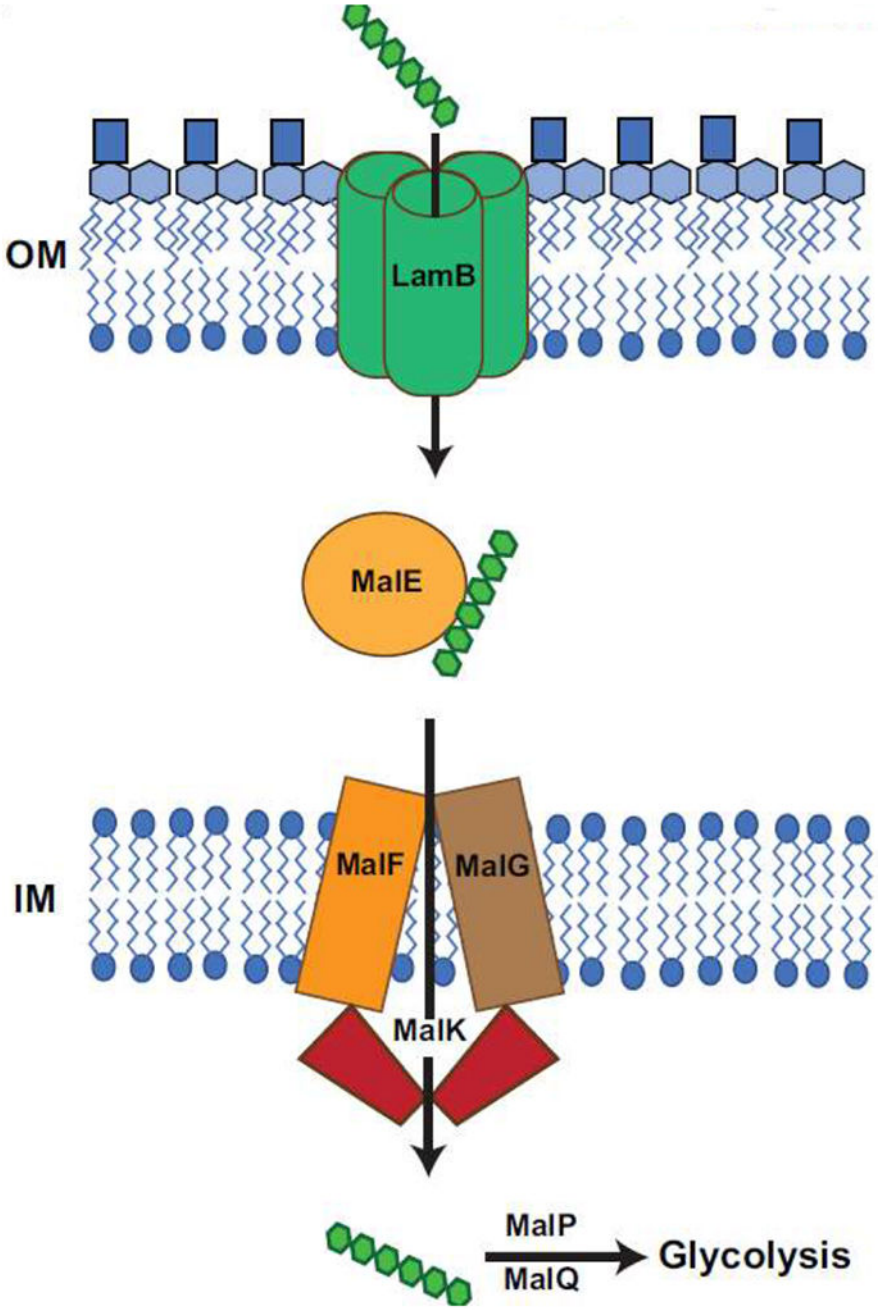

Fig. 3. Cellular import of maltose.

Although maltose can cross the OM through general porins, large maltodextrins (maltose oligomers) require LamB, the OM maltoporin, to enter the periplasm [19]. After entering the periplasm, maltose and maltodextrins are bound by the periplasmic MalE protein, which brings them to the IM import pathway [36]. Binding of MalE and maltose to the periplasmic face of MalFG causes ATP hydrolysis by MalK and import of the maltose or maltodextrin to the cytoplasm [102]. MalP and MalQ release glucose monomers from the maltodextrins to be used in glycolysis [103,104].

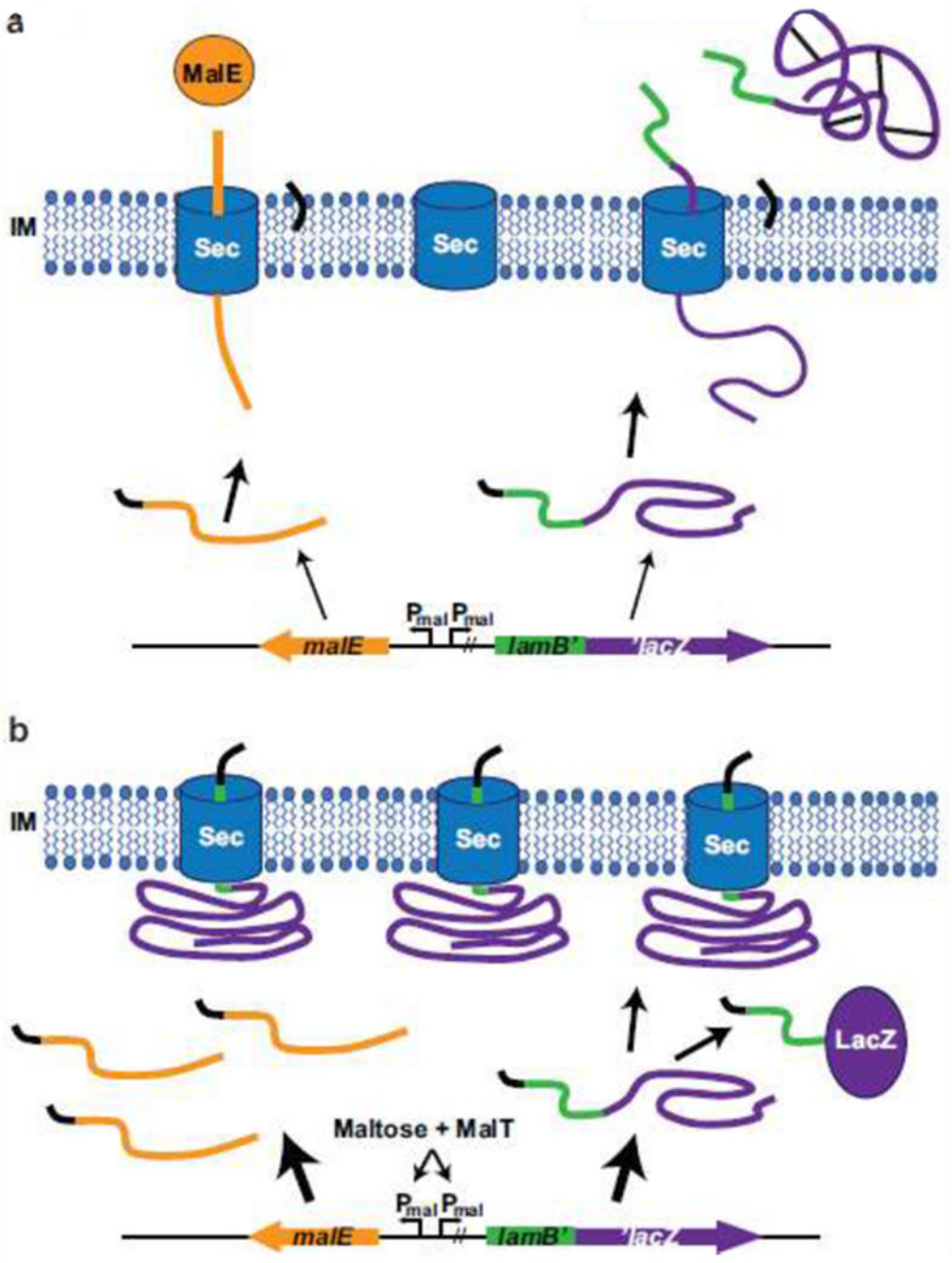

Fig. 4. Phenotypes of LamB-LacZ fusions.

(a) Without induction, the *mal* operons are transcribed at a low level. The low levels of hybrid protein produced allows adequate time for the LamB-LacZ fusion to be secreted despite the propensity for LacZ to fold in the cytoplasm [42]. In the periplasm, aberrant disulfide bonds prevent LacZ activity [48]. The secretion of MalE is not affected. **(b)** When maltose is present, the transcription of the *mal* operon is activated by maltose bound MalT [101]. The protein secretion machinery can no longer keep up with the increased levels of LamB-LacZ and LacZ begins to fold in the cytoplasm. The folded LacZ domain clogs the

Sec translocation machinery, which is eventually degraded causing cell death [46,42]. The clogging and degradation of Sec prevents the translocation of MalE.

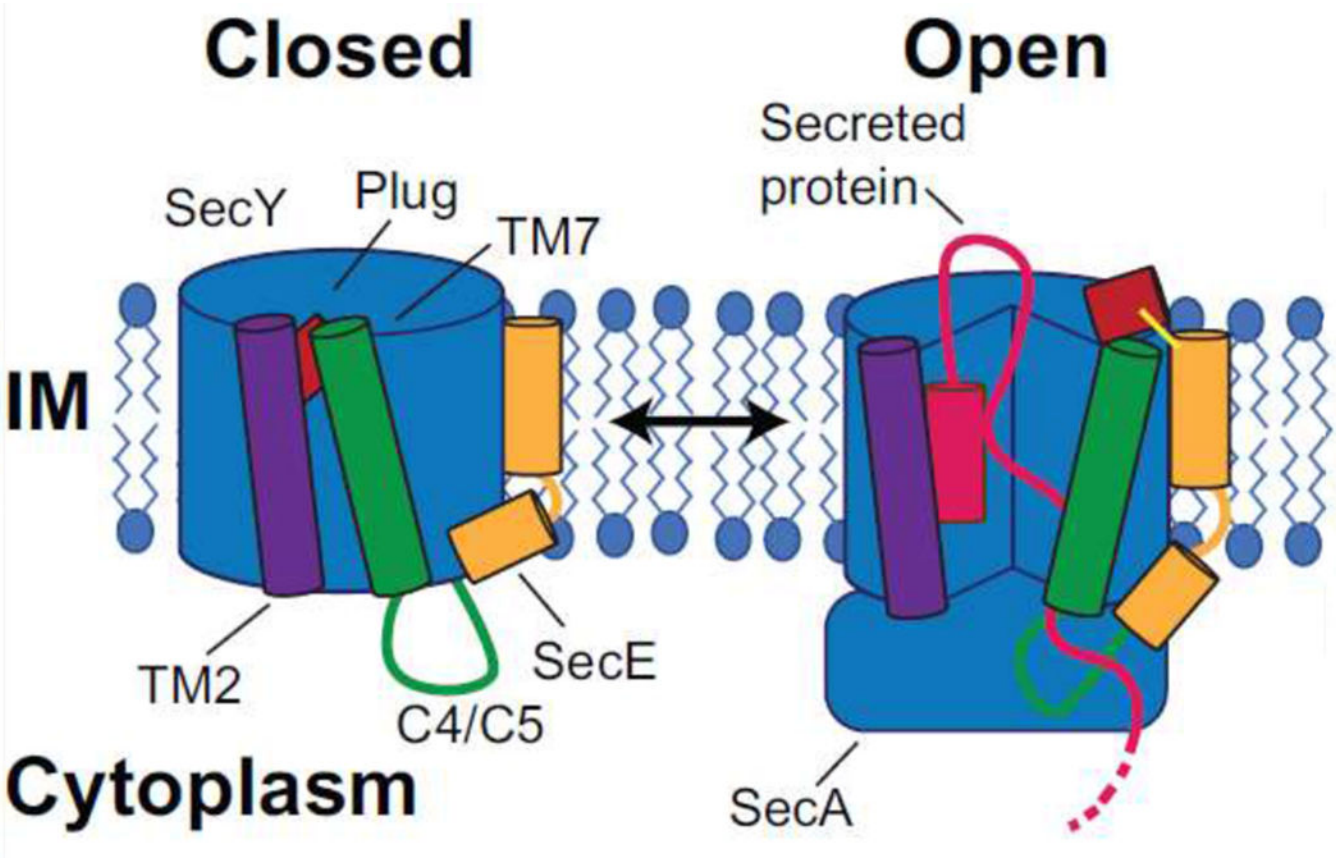

Fig. 5. Conformational states of SecYEG.

In the closed, inactive conformation of SecYEG, transmembrane helix (TM) 2 and TM7 are located close together and the protein translocation channel is blocked by the plug. SecA binds to the open conformation of SecYEG through interactions with the C4/C5 loop and SecE. TM2 and TM7 separate and the plug is moved out of the protein translocation channel, allowing translocation of the secreted protein [74]. The yellow line in the diagram of the open conformation indicates synthetic lethal cross-linking formed by cysteine mutations in SecY and SecE [73]. Adapted from Corey, et al. 2016 [105].